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EDITORIAL

Surgical recurrence in Crohn's disease: Are we getting better?

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Abstract

Crohn's disease (CD) still remains a challenging chronic inflammatory disorder, both for colorectal surgeons and gastroenterologists. The need for recurrent surgery following primary intestinal resection is still considerable, though recent evidence suggested a declining rate of recurrence. Several conflicting surgical parameters have been identified that might impact on the postoperative outcome positively, such as access to the abdomen, anastomotic configuration or type of disease. Additionally, promising results have been achieved with the increased use of immunosuppressive medications in CD. Consequently, the question arises if we are getting better as a result of novel medical and surgical strategies.

Key words: Crohn's disease; Surgical recurrence; Recurrence; Inflammatory bowel disease

Core tip: Crohn's disease still remains a challenging chronic inflammatory disorder, both for colorectal surgeons and gastroenterologists. There is still a considerable risk for recurrent surgery following intestinal resection, although recent evidence suggests a declining number of recurrence rates. Consequently, the question arises if we are getting better as a result of novel medical and surgical strategies.

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INTRODUCTION

Crohn's disease (CD) is a chronic inflammatory bowel disease, and patients have a lifetime risk of intestinal resection of up to 80%[1]. Although a population-based study reported an early use of immunosuppressants over an 18-year period was associated with a significant reduction in the cumulative probability of surgery[2], other studies revealed that the number of surgical procedures remained unchanged over the time, despite the more frequent use of infliximab[3,4].

Unfortunately, surgery is not curative and clinical

as well as endoscopic disease recurrence occurs frequently soon after first surgical intervention. In addition, the need to undergo a repeat operation for CD can reach 50% during a follow up period of over 10 years[5]. Notably, most of the available data concerning re-operation rates was collected retrospectively, before the era of biologicals, thus might not entirely reflect the current situation.

In contrast to a number of previous published studies, Riss *et al*[6] recently reported a surgical recurrence rate of 8.6% after a mean follow-up period of over 8 years. So the question arises whether novel treatment strategies alter the process of recurrence and give hope to affected patients. To date, there is a lack of data on predictors of recurrent surgery in CD. Current predictors such as smoking, young age of onset, family history, jejunal involvement and specific types of fistula, which correlate with a higher rate of surgical recurrence, might be complemented by predictive markers related to therapy[7,8].

CHANGING SURGICAL STANDARDS

CD is a chronic disorder and can usually be managed conservatively for a certain time period. However, patients refractory to medical treatment will require surgery to remove affected bowel segments or strictureplasty to overcome a stenosis. Although these surgical standards have not changed for years, several studies revealed that the timing of surgery, the type of access to the abdomen and the surgical technique itself could have an impact on the postoperative outcome and the course of disease.

In a series of 116 consecutive patients, who underwent primary ileocolic resection for CD, urgent indication for surgery was significantly associated with the necessity of repeated intestinal resection[6,9]. Additionally, Greenstein *et al*[10] showed that perforating CD represented a more aggressive type of disease leading to a higher number of reoperations and even shorter time-periods between the procedures.

Laparoscopic surgery in CD can be challenging but is being more commonly performed, especially for ileocolic resections[11-13]. It has proven short-term benefits such as decreased wound-infection rates, shorter hospital stay and faster recovery of bowel function even in complicated CD[14]. However, its long term effect on disease recurrence and late bowel obstruction is still under debate[15]. A meta-analysis comparing laparoscopic *vs* open surgery for CD indicated a reduced rate of surgery for recurrence in the laparoscopic group[16]. Given that statistical significance was driven by one study mainly[17], further well-designed clinical trials are mandatory to define the impact of laparoscopic surgery in CD on the need to undergo repeat surgery for disease recurrence.

Both the elective as compared to the acute indication and the laparoscopic *vs* the open procedure are known to reduce the systemic inflammatory response syndrome (SIRS). It could be envisioned that a reduced SIRS in the perioperative course could reduce CD-associated pathological hyper-inflammation affecting the healing process of the anastomoses.

The type of anastomotic configuration following bowel resection in CD also seems to influence the postoperative outcome. Although, a stapled side-to-side anastomosis was previously widely considered as an inappropriate technique in CD, due the inflamed and thickened tissue and the potential higher risk for leakage, its safe use and its feasibility was demonstrated in large series[11]. In addition, it was discussed whether a wide anastomotic lumen with less stool stasis and better blood supply, leads to an improved postoperative course with a reduced rate of anastomotic disease recurrence. Notably, a case-controlled study comparing wide-lumen stapled anastomosis with conventional sutured end-to-end anastomosis found a significant lower rate of symptomatic recurrences and reoperations in the stapled group[18]. Furthermore, Simillis *et al*[19] conducted a meta-analysis comparing end-to-end hand sewn anastomosis with other types of anastomotic configuration after intestinal resection for CD. Overall postoperative complications, including anastomotic leaks and length of hospital stay, were reduced in the side-to-side stapled group, although there was no significant difference in terms of surgical recurrences. Consequently, a wide-lumen stapled side-side anastomosis offers potential short-term benefits, but its beneficial effect on disease recurrence in Crohn's patients is not sufficiently confirmed.

CHANGING MEDICAL PARADIGM

Personalized medical therapy represents a keystone in the treatment of patients with CD. Currently, postoperative medical prophylaxis should be chosen individually for each single patient, according to a specific risk profile, such as smoking, penetrating disease or early endoscopic recurrence after routine ileocolonoscopy 6 to 12 mo following surgery. No clear guidelines recommending the routine application of specific immunosuppressive medication currently exist. This situation highlights the important role of close follow-up examinations by specialized gastroenterologists.

The introduction of immunosuppressants has led to significant improvements in the treatment of CD[20]. It has been demonstrated that the postoperative exposure to azathioprine/6-mercaptopurine for more than 36 mo led to a significant reduction of reoperation rates after intestinal resection[6,21]. This treatment benefit was effective even in the high-risk group of smokers. A recent meta-analysis reported Azathioprine and 6-mercaptopurine to be more effective in the prevention of postoperative clinical and endoscopical

recurrence in CD compared to placebo. Unfortunately, they were also associated with considerable adverse events[22]. Surgical recurrence was not addressed in this investigation. In contrast, Ardizzone *et al*[23] conducted a randomized controlled trial and did not observe any difference between azathioprine and mesalamine in preventing recurrent surgery Further studies will be required to compare the effectiveness of azathioprine and 6-mercaptopurine with anti-tumor necrosis factor-α (TNF-α) antibodies in preventing disease relapse after intestinal resections.

Targeting TNF-α has become a major issue in maintaining long-term remission and significantly enriched therapeutic strategies in CD within the last decade. Infliximab, a monoclonal chimeric anti-TNF-α antibody, is effective in CD[24] and induces remission even in patients not responding to conventional treatment[25]. There are promising data that targeted therapy prevents postoperative endoscopic and clinical recurrence, which would further imply that escalation of disease could be limited at an early point, and eventually decreasing surgical interventions to reset disease activity. Regueiro *et al*[26] randomly assigned 24 patients either to postoperative infliximab or placebo treatment. After 1 year, 9.1% had endoscopic recurrence compared to 84.6% in the placebo group. Furthermore, these encouraging results were confirmed by Sorrentino *et al*[27], by performing a prospective study in 12 consecutive patients that were free of clinical and endoscopic disease recurrence at 24 mo under infliximab treatment. Once biological therapy was stopped, 10 out of 12 patients developed endoscopic recurrence, which could be limited by low-dose maintenance therapy.

Another milestone in biological therapy of CD was achieved with the implementation of adalimumab, a human, monoclonal anti-TNF-α antibody, which was expected to be less immunogenic than infliximab. Notably, adalimumab was more effective than azathioprine and mesalamine at preventing postoperative endoscopic recurrence of CD in a randomized trial[28].

Nevertheless, there is a considerable lack of efficient data regarding the usefulness of biologicals (infliximab/adalimumab) in postoperative CD. Most published studies did not address the end-point surgical recurrence and enrolled a low number of patients only. Its routine prophylactic use after surgery to prevent recurrence is debatable taking into account missing large randomized controlled trials, its high medical cost and the potential side effects. The decision to use biologicals postoperatively should be made individually, according to the clinical history and risk stratification of each patient.

CONCLUSION

The impact of surgery on postoperative disease recurrence is still under debate. Most available studies were designed retrospectively and do not provide high quality data to justify absolute recommendations. Few reports exist which indicate that a minimal invasive approach does not only offer short-term benefits, but might also delay repeat surgery for recurrence. A wide-lumen stapled side-to-side anastomotic configuration could potentially reduce the postoperative complication rate and the need for recurrent resection. Additionally, it is important to avoid postoperative complications, which represents another strong risk factor for surgical recurrence.

In our study, we have intended to implement all those strategies. We have used the technique of side-to-side anastomoses, endeavoring to perform elective rather than emergency surgery and a high rate of laparoscopic approaches. We now suggest that those factors could contribute to the favorable outcome with a low re-operation rate as this combination showed some success. It must certainly be stated that careful clinical observation after surgery and risk-stratified individual medical prophylactic therapy guided by experienced gastroenterologists is a keystone to providing the best care for these complex patients. In this line, the introduction of anti-TNF agents has definitely changed the treatment strategies in CD patients and improved the course of disease in a number of patients. Further randomized controlled trials will define the exact role of routine use of postoperative immunosuppressive medications.

In the future, it will be of great importance to further identify predictive factors for recurrence to allow us to select the appropriate patients who may benefit most from prophylactic medical treatment.

Finally, we are getting better in treating our patients, both surgically and medically, but we are still looking for a way to see a light in the end of the tunnel.

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REVIEW

Surgical strategies in paediatric inflammatory bowel disease

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Abstract

Inflammatory bowel disease (IBD) comprises two distinct but related chronic relapsing inflammatory conditions affecting different parts of the gastrointestinal tract. Crohn's disease is characterised by a patchy transmural inflammation affecting both small and large bowel segments with several distinct phenotypic presentations. Ulcerative colitis classically presents as mucosal inflammation of the rectosigmoid (distal colitis), variably extending in a contiguous manner more proximally through the colon but not beyond the caecum (pancolitis). This article highlights aspects of the presentation, diagnosis, and management of IBD that have relevance for paediatric practice with particular emphasis on surgical considerations. Since 25% of IBD cases present in childhood or teenage years, the unique considerations and challenges of paediatric management should be widely appreciated. Conversely, we argue that the organizational separation of the paediatric and adult healthcare worlds has often resulted in late adoption of new approaches particularly in paediatric surgical practice.

Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Paediatric; Surgery

Core tip: Approximately 25% of patients with inflammatory bowel disease have onset of symptoms in childhood or adolescence. The unique and often severe features of childhood presentation make treatment decisions challenging. The dogma of surgical conservatism in Crohn's disease is challenged in the specific instance of left sided colitis. Furthermore we argue that the separation of adult and paediatric inflammatory bowel disease practice may disadvantage children, delaying adaption of innovative treatments and timely transition.

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INTRODUCTION

Inflammatory bowel disease (IBD) comprises two distinct but related chronic relapsing inflammatory

conditions affecting different parts of the gastrointestinal tract. Crohn's disease (CD) is characterised by a patchy transmural inflammation affecting both small and large bowel segments with several distinct phenotypic presentations. Ulcerative colitis (UC) classically presents as mucosal inflammation of the rectosigmoid (distal colitis), variably extending in a contiguous manner more proximally through the colon but not beyond the caecum (pancolitis) except rarely when backwash ileitis features. This article highlights aspects of the presentation, diagnosis, and management of IBD that have relevance for paediatric practice with particular emphasis on surgical considerations. Since 25% of IBD cases present in childhood or teenage years, the unique considerations and challenges of paediatric management should be widely appreciated. Conversely, we argue that the organizational separation of the paediatric and adult healthcare worlds has often resulted in late adoption of new approaches particularly in paediatric surgical practice.

INCIDENCE

A prospective United Kingdom survey of childhood IBD showed that the incidence was 5.2 per 100000 children per year. In terms of disease distribution 60% were diagnosed as CD and 28% as UC. The remaining 12% could not be classified, and were labelled as indeterminate colitis (IC)[1]. The mean age at diagnosis was 12 years with 5% presenting at less than 5 years of age[2]. The United Kingdom data are similar to a systematic review of North American paediatric cohorts suggesting an incidence of 3-4 per 100000[3]. A recent systematic review comparing time-trend analyses across the age spectrum demonstrated that in 75% of CD studies and 60% of UC studies, the incidence of IBD had significantly increased[4].

PATHOGENESIS

A detailed analysis of progress in understanding the aetiology of IBD is beyond the scope of this article. Suggested mechanisms include the influence of a variety of possible environmental factors triggering an inflammatory response which then persists in a genetically susceptible individual, according to the Knudsen "two-hit" hypothesis. A number of candidate IBD genes have been identified since the *CARD15/NOD2* gene was identified on chromosome 16[5]. By 2011, genome-wide association studies had demonstrated 99 non-overlapping gene loci associated with CD and UC, including 28 that are shared, suggesting common mechanisms of pathogenesis[6]. These studies have provided "molecular pointers" to the underlying pathophysiological processes which might be implicated in IBD, including epithelial barrier function, epithelial cell regeneration, microbial defence, innate immune regulation, generation of reactive oxygen species, autophagy, and regulation of adaptive immunity. Even at the level of the individual candidate gene locus, these interactions are complex. For example *NOD2* is currently implicated in autophagy, viral recognition and T cell activation[7]. The genes implicated in UC and CD overlap, as do those implicated in childhood- and adult-onset IBD, indicating both common mechanisms of pathogenesis and genetic predisposition. Disease concordance rates in monozygotic twin studies are 10%-15% in UC and 30%-35% in CD, suggesting that non-genetic factors may have greater influence in the pathogenesis of UC[8]. The relationship between genotype and either locational phenotype or behaviour of disease are complex and have at times yielded conflicting results.

The *NOD2/CARD15* gene association with CD has been most extensively studied and has been linked to defective Toll receptor-mediated macrophage opsonisation of pathogenic bacteria. *NOD2* variants have been associated with the fibrostenosing phenotype, more aggressive disease progression, and ileocaecal presentation[9], although their relation to surgical recurrence has produced contradictory results. "Wild type" gene expression at this locus has been correlated with more favourable response of Crohn's fistulae to antibiotics[10]. In respect of perianal disease phenotype, the influence of dysfunctional gene expression for both the carnitine/organic cation transporter *OCTN*[11], and immunity-related GTP-ase family M protein (IRGM)[12,13] on chromosome 5q31 (IBD5) seem to be more specifically related to both fistula and abscess incidence, possibly *via* defective oxygen burst-mediated bactericidal function, and defects in bacterial autophagy respectively. Although IBD has a multigenic aetiology, each of modest contribution to overall pathogenesis, it remains possible that future advances in genotype studies will identify behavioural subtypes which have significant therapeutic consequence.

Basic science research has focussed on the gut/environmental interface and the various mechanisms maintaining its integrity, the inflammatory process including cell signalling, cytokine responses, the specific gut microbiome and cellular immune defences. Various epidemiological studies have implicated diet, ethnicity, socioeconomic status, smoking, migration and vaccination status in the pathogenesis of CD[14].

DIAGNOSIS AND MEDICAL MANAGEMENT

The configuration of United Kingdom medical services has resulted in the paediatric gastroenterologist being the focal point of referral for children with suspected IBD. Moreover, the specialist endoscopic skills required for diagnosis are now less readily available within paediatric surgical departments[15]. Thus the diagnosis of IBD in childhood is largely the domain of the gastroenterologist. Despite this it is

vitally important that any surgeon managing children should be aware of the common presenting features of inflammatory bowel disease, both to ensure that the child is appropriately investigated and to avoid premature ill-conceived surgical intervention. The most common presentations to surgical services are for investigation of rectal bleeding, anal pain, and acute exacerbations of abdominal pain. It is very uncommon for surgical intervention to be required before appropriate diagnostic endoscopic and radiological tests have established the type and extent of IBD, allowing opportunity for appropriate targeted medical management.

Any surgeon treating children with IBD should have a working knowledge of the medical treatment options, their efficacy, side effects and psychosocial impact, to ensure that when a surgical option is under consideration with its potential attendant morbidity, the child and his parents are fully aware of the advantages and disadvantages of all management strategies. Joint clinics staffed by a gastroenterologist, specialist surgeon, dietician and nurse specialist constitute the ideal venue for difficult discussions where clinical decision making is often highly nuanced.

DIAGNOSIS

All children suspected of IBD require full history and examination including assessment of growth velocity and pubertal staging. A minority (25%) of children with CD present with the classic triad of abdominal pain, diarrhoea and weight loss. A high index of suspicion should be maintained in children presenting with vague complaints such as lethargy, anorexia and impaired growth or delayed puberty[2]. The development of standardised diagnostic criteria (the Porto criteria) by the IBD working group of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) has done much to ensure uniformity in diagnosis and management[16].

The gold standard in diagnosis is combined upper and lower gastrointestinal endoscopy (including ileal intubation), together with small bowel radiology. Additional laboratory investigations are adjunctive, but should always include inflammatory markers (ESR, platelets and CRP), nutritional markers (albumin), and liver function tests because of the association of primary sclerosing cholangitis with UC. Stool cultures are mandatory to exclude an infective colitis. Absence of elevated faecal inflammatory surrogate markers calprotectin and lactoferrin make active bowel inflammation unlikely. In children younger than 2 years and in those with atypical presentation immunological tests are required to exclude chronic granulomatous disease, common variable immune deficiency, Wiskott-Aldrich syndrome and other immunodeficiency states. Increasing experience with MR enterography, the lack of exposure to radiation, and the utility of MRI in the evaluation of perianal CD, all favour this study over contrast meal and follow through for the radiological evaluation of the small bowel. Capsule endoscopy is showing promise as an adjunct in cases of diagnostic difficulty, but carries the risk of obstruction if the capsule becomes impacted at an occult area of narrowing. Lack of sensitivity of technetium leukocyte scintigraphy has largely consigned this test to being of historical interest only.

CLASSIFICATION

The Montreal classification of IBD classifies CD by virtue of age, location and behaviour (inflammatory, structuring or fistulating)[17]. It subdivides UC based on the extent and severity of the colitis. More detailed scoring systems have both prognostic and comparative value for research purposes. The paediatric CD activity index (PCDAI) is based on clinical parameters from recent history, examination findings, laboratory results, growth parameters and extra-intestinal manifestations[18]. The paediatric ulcerative colitis activity index (PUCAI) incorporates 6 clinical items and is therefore easy to use[19]. Any classification for CD can be particularly frustrating for the surgeon. The issue for the surgeon is the difference between macroscopic and microscopic disease. The danger of the classification system is that equal weight is given to asymptomatic microscopic disease, and the macroscopic disease which is actually causing the symptoms. Thus, for example, a patient may be labelled as having pan-intestinal CD when he is actually symptomatic from stricturing ileocaecal disease and merely has asymptomatic microscopic disease elsewhere. As with every classification system, its intrinsic usefulness is dependent on its clinical significance for decision making.

MEDICAL MANAGEMENT

Ulcerative colitis

The medical approach to UC in childhood is dependent on the extent and severity of the colitis. Presentation with pan-colitis is twice as common (60%-80%) as adults[20], and for this reason steroid therapy is most often initiated. In less severe colitis, oral or rectal therapy with aminosalicylic acid (5-ASA) derivatives would represent both first line and maintenance therapy after induction of remission. Oral ASA therapy may be combined with topical treatment but often the rectal route is unacceptable to the child. Steroids do not have a role in maintaining remission, and therefore thiopurines (azathioprine or mercaptopurine) are used as immunomodulators either alone or alongside 5-ASA. Thiopurines may take up to 10-14 wk to achieve therapeutic effect and are usually started alongside steroids for this reason. Infliximab should be considered in steroid-dependent UC, resistant to 5-ASA or thiopurines. Adalimumab may be substituted in patients who have lost response to or are intolerant

of infliximab. Less commonly, cyclosporin or tacrolimus may be used in acute severe colitis as a bridging therapy before thiopurine efficacy. There is currently no evidence to support using methotrexate, antibiotics or probiotics for the induction or maintenance of remission in children. Similarly plasmapheresis remains controversial as a therapeutic option for severe childhood UC[21].

CD

Since CD has many phenotypic variants, medical therapeutic strategies are, to a certain extent, tailored both to disease location and to the severity of symptoms. Perianal CD (PACD) is discussed separately in this article since it is of particular interest to the surgeon, and only the broad principles of medical management of paediatric CD are discussed below. Both steroids and exclusive enteral nutrition (EEN) are considered equally effective in inducing remission. There is no difference in efficacy of either elemental or polymeric formula feeds which both avoid the unpleasant side-effects of steroids and promote nutrition. Prolonged courses of EEN are difficult to sustain both due to taste and dietary boredom. In common with UC, remission is maintained with thiopurines which should be initiated at the onset of either dietary of steroid therapy. Unlike UC, methotrexate is effective in maintaining remission in cases of thiopurine toxicity. 5-ASA treatment cannot be recommended in the management of CD, both because of lack of paediatric data, and lack of efficacy in adults. Infliximab may be used as second line induction and maintenance therapy in relapsing CD where EEN or steroids are losing effect, or where side effects of steroids are intolerable[22,23]. Infliximab is controversial in the setting of the stricturing CD phenotype, with some studies suggesting lack of efficacy[24], and others favouring its use[25]. It is entirely possible that the distinction lies between fibrous and inflammatory strictures with lack of efficacy in the former group. It has been the author's experience that acute obstructive symptoms may rapidly respond to steroid therapy suggesting a significant inflammatory component in the aetiology of symptoms. Second line biological therapy may be effective in case of either loss of effect of infliximab or intolerance. The rates of surgical treatment of IBD in the "biologic era" have recently received attention in the literature, suggesting that the overall incidence of surgery has declined in those with mild disease, but that the incidence of surgery is unchanged in patients with severe disease[26].

SURGICAL MANAGEMENT OF ULCERATIVE COLITIS

The objective in both elective and emergency surgical interventions for UC is the removal of the colon. The most common indication for colectomy is failure of medical therapy whether due to frequent disease activation with relative short periods of remission, or unacceptability of the side-effects of medical therapy. Inadequate disease control may also be reflected by reduced growth velocity, delayed puberty, inadequate nutrition, poor bone mineralisation and loss of time from school. Although some extra-intestinal features of UC are improved by surgery, primary sclerosing cholangitis and sacroiliitis are not[27]. Colonic cancer per se does not feature in the indications for colectomy in childhood since the quoted risk is 2% at 10 years, 8% at 20 years and 18% at 30 years[28]. However, a French study noted a 3-fold increased risk of neoplasia (including colon cancer 2/698) in paediatric onset IBD patients over a median follow up of 11.5 years[29], and the Porto IBD group noted a number of treatment-related malignancies (mainly lymphoma)[30]. Body image concerns are significant barriers to surgery for many children and careful support form psychologists and stoma therapists may be necessary for them to accept even a temporary stoma. Colectomy rates at five years from diagnosis range between 14%[31] and 24%[32,33] in children.

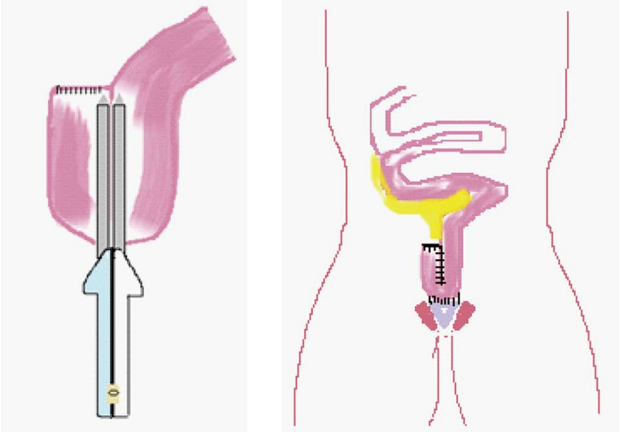

Figure 1 Ileal pouch anal anastomosis being fashioned, stapling a small bowel J-pouch, and its anastomosis to the anus and sphincter complex.

Reconstructive (continent) surgery for UC was transformed in 1978 by the development of the ileal pouch anal anastomosis (IPAA) (see Figure 1) by Parks and Nicholls which is now the gold standard[34]. Since paediatric surgeons were at that time already familiar with straight ileoanal pull as an option for treatment of total colonic Hirschsprung's disease[35], and also because of low patient numbers requiring colectomy for UC, they were generally late adopters of the IPAA. The most conservative approach for elective surgery is a 3-stage procedure performing total colectomy with end ileostomy, delaying completion proctectomy to the time of construction of IPAA, and covering this with a temporary ileostomy. Two-stage surgery either involves total colectomy and avoiding a covering ileostomy at the time of pouch formation (authors preference), or fashioning a primary pouch

at the time of panproctocolectomy and covering this with an ileostomy. In the elective setting, total colectomy and end ileostomy was the most widely performed procedure in adult practice (guidelines American Society of Colon and Rectal Surgeons)[36], although panproctocolectomy, and primary pouch construction with covering ileostomy is rapidly gaining acceptance[37-39]. Clearly single stage surgery is feasible[40], but is associated with a higher risk of major complications such as anastomotic dehiscence, sepsis and late pouch failure[37]. Since the median number of pouch operations performed per year by United Kingdom paediatric surgeons specializing in IBD is 1 (range 0-4)[15], there is a natural tendency to opt for the most conservative elective operation and perform colectomy and end ileostomy. Children are usually transformed by this procedure and rapidly resume normal activity, including, most importantly, school attendance. Since significant symptoms from the retained rectum are infrequently encountered[41], the driver for restorative surgery is the child's desire to lose the end ileostomy, which is usually counter-balanced by individual educational pressures and the child's general sense of well-being.

The greater experience of IPAA within the adult sector and the fact that most children undergoing continent reconstruction will soon be transitioned into adult care, have prompted many surgeons to joint operate with adult colleagues at the time of pouch formation. Such cooperation both facilitates later transition and enables the sharing of technical expertise. Advances such as the double-stapled IPAA have been incorporated into paediatric practice with considerable reduction in operating time when compared with traditional hand-sewn IPAA. The complication rate has been shown to be unchanged and the functional performance of the reservoir marginally improved in the shift from the hand-sewn to the double-stapled anastomosis[42].

Large volume outcome data for IPAA within the paediatric sector are sparse with studies tending to compare the IPAA with straight pull through. A paediatric meta-analysis suggested a higher failure rate for straight (15%) over pouch (8%) pull through procedures, associated with both higher daily stool frequency and post-operative sepsis rates[39]. A multicentre analysis of 203 children undergoing straight (SIAA 112) and J pouch (JPAA 91) ileoanal anastomosis (mainly for UC) demonstrated significantly reduced daily stool frequency in the JPAA, although after 24 months the difference became less apparent (SIAA 8.4 *vs* JPAA 6.2)[43]. The mean daily defecation frequency 24 months after IPAA in a Finnish study was 3.3 ± 0.5, demonstrating that excellent short term functional results can be achieved in children[44].

Morbidity

The IPAA is associated with a high surgical complication rate. The Mayo clinic experience of intraoperative abandonment of IPAA (1789 cases) was 4.1%[45]. One large study including 151 children reported that one fifth of patients will have at least one complication in the first month after surgery[46]. This study focussed on pouchitis demonstrating a single episode in 48%, chronic refractory pouchitis in 7%, and pouch failure in 9%. The authors demonstrated that late diagnosed Crohns disease (15%) was an important determinant of poor outcome. In another paediatric series the complication rate was as high as 21/37 (57%); including stenosis of the IAA (2/37), pelvic abscess/ sepsis (4/37), late fistula (3/37), early intestinal obstruction (7/37), late intestinal obstruction (11/37), pouch prolapse (1/37), wound complications 6/37, pouchitis (23/37), and recurrent pouchitis (13/37)[47]. Another paediatric study reported a high incidence (19%) of intestinal obstruction[48]. There is no reason to assume that the complication rates of IPAA in children should be different from those seen in adult practice where anastomotic dehiscence is observed in 5%-10%, pouch-vaginal fistula in 3%-16%, pouchitis in 24%-48%, and pouch failure at 5 years in 8.5%[38], as well as the risk of reduction in fertility in females, discussed in the next section. Long term follow up suggests overall cumulative pouch failure rates of 15% over 10-15 years. A proportion of these patients with outlet obstruction and low pouch capacity will be improved by abdominal salvage procedures[49]. There is very little experience in the paediatric surgical literature of revision pouch surgery which is a further strong argument for close links with high case load adult specialist units[15].

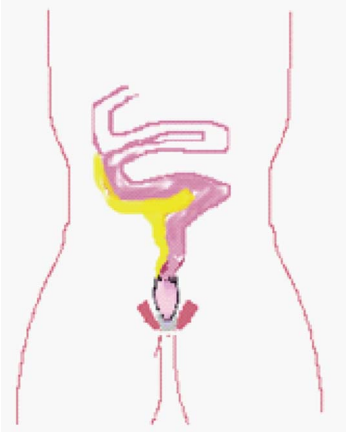

Figure 2 Straight ileo-rectal anastomosis.

Ileorectal anastomosis

The option of ileorectal anastomosis (IRA) (Figure 2) for UC is rarely considered in childhood. Potential advantages include a reduced stool frequency with improved faecal continence[50], improved fertility in females[51], and a reduced likelihood of impaired sexual functioning due to nerve injury during the pelvic

dissection. This has to be balanced with the need for regular endoscopic surveillance and the potential for failure of medical control of the residual disease in the rectum.

The ultimate failure rate of IRA for UC is as high as 57%[52], but this does not argue against the procedure in females if time is gained for pregnancy before later restorative proctectomy. A meta-analysis has demonstrated that the rate of female infertility (15% in medically treated UC) rises to 48% after IPAA[53], although many of these "infertile" women could potentially achieve medically-assisted conception. Another systematic review demonstrated a more modest effect on infertility, with a rate of 12% before IPAA, and 26% thereafter (945 women in 7 studies). The same authors reported rates of sexual dysfunction (dyspareunia) in 8% preoperatively, compared with 25% after restorative surgery (419 women in 7 studies)[54]. One study looked at the effect of restorative proctectomy in childhood on later sexual function in adulthood concluding that rates of dysorgasmia and dyspareunia were not significantly different between girls undergoing surgical or medical management of UC. They authors also noted that sexual satisfaction was inversely correlated with faecal incontinence[55]. A study evaluating quality of life (QOL) after colectomy identified younger age at colectomy, diagnosis and survey to be associated with better QOL scores. The length of time post colectomy did not have any correlation with QOL[56].

ACUTE SEVERE COLITIS

The management of children presenting with acute severe colitis (ASC), identified by the requirement of intravenous steroid therapy, has been subject to little scrutiny in the literature. Cumulative colectomy rates in 99 children with ASC at discharge, 1 year, and 6 years, were 42%, 58% and 61% respectively[57]. Predictive factors significantly associated with corticosteroid failure include C-reactive protein, and the number of nocturnal stools on days 3 and 5. The PUCAI, Travis and Lindgren's indices were strong predictors of failure of response to steroids[57]. In adult practice, the Travis criteria assessed on day 3 of steroid treatment indicate that a C-reactive protein > 45, and bowel action > 8 times per day, carry an 85% likelihood of subtotal colectomy during that admission[58]. The PUCAI has been promoted as a marker for failure of response to intravenous corticosteroids (day 3 > 45 points, and day 5 > 65-70 points) with a predictive accuracy of 85%-95%, allowing rapid introduction of second line medical therapies[57-59]. Early introduction of salvage medical therapies (cyclosporine, tacrolimus and infliximab) has reduced the emergency colectomy rate in ASC from 30%-70% to the current 10%-20%, with concomitant reduction in mortality[60]. Upper limits of normal colonic width in children with ASC should take age into consideration (4 cm < 11 years, and 6 cm in older children)[57]. Absolute indicators for surgery in the setting of unresponsive ASC include perforation and significant haemorrhage. Otherwise the risks, benefits and potential psychological morbidity of colectomy should be considered alongside salvage medical therapies.

The surgical procedure of choice is colectomy and end ileostomy preserving the rectal stump[60,61]. The options for managing the rectal stump include formation of a mucous fistula, division at the sacral promontory, or subcutaneous placement beneath the laparotomy wound. The presence of a mucus fistula is associated with mucoid discharge which may be unacceptable[41]. Although subcutaneous placement increases wound infection rates, the rate of pelvic sepsis is decreased when compared with division at the rectosigmoid junction[62,63].

LAPAROSCOPIC COLECTOMY AND POUCH ANAL ANASTOMOSIS

The adult sector now has a large experience of the application of laparoscopic approaches to surgery for UC. A meta-analysis comparing open and laparoscopic subtotal colectomy identified a conversion rate of 5%. Significant benefit was shown for laparoscopic surgery in terms of wound infection, intra-abdominal abscess, and length of stay[64]. Laparoscopic IPAA is increasingly being performed in adult IBD centres. The application of these techniques to paediatric practice has been slower. Diamond *et al*[65] identified a 7% conversion rate in 42 children undergoing subtotal colectomy. Fraser *et al*[66] compared overall morbidity seen in 44 children undergoing different variations on a theme of total colectomy (including 27 children with UC) noting an overall major complication rate of 43% unaffected by laparoscopic or open approaches. Linden *et al*[67] compared open (39) and laparoscopic-assisted (68) IPAA, demonstrating a significantly reduced incidence of small bowel obstruction at 1 year follow up in the laparoscopic group. Sheth *et al*[68] compared open (37) and laparoscopic (45) IPAA (including 56 children with UC) demonstrating comparable outcomes and surgical morbidity. Flores *et al*[69] compared open and laparoscopic colectomy in 32 consecutive patients finding significantly shorter lengths of stay in the laparoscopic group[69].

SURGICAL MANAGEMENT OF CD

The life time risk of surgery for CD is approximately 80%[70]. Indications for surgical management of children with CD include failure of medical therapy, growth failure despite full medical therapy, associated extra-intestinal manifestations (especially eye and joint pathology), and complications of the disease (fistula, obstruction, perforation, abscess formation, and bleeding). Timely surgical intervention has been

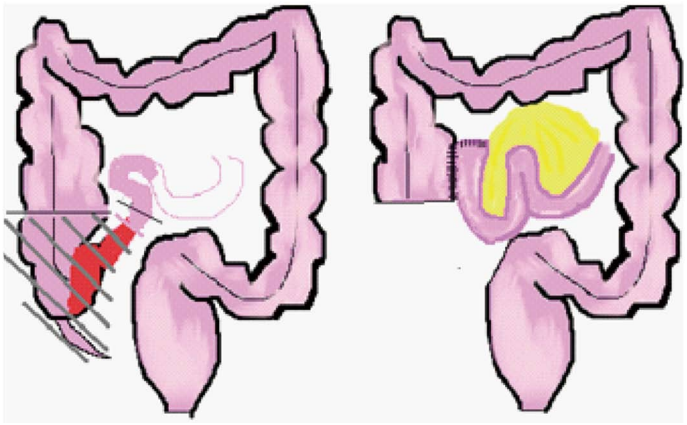

Figure 3 Ileocaecal resection for terminal ileal Crohn's disease.

demonstrated to improve height velocities in patients refractory to medical therapy[71,72]. Location of disease also has a significant bearing on the decision to operate or to persevere with medical management, since a local resection with primary anastomosis is less psychologically debilitating than a major colonic resection and possible permanent stoma.

Using the Paris classification (modification of the Montreal classification)[73], applied to 582 children on the EUROKIDS registry, 16% had isolated terminal ileal disease (± limited caecal disease), 27% had isolated colonic involvement, ileocolonic disease was seen in 53%, and 4% had a disease distribution at presentation localised to the upper gastrointestinal tract[74]. A radiological study suggested an increased propensity for left sided colitis in children compared with adults[75].

The author's surgical perspective is of clear phenotypic subtypes (relating to macroscopic disease location), which greatly simplify decision making. Thus ileocaecal distribution including contiguous disease to the just beyond hepatic flexure (watershed of ileocolic arterial blood supply), and left sided colitis, are distinct and usually mutually exclusive phenotypes. Isolated proximal small bowel disease is rare in paediatric practice[76].

Resectional surgery

Requirement for surgical resection ranges from 20%-29% at 3 years, and 34%-50% at 5 years from diagnosis in paediatric practice[77-79]. The fundamental surgical consideration is localization of the macroscopic disease in accordance with one of the "five golden rules" of Alexander-Williams and Haynes[80], which may be paraphrased thus; "resect only symptomatic macroscopic disease". Children with ileocolic disease (with colonic involvement proximal to the mid-transverse colon) are readily managed by right hemicolectomy and primary anastomosis with low associated morbidity[76] (Figure 3).

Debate continues regarding the management of colonic disease distal to the transverse colon, and whether this might break the golden rule of surgical conservatism. The phenotype associated with left sided colitis in childhood has been shown to relapse early following segmental resection[81], or develop significant complications from anastomotic failure after segmental resection[76]. An adult meta-analysis (448 patients) suggested that segmental resection was not associated with increased overall recurrence rates, complications, or need for a permanent stoma, but that time to recurrence was longer by 4.4 years in the subtotal resection group[82]. However, segmental resection for left sided colitis continues to be advocated by some surgeons, citing preservation of anorectal function and decreased post-operative symptoms[83]. Another study advocated segmental resection over subtotal colectomy and IRA, based on favourable clinical recurrence rates, reoperation rates and risk of permanent stoma requirement. Risk factors for recurrence/reoperation included both perianal CD and colocolic anastomosis, which implies that ileocolic anastomoses were included in the evaluation and thus that cases of right sided colitis were included in the evaluation[84]! A possible explanation for the difference between the paediatric and adult experience is that subtotal colectomy with end ileostomy was favoured in children compared to IRA in adults. Defunctioning the rectum in Crohn's colitis is potentially therapeutic for associated CD within the retained rectum[85], but might predispose to later disuse proctitis. Prospects for restoration of continuity are limited, and the adolescent needs to know that the stoma may be permanent and that half of all patients will eventually come to proctectomy[86].

Disease recurrence

Recurrence following surgery in paediatric CD has received little attention in the paediatric surgical literature since transition to adult care has usually taken place before repeat surgery is required. A small paediatric series comprising 82 children undergoing surgery for CD concluded that early recurrence was associated with extensive colonic disease, long duration of symptoms (> 1 year) before surgery, and failure of medical therapy as underlying reason for surgery[72]. Another paediatric series found significantly earlier recurrence in children with colonic rather than

ileocaecal disease. The same report also showed that high PCDAI scores were correlated with a shorter remission periods[87]. In a study of 1936 adult patients, surgical re-intervention was required in 25%-35% of all patients at 5 years and 40%-70% at 15 years[88]. The only patient-related factor consistently associated with early recurrence is smoking[89]. Studies analysing the influence of disease location on recurrence have shown no particular correlation, but the perforating/fistulating phenotype increases both clinical and surgical recurrence[89]. Studies looking at the influence of resection margins on disease recurrence have provided conflicting results. Most surgeons would advocate conservative resection to achieve margins free of macroscopic disease, with support from a randomized controlled trial[90]. There remains controversy surrounding the effect of anastomotic configuration (side to side *vs* end to end) on disease recurrence. The most recent meta-analysis suggested that side to side anastomosis was associated with reduced rate of recurrence[91], while an earlier meta-analysis and a randomized controlled trial failed to demonstrate any difference[92,93]. Recommendations on postoperative drug prophylaxis to prevent recurrence are lacking[89], but given the efficacy of thiopurine therapy in maintaining remission[94], there is a broad consensus in favour of maintaining this treatment in children after resectional surgery.

Anastomotic technique

The meta-analysis conducted by Simillis *et al*[92] concluded that side to side configuration was associated with fewer postoperative complications. The Cochrane review of ileocolic anastomoses concluded that stapled anastomosis were associated with fewer anastomotic leaks than handsewn, although subgroup analysis did not achieve significance in non-cancer patients[95]. Individual large volume cohort studies comparing stapled and hand sewn anastomoses in the treatment of CD have suggested a significant reduction in both anastomotic leak rate[96], and requirement for reoperation for anastomotic recurrence[97]. Lack of cross fertilization between adult and paediatric practice has undoubtedly been a factor in late adoption of stapling approaches to various anastomoses in paediatric IBD practice. Appropriate mentoring is essential in the adoption of any new technique, since use of an unfamiliar technique is associated with a higher incidence of complications including anastomotic failure[98].

Pre-operative optimisation

Risk factors associated with postoperative sepsis include poor nutritional status (where albumin < 30 g/L is a useful proxy), presence of abscess or fistula, preoperative steroids[99,100], and recurrent clinical exacerbations of CD[100]. Since the risk of a septic complication is additive with each of these risk factors[99], foreknowledge should prompt consideration of a temporary stoma rather than an anastomosis[100]. These considerations have fuelled the debate on preoperative optimization in patients with CD. To date there is little strong evidence supporting delaying surgery to allow for a period of pre-operative hyperalimentation by either enteral or parenteral routes[101]. However medical management of sepsis and percutaneous drainage of intra-abdominal abscesses may reduce post-operative septic complications[101].

Strictureplasty

Repeated resectional surgery in CD is clearly associated with a risk of short bowel syndrome in adulthood. For this reason strictureplasty has become an established surgical approach with proven efficacy and safety in adult practice[102]. A meta-analysis (1112 patients) identified septic complications in 4% and a 5 year recurrence rate of 28%[103]. As has been seen elsewhere paediatric surgeons have been slow to adopt strictureplasty into their operative repertoire. A comparative study of strictureplasty (19), resection (13), and combined (8) procedures in children demonstrated that strictureplasty was associated with a significantly earlier recurrence rate[104]. However another paediatric group have successfully used strictureplasty in long segment stenosis without complication and with good symptom control[105].

Balloon dilatation for Crohn's strictures

The evidence for balloon dilatation of strictures in Crohn's Disease is limited to the adult literature only. One third of patients diagnosed with CD develop strictures within 10 years of diagnosis[106]. Dilatation is usually attempted to a diameter of 18-25 mm in gradual increments[107]. Complications include bleeding and perforation. Short term success rates are 86%-94%[107,108], which may be enhanced when used in conjunction with oral corticosteroids[109], or intralesional steroid injection[107]. Intervention-free success rates decline over time, with relapse rates quoted as 46% after a mean of 32 months. However one third of patients require no further treatment ten years after their first dilatation[107].

SPECIAL SITES

PACD

The management of PACD continues to pose a significant challenge to both gastroenterologist and surgeon, despite significant advances in understanding of the epidemiology and natural history, occurring alongside the development of new treatment modalities. As in other areas of paediatric IBD practice, there is a distinct lack of good quality evidence on which to base therapeutic strategy, and reliance is therefore placed on extrapolation from the adult

literature, and from expert consensus.

The incidence of symptomatic PACD at presentation in a cohort of 145 children with CD in the Northwest of England was 25 (17%). The majority (80%) required some form of surgical intervention for their PACD. Paediatric studies suggest a wide range of incidence from 15% at initial presentation[110], to 62% over the course of their disease[111]. This wide disparity may reflect a degree of inattention (from either patient or doctor) to mild PACD in the setting of more debilitating disease at other sites, or reporting bias depending on medical or surgical authorship. PACD represents a spectrum of pathologies which can follow a relatively benign course, through to a locally aggressive process relentlessly progressing towards proctectomy. The presence of PACD has been associated with young age at presentation, ileal disease distribution and defective expression of neutrophil cytosolic factor (NCF4), again linking pathogenesis with defective invasive bacterial defensive mechanisms[112].

The most commonly used descriptive assessment of PACD is the Cardiff classification which reflects the severity of three components of the disease spectrum; ulceration, abscess/fistula and anal stricture. Additional information is given concerning associated anal disease (A), proximal intestinal CD location (P), and inflammatory activity in the perianal disease (D)[113]. The Parks classification can only be applied to fistulating PACD and is a precise anatomical description of the fistula track in relation to the sphincter complex (superficial, intersphincteric, transphincteric, suprasphincteric and extrasphincteric), remembering that there may be multiple separate fistulae to describe in this way[114]. More recently an attempt has been made to simplify the classification of fistulae into simple and complex disease based on anatomy of the fistula, and the presence or absence of abscesses, strictures, and of significant rectal disease[115]. The perianal CD activity index, abbreviated PACDAI for the purposes of this article to distinguish it from the paediatric CD activity index (PCDAI), enables quantitative assessment of treatment efficacy[116], and has largely superseded the earlier classification of Irvine[117]. The PACDAI has no quality of life component, unlike Irvine's score in which two of the five categories reflect interference with social and sexual activity. Both scoring systems were derived for adult patients, but since the former has as its focus the pathology of PACD, it can be applied to paediatric practice. As with many medical scoring systems, the PACDAI assigns a weighted numeric value to each component of the clinical picture assuming that the derived cumulative total has a value in quantifying disease severity. The result is a cumbersome tool whose value is less in the overall score and more in the precise description of each component of PACD. The authors consider the differentiation of PACD into ulcerating, fistulating and stenosing disease (after Cardiff) to have prognostic significance in terms of the likelihood of proctectomy. Our experience is that ulcerating PACD is often difficult to control, and if progressive can result in the need for proctectomy. Others, like us, have also concluded that stenotic PACD is associated with an unfavourable prognosis[118].

Effective management of PACD requires close collaboration between gastroenterologist and surgeon with appropriate use of imaging techniques. Three essential principles guide management; the prompt identification and drainage of any septic focus, conservative surgery, and appropriate medical therapy. The locally destructive effects of abscess formation and often significant pain both mandate early surgical drainage. The impaired wound healing commonly seen in patients with CD, should make a surgeon think twice about embarking on extensive perianal surgery. Fistulotomy may be undertaken in simple PACD with extra-sphincteric or short low inter-sphicteric fistula tracks in medically well-controlled disease and in the absence of significant rectal disease. However most surgeons favour the use of non-cutting setons to control septic complications from fistulae, especially in complex PACD. Setons are extremely well tolerated and, after drainage of pus, represent the principal adjunctive surgical contribution to medical management. Antibiotics (metronidazole and ciprofloxacillin) are effective in controlling acute exacerbations of PACD[119], and together with maintenance immunosuppression using thiopurines (azathioprine or mercaptopurine) comprise the first line of medical management. Steroids have no place in the treatment of PACD[120].

Since its introduction to the medical armamentarium against CD in 1999[121], the chimeric anti-TNF monoclonal antibody infliximab has provided an effective second line of medical management in recalcitrant Crohn's perianal fistulae. Paediatric studies have confirmed its efficacy in the management of PACD[122,123]. Before embarking upon such biological therapy, the exclusion of occult sepsis should be mandatory and pelvic MRI should be undertaken, both for this purpose, and to document the extent of PACD at the start of therapy[124]. Early optimism associated with infliximab has persisted into more recent paediatric reports of control of fistulating disease in 76% at 1 year[123], and 56% at 2 years[125]. This experience has translated into some advocating infliximab as first line therapy in children with fistulating PACD rather than the "step up" approach outlined above[126]. Whilst expert opinion is tending to favour combined use of setons and infliximab in the treatment of complex Crohn's perianal fistulae[127], there is no consensus regarding timing of removal of the seton and subsequent duration of biological therapy, although there is general agreement that full therapy should continue for a year[94]. Some authors are reluctant to recommend discontinuation of infliximab therapy because of the high relapse

rate of fistulating PACD[128]. Similarly, others prefer to emphasize tolerability and efficacy of a long-term indwelling seton in control of complex PACD[129].

Appropriate evaluation of PACD should include recent anatomical localization of active disease burden by endoscopy and contrast-, or MR-enteroclysis. Pelvic MRI should be undertaken prior to EUA and rectosigmoidoscopy, for reasons identified above and also as a guide to informed surgical consent. Abscesses pointing to the perianal skin should be drained externally. Pelvic abscesses can be effectively drained using the trans-rectal route in the hope of avoiding formation of an iatrogenic extra-sphincteric fistula. Horse-shoe abscesses represent a particular challenge and may be drained *via* a trans-anorectal route. If control cannot be achieved by effective internal drainage and intensive medical management including antibiotics, thiopurine immunosuppressive maintenance, and infliximab, then a defunctioning stoma accompanied by laying open of the post anal space together with seton insertion will be required to achieve control of sepsis and hopefully avoid proctectomy. More complex fistulating disease involving the vagina or urinary tract is rarely seen in paediatric practice, and will usually require a defunctioning stoma in the first instance. In this circumstance fistula eradication is only going to be successful if there is no significant inflammatory involvement of the anorectum apart from that associated with the fistula itself. In the absence of significant proctitis, rectal mucosal advancement flaps[130], and interposition techniques such as graciloplasty[131] have all been employed with limited success.

A defunctioning stoma may provide significant temporary relief in advanced ulcerating, fistulating or stenotic PACD (usually seen in advanced Crohn's proctitis), but healing of perianal disease, if it occurs to any meaningful extent, is usually reversed on restoration of continuity. This approach often only delays eventual proctectomy, which is required within 8 years in 50% of patients with locally aggressive PACD[132].

Proctectomy is not the end of the surgical challenge in this situation, since healing of the resultant perineal wound is often the exception rather than the rule. The use of topical negative pressure dressings to control sepsis, and, where necessary, delayed application of transposition muscle/myocutaneous flaps, can result in an acceptable cosmetic result.

In recent years novel local treatments directed at the fistula track in PACD have included the use of the CO_2 laser[133], fibrin glues[134], and fistula plugs[135]. Local injection of infliximab into the fistula track[136], and the delivery of adipose tissue-derived stem cells either by direct injection[137] or by incorporating them into fistula plugs are current research developments that are showing some promise.

Upper GI CD

Upper GI endoscopy is mandatory in the investigation of CD since inflammatory changes have been demonstrated in up to 40% of children[138]. The incidence of gastroduodenal CD was only 10% in a group of 196 children in whom gastroduodenoscopy was solely performed in children with suggestive symptoms. Most cases with upper GI involvement are therefore asymptomatic with respect to the gastroduodenal inflammatory changes[139]. Endoscopic and histological findings are variable and often non-specific[140]. Medical treatment is rarely directed at the gastroduodenal disease alone since symptomatic disease is usually present distally. If treatment is deemed necessary then proton-pump inhibitors should be included with standard first line treatment[140]. There is no body of literature describing surgical intervention in childhood. The benign outlook in children is not reflected in the adult literature which probably reflects the fact that the huge adult caseload allows rare cases of clinically significant macroscopic gastroduodenal disease to come to the fore. Yamamoto *et al*[141] describe 54 patients with gastroduodenal disease in whom 33 required surgery mainly for gastric outlet obstruction (16 bypass, 10 strictureplasty and 4 gastrectomy). One third required re-operation for recurrent obstruction or stomal ulceration.

NEW DEVELOPMENTS IN SURGERY IN CHILDREN WITH IBD

The low case volume in paediatric IBD surgery has led to this subspecialty service being late adapters of technological and other advances coming out of adult colorectal practice as has been argued frequently in this article. The benefits of collaborative practice may seem self-evident, but take considerable effort to accrue, given the artificial separation of adult and paediatric medicine. In concluding this review we consider the potential benefits to paediatric practice of accelerated recovery programmes which are now commonplace in adult units, and mechanisms that ensure continuity of care as adolescents with IBD reach the interface between paediatric and adult services.

ENHANCED RECOVERY AFTER SURGERY

The recent widespread adoption of fast-track protocols in adult colorectal surgery has led to reduction in length of hospital stay with concomitant cost-reduction[142-145]. Enhanced recovery after surgery (ERAS) protocols focus on drawing together preoperative, intraoperative and postoperative considerations, which might enhance recovery, into a cohesive care package designed to effect significant gains by delivering

multiple small increments of improved care. Studies of ERAS application in major colonic resections predominantly for inflammatory bowel disease have demonstrated either comparable[146], or reduced morbidity[147]. These benefits remain to be confirmed in children, but a comparative study of "conventional management" of children undergoing resectional surgery for inflammatory bowel disease, and of young adults (less than 25 years old) undergoing matched procedures according to an ERAS protocol, has demonstrated a significant reduction in hospital stay in the ERAS group without increase in morbidity[148]. This matched cohort study lacks the power of a RCT, but strongly suggests that the gains obtained from accelerated recovery programmes can be translated into paediatric IBD surgical practice.

Laparoscopy is considered to be an area in which gains might be achieved in earlier recovery, reduced pain and improved cosmesis, and is an integral part of many ERAS protocols. Demonstrating significant benefit for laparoscopy in any area other than cosmesis is highly controversial. This however does not detract from its potential value within a highly structured care package.

TRANSITIONAL CARE

The unique problems that arise in children with IBD, and the models of delivery of care in children's services probably both conspire to delay referral into the adult sector. However, a recent United Kingdom study of paediatric IBD services suggested that transitional care arrangements were well established in most tertiary centres, and indeed that shared care with adult surgeons was a feature of practice, especially in relation to ileoanal pouch surgery. This is probably explained both by the lack of exposure to IBD surgery in the training programmes of United Kingdom paediatric surgeons, and by the small case load even in the larger centres[15].

The fact that most children requiring colectomy and IPAA for UC are approaching the age at which transition into adult care is feasible, strengthens the argument for adult surgical involvement, since the ethics of undertaking surgery with a high complication rate and then transferring responsibility for follow up within a short time frame is certainly open to question. Furthermore case volume considerations, and the allocation of research funding and scarce health care resources, maximise the options available for the young patient with IBD to access the newest treatment when shared care is available.

Consensus guidelines issued jointly by the European Crohn's and Colitis Organization and the ESPGHAN[149], state that every adolescent should be included in a transitional care programme which is adapted to fit local paediatric and adult health care models, and that within the paediatric setting adolescents should be encouraged to take increasing responsibility for their treatment and visit the clinic at least once without their parents. Endoscopic interventions in children with IBD are performed under general anaesthesia and are largely diagnostic. Adult endoscopy is performed almost exclusively under sedation and is performed more frequently given the increasing emphasis on surveillance with duration of disease. Other details of care which enhance the perception that paediatric health care delivery is more child-friendly, include the emphasis on nutrition, growth, puberty and psychological well-being, including integrating health care into the educational needs of the child. These factors are therefore potential barriers to timely transition, which need to be overcome to ensure that each adolescent can access the full range of treatment options.

There are no research studies with confirm the suggested benefits of well-organized transition services in IBD[150]. However it is likely that the perceived benefits of structured transition programmes seen in other chronic paediatric conditions will translate into the setting of adolescent IBD[151].

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REVIEW

Contemporary concepts of the medical therapy of portal hypertension under liver cirrhosis

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Abstract

Severe complications of liver cirrhosis are mostly related to portal hypertension. At the base of the pathogenesis of portal hypertension is the increase in hepatic vascular resistance to portal blood flow with subsequent development of hyperdynamic circulation, which, despite of the formation of collateral circulation, promotes progression of portal hypertension. An important role in its pathogenesis is played by the rearrangement of vascular bed and angiogenesis. As a result, strategic directions of the therapy of portal hypertension under liver cirrhosis include selectively decreasing hepatic vascular resistance with preserving or increasing portal blood flow, and correcting hyperdynamic circulation and pathological angiogenesis, while striving to reduce the hepatic venous pressure gradient to less than 12 mmHg or 20% of the baseline. Over the last years, substantial progress in understanding the pathophysiological mechanisms of hemodynamic disorders under liver cirrhosis has resulted in the development of new drugs for their correction. Although the majority of them have so far been investigated only in animal experiments, as well as at the molecular and cellular level, it might be expected that the introduction of the new methods in clinical practice will increase the efficacy of the conservative approach to the prophylaxis and treatment of portal hypertension complications. The purpose of the review is to describe the known methods of portal hypertension pharmacotherapy and discuss the drugs that may affect the basic pathogenetic mechanisms of its development.

Key words: Liver cirrhosis; Portal hypertension; Pathogenesis; Medical therapy

Core tip: The purpose of the review is to describe the known methods of portal hypertension pharmacotherapy and discuss the drugs that may affect the basic pathogenetic mechanisms of its development.

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INTRODUCTION

Severe complications of liver cirrhosis are mostly related to portal hypertension. At the base of the pathogenesis of portal hypertension is the increase in hepatic vascular resistance to portal blood flow. It is now established that the reason for this, in addition to gross structural changes in the liver due to diffuse fibrosis and the formation of nodules of regenerating hepatocytes, remodeling and capillarization of the hepatic sinusoids, is endothelial dysfunction and the disorder of paracrine interactions between damaged hepatocytes, sinusoidal endothelial cells (SEC), Kupffer cells and activated hepatic stellate cells (HSC) of the liver[1]. Further development of splanchnic hyperemia, the formation of collateral circulation and established hyperdynamic circulation, as a result of complex processes of angiogenesis, vascular remodeling and endothelial dysfunction, contributes to the progression of portal hypertension[2]. It is obvious that the aim of pharmacotherapy should be to correct these disturbances, while striving to reduce the hepatic venous pressure gradient (HVPG) to less than 12 mmHg or 20% of the baseline. In addition, preventing arterial hypotension, it is necessary to reduce the inflow of splanchnic blood to the portal vein, while maintaining portal circulation, which participates in hepatic perfusion[3].

Dissatisfaction with the existing methods of pharmacotherapy, as well as advances in understanding the pathogenesis of portal hypertension under liver cirrhosis, make finding effective drugs for the prevention and treatment of its complications a crucial task.

The purpose of the review is to describe the known methods of portal hypertension pharmacotherapy and discuss the drugs that may affect the basic pathogenetic mechanisms of its development.

CURRENT PHARMACOTHERAPY OF PORTAL HYPERTENSION

Most drugs used in clinical practice against portal hypertension are splanchnic vasoconstrictors, whose effect is based on reducing splanchnic blood flow and hyperdynamic circulation.

Vasopressin derivatives

Terlipressin (N-triglycer-8-lysine-vasopressin) is a synthetic analogue of vasopressin with a longer biological activity and a better safety profile, administered to liver cirrhosis patients with bleeding from esophageal varices and type Ⅰ hepatorenal syndrome. The drug affects specific V1 receptors of smooth muscles of arteries. Its effects encompass a marked vasoconstriction of the splanchnic circulation, an increase in arterial blood pressure and systemic vascular resistance, and a decrease in cardiac output.

Møller *et al*[4], having studied the influence of terlipressin on the hemodynamics in liver cirrhosis patients with portal hypertension, showed that an intravenous bolus of 2 mg of the drug leads to a fast reduction in portal pressure and hepatic blood flow (17% and 29%, respectively). It also increases blood pressure and systemic vascular resistance (26% and 61%, respectively), and reduced cardiac output, heart rate and elasticity of arteries (18%, 11% and 32%, respectively).

The effect of terlipressin lasts for up to 4 h, which allows administering it in periodic intravenous injection, but, if necessary, continuous infusion is also possible[5].

In case of bleeding from esophageal varices in adults weighing over 40 kg, terlipressin is injected every 4 h, 2 mg in the first 1-2 d and 1 mg for 2-5 following days[6]. Treatment of patients with type Ⅰ hepatorenal syndrome starts with a 0.5-1 mg intravenous bolus every 4-6 h, or a continuous intravenous infusion at 2 mg/d. If the creatinine level is not reduced by more than 25% by the third day, the amount of drug injected by intravenous injection is increased to 2 mg every 4 h or to 12 mg/d with continuous infusion[7].

The most frequent side effects associated with the use of terlipressin are moderate abdominal pain, arterial hypertension, hyponatremia; these generally have reverse development after its cancellation. Severe cardiovascular and ischemic disorders occur in about 15% of patients. In this regard, terlipressin is not recommended for patients with a history of ischemic heart or cerebral disease, limb or gut vascular disease, cardiomyopathy, bronchial asthma, chronic obstructive pulmonary disease, or having cardiac rhythm disturbance; caution should be used for elderly and/or hypertensive subjects[8].

Somatostatin and long-acting somatostatin analogues

Somatostatin is a 14-amino-acid peptide secreted by neural, endocrine, and enteroendocrine cells in the hypothalamus and in the digestive system (in the stomach, intestine, and pancreatic delta cells of the pancreas). Somatostatin and its synthetic analogues (octreotide, vapreotide and others) are used in patients with liver cirrhosis for the treatment of bleeding from esophageal varices. It affects both intra- and extrahepatic mechanisms of portal hypertension.

Somatostatin reduces hepatic vascular resistance by blocking G-protein coupled receptors ET_A, which prevents the contraction of HSC induced by endothelin-1 (ET)-1 and contributes to the expansion of sinusoids. A similar effect of octreotide is associated with a decrease in intracellular Ca^{2+}[9]. With long-term use, the latter also positively influences hepatic fibrogenesis as a result of inhibiting the proliferative activity of HSC, decreasing the expression of the transforming growth factor β_1 (TGF-β_1), α-smooth muscle actin, intracellular

protein Smad4a and the suppression of transcription factors, in particular C-Jun and SP1[10].

The reduction of portal inflow caused by somatostatin is explained by the weakening of splanchnic hyperemia due to somatostatin's antisecretory effect on the secretion of glucagon and other gastrointestinal vasodilating peptides. The positive effect of octreotide on splanchnic blood flow is due to both the potentiation of protein kinase of C-dependent vasoconstrictors through subtype 2 somatostatin receptors and the suppression of mesenteric angiogenesis at an early stage of portal hypertension[11].

In patients with liver cirrhosis and portal hypertension an intravenous bolus of 250 μg of somatostatin contributes to a 28.4% reduction of wedged hepatic venous pressure and a 15%-71% reduction of the pressure in the esophageal varices. Continuous infusion of the drug reduces wedged hepatic venous pressure by 17% and hepatic blood flow by 17.4%. High doses of somatostatin (500 μg/h) have a more pronounced effect on these indicators, also reducing azygal blood flow by 45% in the case of bolus injection and by 23% with continuous infusion. A positive effect of the drugs in this group on the hemodynamics is short, despite the much larger half-life of synthetic analogues of somatostatin, compared with the natural hormone; this is probably due to the desensitization or tachyphylaxis[12].

With bleeding from esophageal varices, 250 μg of somatostatin is initially injected as a bolus, and then in the form of continuous infusions, 250-500 μg/h for 2-5 d. The first dose of octreotide and vapreotide is 50 μg followed by an infusion of 50 μg/h. Severe complications in the course of this therapy are rare. Approximately 21% of patients may have vomiting and hyperglycemia, which, as a rule, can be easily remedied[13].

Nonselective β-adrenergic blockers alone and combined with vasodilators

Nonselective β-adrenergic blockers, which are drugs of choice for the prevention of bleeding from esophageal varices[14], affect several links of the pathogenesis of portal hypertension in cirrhotic patients: (1) blocking β_2-adrenergic vascular receptors, allowing unopposed α_1-adrenergic activity that results in splanchnic vasoconstriction and the reduction of portal inflow; (2) blocking β_1-adrenergic cardiac receptors reduces cardiac output, which improves the hyperdynamic circulation; (3) reduction of azygos blood flow and variceal pressure; and (4) shortening the intestinal transit time which has been related to decreased bacterial overgrowth and thereby reduced risk of bacterial translocation.

The first nonselective β-adrenergic blocker introduced into clinical practice for the treatment of portal hypertension was propranolol. Currently, its impact on portal and systemic hemodynamics is well studied. It is established that it is able to decrease HVPG by 10%-31%, azygal blood flow by 29%-47%, cardiac output by 10%-31%, mean arterial pressure by 0%-14% and hepatic blood flow by 0%-39%[15].

It is recommended to start the propranolol therapy with a dose of 20 mg/d, which can be increased, if necessary[16]. However, one should be careful: Because of the possible negative reaction of systemic hemodynamics, there is a higher risk of severe complications and even deaths not related to variceal bleeding[17]. In addition, it is still unclear whether patients with decompensated liver cirrhosis should take nonselective β-adrenergic blockers. In such patients, despite the increased volume of circulating blood, the effective arterial volume decreases, impairing the perfusion of vital organs, causing azotemia and creating a risk of hepatorenal syndrome[18].

Clinical efficacy of nonselective β-adrenergic blockers against portal hypertension is variable. A number of studies have reported HVPG not decreasing by more than 20%, and the long term weakening of the therapeutic effect was observed in 50%-70% of patients[19]. To improve the results of the treatment, it is possible to combine nonselective β-adrenergic blockers with drugs that reduce hepatic vascular resistance. Some of them are exogenous NO donors, nitrates; in particular, isosorbide-5-mononitrate, even lower doses which (10 mg/d) are able to blunt the postprandial increase in HVPG, not changing the perfusion of the liver[20]. Although monotherapy of portal hypertension with nitrates in cirrhotic patients resistant to nonselective β-adrenergic blockers has been inconclusive, combining the medications proved to be effective[21].

It was assumed that another combination could be the simultaneous use of nonselective β-adrenergic blockers and α_1-adrenergic blocker prazosin; not only can the latter reduce the HVPG, but also improve the perfusion of the liver. Indeed, their combined use has led to a greater reduction in portal pressure than the combination of propranolol with isosorbide-5-mononitrate. However, prazosin's lack of selectivity caused a significant decrease in arterial pressure and systemic vascular resistance, and induced stimulation of endogenous vasoactive systems led to increased plasma volume, the retention of sodium and water. Also, a potential drawback of long-term medication is the development of true tolerance associated with a decrease in the expression of α1-adrenergic receptors in response to arterial hypotension[22].

Carvedilol

In the past decade, there have been a number of reports about the use of the nonselective β-adrenergic blocker carvedilol to treat cirrhotic patients with portal hypertension. This drug has weak anti-α_1-adrenergic activity, which makes its effect similar to the

combination of propranolol and prazosin. It was found that carvedilol (12.5 mg/d) reduces HVPG significantly more than propranolol. Carvedilol is effective in 56% of patients resistant to propranolol, and surpasses it during primary prevention of bleeding from esophageal varices[23]. However, in a systematic review and meta-analysis, Aguilar-Olivos *et al*[24] showed limited evidence suggesting that carvedilol is more effective than propranolol for improving the haemodynamic response in cirrhotic patients with portal hypertension. Moreover, it had no advantages over the combination of nadolol and isosorbide-5-mononitrate used to prevent recurrent bleeding[25]. The most common adverse reaction to carvedilol was arterial hypotension, and in rare cases, due to the delay of sodium and water, ascites and edema occurred[26]. Thus, it is believed that in the absence of contraindications carvedilol may be used for the primary prevention of bleeding from esophageal varices in cirrhotic patients with portal hypertension tolerant to the action of propranolol. Further studies are needed before it enters routine clinical practice.

Antibiotic prophylaxis

Endotoxemia due to the translocation of gram-negative bacteria from the intestine plays an important role in the pathogenesis of complications of portal hypertension under liver cirrhosis, in particular, bleeding from esophageal varices[27]. To prevent early recurrences of such bleeding, all modern guidelines and consensus decisions point to the need for including antibiotic therapy in treatment. It is recommended to introduce norfloxacin orally, 400 mg every 12 h for 7 d, or, in patients with decompensated liver cirrhosis, introduce ceftriaxone intravenously, 1-2 g/d for 7 d[28]. In a recent systematic review and meta-analysis, Chavez-Tapia *et al*[29] showed that the use of antibiotics can significantly reduce overall mortality, the frequency of recurrent bleeding and the duration of hospitalization.

DRUGS POSITIVELY AFFECTING PORTAL HYPERTENSION, WHOSE CLINICAL EFFECTIVENESS IS NOT FULLY PROVEN

Antifibrotic therapy

The object of current research is the search for drugs that affect the basic mechanisms of development of portal hypertension under liver cirrhosis, and, above all, that are able to suppress hepatic fibrogenesis at its early stages. This universal pathophysiological process is a response to damage of various etiologies, resulting in necrosis and apoptosis of hepatocytes, oxidative stress, induction of the inflammatory response by chemokines and cytokines, and recruitment of immune cells. Activated HSC proliferate and migrate into diseased areas of the parenchyma, producing there excessive amounts of extracellular matrix components. Increased production of matrix metalloproteinases in this pathological situation is blocked by the hypersecretion of their tissue inhibitors. Leading regulators of fibrogenesis are TGF-β_1, platelet-derived growth factor (PDGF), connective tissue and fibroblast growth factors[30].

Diffuse fibrosis, the formation of nodules of regenerating hepatocytes, as well as the capillarization of sinusoids impair the delivery of oxygen to the cells of the liver. Hypoxia, developed as a result of the stimulation of hypoxia-inducible factor (HIF) 1α, contributes to the production of angiogenic factors [placental growth factor (PIGF), vascular endothelial growth factor (VEGF), NO, *etc.*] by activated HSC. This leads to the formation of new blood vessels that bypass sinusoids, leading to the progression of the disease[31].

Etiological treatment

There are some publications about the positive impact on portal hypertension under liver cirrhosis of antiviral drugs with antifibrotic properties. Pozzi *et al*[32] described a patient suffering from HBV-related liver cirrhosis (Child-Pugh A), whose HVPG was reduced by 17% as a result of three-month treatment with entecavir.

Correction of the increased hepatic vascular tone

Colmenero *et al*[33] reported that prolonged treatment of patients with chronic hepatitis C with losartan, a type Ⅰ specific antagonist of angiotensin Ⅱ (AT Ⅱ) receptors (50 mg/d for 18 mo) reduces the activity of NADPH-oxidase, the enzyme that generates oxidative stress. It also reduces gene expression of the main glycoprotein of the extracellular matrix of collagen Ⅰ, with a positive effect on fibrogenesis. In a systematic review and meta-analysis, Tandon *et al*[34] noted that reducing hepatic vascular resistance with the antagonists of the renin-angiotensin-aldosterone system (type Ⅰ blockers of AT Ⅱ receptors or inhibitors of the angiotensin-converting enzyme) in patients with compensated liver cirrhosis (Child-Pugh A), leads to a slightly smaller reduction of the HVPG than in the case of treatment with non-selective β-adrenergic blocker (17% and 21%, respectively), without significant side effects. However, in decompensated patients, the activation of the systemic renin-angiotensin-aldosterone system caused arterial hypotension, which aggravated hemodynamic impairment and led to the development of renal failure. Similar results were obtained in a randomized controlled clinical trial involving thirty patients with Child-Pugh B liver cirrhosis with large varices. Losartan, like propranolol, improved portal hypertension, but also adversely affected arterial blood pressure with no statistical difference between the two groups[35].

One of the main causes of endothelial dysfunction

in liver sinusoidal endothelial cells under liver cirrhosis is the deterioration of the bioavailability of the key relaxing factor NO in the hepatic microcirculation. The mechanisms of this phenomenon are diverse. Asymmetrical dimethylarginine, inhibiting the activity of endothelial NO synthase (eNOS), generates peroxynitrite, while reduced expression of tetrahydrobiopterin leads to eNOS producing oxygen instead of NO. The cyclooxygenase (COX) involved in the synthesis of thromboxane A_2 (TXA_2) as well as excessive stimulation of Rho-kinase, inhibit Akt phosphorylation in endothelial cells and significantly inhibit Akt-eNOS signaling. Also, impaired bioavailability of NO may be caused by the weakening of the activity of the superoxide dismutase and increased serum levels of homocysteine due to reduced expression of the enzymes cystathionine-γ-lyase and cystathionine-β-synthase[36].

Statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, in addition to the hypolipidemic properties can improve the endothelial dysfunction of sinusoids[37]. This is due to the blockade of RhoA/Rho-kinase and the activation of the Akt-eNOS signaling, which increases the bioavailability of NO in cirrhotic livers, leads to a decrease in hepatic vascular resistance and reduction of portal pressure without an adverse effect on systemic hemodynamics[38]. In addition, it is possible that the positive effect of statins on portal hypertension is at least partially caused by decreased portal-systemic collateral vascular resistance through NO-mediated vascular hyporesponsiveness to ET-1[39].

In the prospective, randomized, multicenter trial by Abraldes *et al*[40], simvastatin treatment (20-40 mg/d for 1 mo) of cirrhotic patients resulted in an effective reduction of portal pressure, was safe, and improved perfusion and liver function. This suggests the possibility of clinical use of statins for the treatment of portal hypertension in patients with liver cirrhosis, especially in combination with non-selective β-blockers.

Experiments on rats with a model of biliary cirrhosis showed that inhibitors of phosphodiesterase-5, as a result of increased expression of tetrahydrobiopterin, activity of GTP COX-1, protein levels of phospho-Akt, phospho-eNOS and soluble guanylate cyclase, improved the bioavailability of NO in the liver, eliminated endothelial dysfunction, increased sinusoidal flow and reduced hepatic vascular resistance[41]. However, in clinical practice, drugs of this type (sildenafil, tadalafil, vardenafil) did not have a positive impact on portal hypertension in most cirrhotic patients, and the resulting deterioration of systemic hemodynamics contributed to kidney dysfunction[42].

Fiorucci *et al*[43] comprehensively studied the effect of NCX-1000 (or "Urso-NO"), the liver-specific NO donor, on microcirculation in the cirrhotic liver. In an *in vitro* study, the drug increased cGMP synthesis and the level of nitrite/nitrate in the homogenates of the liver, as well as the number of total bile acids and tauroursodeoxycholic acid in the bile. In the model of an isolated portal perfused rat liver it raised the sensitivity to α-adrenergic stimuli, and *in vivo* it reduced portal pressure. The authors suggested that, once in the liver, NCX-1000 is included in the metabolism and stimulates the production of biologically active NO. However, despite the good results of experimental studies, clinical trials of the drug in cirrhotic patients showed only systemic hemodynamic effect without affecting portal hypertension[44].

The selective inhibitor of Rho kinase fasudil can reduce hepatic vascular resistance and HVPG in cirrhotic patients with portal hypertension. However, its effect was accompanied by an expressed arterial hypotension[45].

Theoretically, it is possible to improve the endothelial dysfunction in liver sinusoidal endothelial cells under liver cirrhosis by eliminating the influence of the potent vasoconstrictor ET-1. However, while the nonselective antagonist of endothelial receptors of types ET_A and ET_B bosentan effectively reduced portal pressure in experiments on rats with a liver cirrhosis model[46], its analogue tezosentan (infusion of 3 mg/h during 2-3 h) did not have a considerable effect on clinically significant portal hypertension in patients with liver cirrhosis in a randomized, double-blind, placebo-controlled multicenter study[47].

Correction of hyperdynamic circulation and inhibiting the formation of portosystemic collaterals

Disturbance of organ and systemic hemodynamics and the formation process of portosystemic collaterals under portal hypertension begin with splanchnic vasodilation and neovascularization due to the hypoxia of the small intestine mucosa. In this connection, the goal of comprehensive treatment may be to affect the proinflammatory cytokines, chemokines and angiogenic factors (VEGF, PIGF, PDGF and others) that contribute to the development of these disorders[48].

The orally active multikinase inhibitor sorafenib, used in clinical practice for the treatment of hepatocellular carcinoma, was studied in experiments on rats with models of intra- and extrahepatic portal hypertension. Sorafenib administered orally once a day for 2 wk effectively inhibited VEGF, PDGF, and Raf signaling pathways, and produced several protective effects by inducing an approximately 80% decrease in splanchnic neovascularization and a marked attenuation of hyperdynamic splanchnic and systemic circulations, as well as an 18% decrease in the extent of portosystemic collaterals. In cirrhotic rats, sorafenib treatment also resulted in a 25% reduction in portal pressure, as well as a remarkable improvement in liver damage and intrahepatic fibrosis, inflammation, and angiogenesis. Notably, beneficial effects of sorafenib against tissue damage and inflammation

were also observed in splanchnic organs[49]. It was also found that the positive effect of sorafenib on portal hypertension was more significant when combined with propranolol[50].

Pinter *et al*[51] assessed the effect of sorafenib on portal hypertension in 13 patients with liver cirrhosis and hepatocellular carcinoma (Child-Pugh A and B). The drug was administered in a daily dose of 800 mg twice a day for two weeks. A reduction of the HVPG by over 20% from the baseline was achieved in four patients, with no serious dysfunction of the liver. Despite the positive results, studies on the safety and efficacy of lower doses of the drug in cirrhotic patients with portal hypertension without hepatocellular carcinoma, are not yet available.

The ability to influence extrahepatic mechanisms of portal hypertension pathogenesis was found in some natural compounds with antioxidant activity. It turned out that ascorbic acid and dark chocolate can reduce the postprandial increase in portal pressure, and the green tea made from the leaves of *Camellia sinensis*, decreases the severity of portosystemic collaterals and mesenteric angiogenesis in rats with a liver cirrhosis model[52-54].

Correction of endotoxemia

Portal hypertension is most severe in cirrhotic patients with concomitant manifestations of the systemic inflammatory response syndrome. The associated endotoxemia due to the translocation of gram-negative bacteria from the intestine occurs in approximately 30%-40% of decompensated patients, who are classified in this clinical situation as "critically ill cirrhotics". The endotoxemia is the cause of many complications of portal hypertension; it increases the mortality by a factor of four, which is often due to the spontaneous bacterial peritonitis and hepatorenal syndrome[55]. Stimulating innate immune signals with pathogen-associated molecular patterns (PAMPs), leads to the activation on the cell surface of Toll-like receptors (TLR) that are widely present in the liver. The first to react to the exposure to PAMPs are Kupfer cells, which because of TLR-signaling acquire a proinflammatory phenotype and produce excessive amounts of cytokines, which exacerbates portal hypertension under liver cirrhosis[56]. On the contrary, the high-density lipoprotein administration attenuates liver proinflammatory response, restores liver eNOS activity, and lowers portal pressure in rats with a liver cirrhosis model[57].

Once in the portal circulation, endotoxin enters the systemic circulation through the network of portosystemic collaterals or by-passing the Kupfer cells. Therefore, bacterial translocation in patients with liver cirrhosis leads not only to infectious complications, but also to blood circulation disorders typical for portal hypertension. These are caused by the endotoxin stimulating NO production in the arterial bed, contributing to splanchnic and systemic vasodilation, exacerbating hyperdynamic circulation and increasing portal pressure[58]. In particular, a direct correlation was observed between the level of endotoxemia in cirrhotic patients, the degree of esophageal varices and whether there is bleeding from them[59].

In connection with that, there is some discussion about the practicality of treating portal hypertension with medications normalizing the intestinal flora and preventing its translocation. Indeed, in patients with alcohol-related liver cirrhosis, oral administration of norfloxacin (800 mg/d for 4 wk) reduced the level of serum endotoxin, which contributed to the reduction of portal pressure and improved the hyperdynamic circulation[60]. In addition, its long-term administration (400 mg/d for one year) to decompensated patients prevented the development of hepatorenal syndrome and significantly improved survival rate[61].

Reduced severity of endotoxemia and an 18% decrease in the HVPG was observed in a prospective study including patients with alcohol-related decompensated liver cirrhosis. The decontamination of the intestine in these patients was done with the nonabsorbable antibiotic rifaximin (1200 mg/d for 28 d)[62]. In addition, long-term rifaximin administration in these patients is associated with reduced risk of developing complications of portal hypertension and improved survival[63]. Apart from affecting the intestinal microflora, the positive effect of rifaximin on portal hypertension can be explained by the inhibition of the binding of lipopolysaccharide with TLR4 on the surface of the HSC, which contributes to their inactivation, the breaking of the fibronectin-mediated interaction with the SEC and eventually the suppression of fibrogenesis and angiogenesis in the liver[64].

The therapeutic effect of probiotics under portal hypertension is ambiguous. In particular, the combined probiotic VSL#3, which contains eight different strains (*Bif. breve, Bif. longum, Bif. infantis, L. acidophilus, L. plantarum, L. casei, L. bulgaricus, Streptococcus thermophilus*) can stabilize the intestinal epithelial barrier, reduce the bacterial translocation and systemic endotoxemia. This reduces the production of proinflammatory cytokines and NO, eliminates endothelial dysfunction of mesenteric arteries caused by vascular oxidative stress and inactivates the local renin-angiotensin system[65].

In a pilot study involving 8 patients with compensated liver cirrhosis (Child-Pugh A)[66] and in a randomized, double-blind, placebo-controlled study, including 7 patients with decompensated liver cirrhosis (Child-Pugh B and C)[67], monotherapy with the probiotic VSL#3 at a dose of 3600 billion CFU/d for 2 mo had no significant impact on clinically important portal hypertension.

In a randomized double-blind placebo-controlled trial in parallel groups, including 94 cirrhotic patients having large esophageal varices without history of variceal bleeding, changes in the HVPG were studied after administering propranolol, singly or in

Table 1 Drugs that can affect the portal hypertension, the effect of which was studied in the experiment

Ref.	Drugs	Experimental model	Effects
Zhao *et al*[69]	Diammonium glycyrrhizinate	CCl_4/IPPRLs	Improves the bioavailability of NO in portal triads
Di Pascoli *et al*[70]	Resveratrol	CCl_4	Improves vasodilatory response to acetylcholine, decreases TXA_2 production, increases endothelial NO and reduces hepatic fibrosis
Yang *et al*[71]	Ursodeoxycholic acid	BDL	Suppresses hepatic TXA_2 production and lipid peroxidation. An increase in antioxidative defence leading to the prevention of hepatic fibrosis
Rodríguez-Vilarrupla *et al*[72]	Fenofibrate	CCl_4	Reduces hepatic fibrosis, improves vasodilatory response to acetylcholine, reduces COX-1 expression and TXB_2 production, increases NO bioavailability in SEC
Hsieh *et al*[73]	Aliskiren	BDL	Ameliorates the angiotensin Ⅱ induced intrahepatic vasoconstriction
Luo *et al*[74]	Spironolactone	BDL	Inhibits hepatic fibrosis, ROCK-2 activity and activates NO/PKG pathway
Gao *et al*[75]	Celecoxib	TAA	Inhibits hepatic fibrosis and angiogenesis. The anti-angiogenesis effect associates with the modulation of VEGF/VEGFR-2
Rosado *et al*[76]	Terutroban	CCl_4/BDL	In CCl_4-cirrhotic rats decreases hepatic fibrosis, in BDL-rats enhances eNOS-dependent vasodilatation
Wang *et al*[77]	Rapamycin	BDL	Ameliorates intrahepatic inflammation and fibrosis, improves liver function
Laleman *et al*[78]	NitroflurbiprofenFlurbiprofen	TAA/IPPRLs	Decreases hepatic TXA_2 production and increases intrahepatic nitrate/nitrite level
Yang *et al*[79]	Vitamin E	BDL	Asymmetric dimethylarginine improves hepatic endothelial dysfunction by a vitamin E through an increase of NO bioavailability
Liu *et al*[80]	Blebbistatin	*In vitro*	Inhibits the contraction and accelerates migration of HSC
Verbeke *et al*[81]	Obeticholic acid	BDL	Decreases hepatic vascular resistance by increasing eNOS activity
Steib *et al*[82]	Montelukast	TAA/BDL	Inhibiting the cysteinyl leukotrienes receptors reduces hepatic vascular resistance
Xu *et al*[83]	Salvianolic acid B	DMN/*In vitro*	Reduces HSCs contractility
Fernandez *et al*[84]	Rapamycin+Gleevec	PPVL	Reduces splanchnic neovascularization
Schwabl *et al*[85]	Pioglitazone	BDL/PPVL	Decreases portosystemic shunting
Fallowfield *et al*[86]	Relaxin	CCl_4/BDL/*In vitro*	Down-regulates HSC- myofibroblast contractile filament expression and contractile function
Lin *et al*[87]	Bivanib alaninate	BDL/ IPPRLs/*In vitro*	Suppresses and ameliorates fibrogenic and angiogenic markers in the serum and liver. Inhibits the $TGF\beta_1$-induced HSCs contraction/migration and VEGF-induced SECs angiogenesis

IPPRLs: Isolated portal perfused rat livers; BDL: Bile-duct ligated rats; TAA: Thiacetamide-induced cirrhosis in rats; DMN: Dimethylnitrosamine-induced cirrhosis in rats; PPVL: Partial portal vein ligation in rats.

combination with either VSL#3 (900 billion CFU/d) or norfloxacin (400 mg/d). The treatment was carried out for 2 mo. The initial propranolol dose of 40 mg/d was increased by 20-40 mg every two days (to the maximum of 320 mg/d) until the heart rate of 55 BPM or the occurrence of side effects. It turned out that the combination of propranolol with the probiotic or the antibiotic was more effective in reducing portal pressure than the exclusive use of a nonselective β-adrenergic blocker (by 19%, 18% and 11%, respectively), was safe and well tolerated by patients[68].

Apart from the mentioned drugs, there are some others whose action on portal hypertension was studied only in experiments (Table 1) and clinical trials are necessary for their final assessment.

In conclusion, it should be noted that dissatisfaction with the current methods of pharmacotherapy of portal hypertension and advances in the study of its pathogenesis under liver cirrhosis have contributed to the search for effective drugs aimed at the prevention and treatment of the complications characteristic of this syndrome. It can be expected that the practical implementation of the new methods will lead to some progress in solving this problem.

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REVIEW

New targeted therapies in pancreatic cancer

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Abstract

Patients with pancreatic cancer have a poor prognosis with a median survival of 4-6 mo and a 5-year survival of less than 5%. Despite therapy with gemcitabine, patient survival does not exceed 6 mo, likely due to natural resistance to gemcitabine. Therefore, it is hoped that more favorable results can be obtained by using guided immunotherapy against molecular targets. This review summarizes the new leading targeted therapies in pancreatic cancers, focusing on passive and specific immunotherapies. Passive immunotherapy may have a role for treatment in combination with radiochemotherapy, which otherwise destroys the immune system along with tumor cells. It includes mainly therapies targeting against kinases, including epidermal growth factor receptor, Ras/Raf/mitogen-activated protein kinase cascade, human epidermal growth factor receptor 2, insulin growth factor-1 receptor, phosphoinositide 3-kinase/Akt/mTOR and hepatocyte growth factor receptor. Therapies against DNA repair genes, histone deacetylases, microRNA, and pancreatic tumor tissue stromal elements (stromal extracellular matric and stromal pathways) are also discussed. Specific immunotherapies, such as vaccines (whole cell recombinant, peptide, and dendritic cell vaccines), adoptive cell therapy and immunotherapy targeting tumor stem cells, have the role of activating antitumor immune responses. In the future, treatments will likely include personalized medicine, tailored for numerous molecular therapeutic targets of multiple pathogenetic pathways.

Key words: Immunotherapy; Pancreas neoplasm; Vaccines

Core tip: Adjuvant therapy in pancreatic cancer has limited efficiency, and low survival rates are related to resistance to gemcitabine. New targeted therapies, such as passive immunotherapy, may have a role in combination with radiochemotherapy by targeting various protein kinases, as well as specific immunotherapies, such as vaccines, adoptive cell therapy and immunotherapy targeting tumor stem cells. In the future, treatments will likely include personalized medicine, tailored for numerous molecular therapeutic targets of multiple pathogenetic pathways.

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INTRODUCTION

Patients with pancreatic cancer (PC) have a poor prognosis with a median survival of 4-6 mo and a < 5% five-year survival rate[1]. Over 80% of patients have advanced disease at presentation (metastatis or invasion of the superior mesenteric artery or celiac trunk in case of locally advanced tumors), which does not allow for surgical resection of the tumor[2]. Even if resection can be achieved, the median survival is still only 18 mo[3]. Despite therapy with gemcitabine (GEM), which represents the first-line therapy for advanced tumors, patient survival typically does not exceed 6 mo for metastatic disease and 9-12 mo for locally advanced disease, likely due to natural resistance to GEM[4,5]. FOLFIRINOX represents an alternative to gemcitabine in first line settings, with better survival, but it is suitable only for good performance status patients. As second line treatment, GEM-platinum-based combination provide the best results[6].Therefore, it is hoped that more favorable results can be obtained by using passive and specific immunotherapies against molecular targets.

PASSIVE IMMUNOTHERAPY

Passive immunotherapy involves *in vivo* infusion of monoclonal antibodies or *in vitro*-activated T cells. Monoclonal antibodies have been created to act on molecules at the cell surface of the tumor and on stromal tissue in connection with PC oncogenesis, tumor growth, and chemotherapy-resistant or immune-response regulation. Currently developed therapies target pre-transcriptional kinases, post-transcriptional level (DNA repair genes, histone deacetylases, microRNAs), antipancreatic tumor tissue stromal elements and antiangiogenic factors (Figure 1).

Anti-kinase therapies

Tyrosine kinases are important in the proliferation, migration, invasion, and resistance to apoptosis of tumor cells, and involve activation of mitogen-activated protein kinase (MAPK; which is responsible for the malignant transformation of pancreatic cells[7]), phosphoinositide 3-kinase (PI3K; which stimulates cell proliferation and chemotherapy resistance[8]), and protein kinase B [Akt; the overexpression of which promotes invasion and expression of insulin growth factor receptor (IGF-1R)[9,10]]. In addition, K-ras is involved in the pathogenesis of PC *via* tyrosine kinase pathways[11,12]. The expression of two tyrosine kinase receptors, epidermal growth factor receptors (EGFRs) B-1 and B-2, has been found in 90% and 21% of PCs, respectively[13,14]. Increased coexpression of EGFR and its ligand in PC is associated with greater liver metastasis and poorer prognosis[15-17].

Anti-EGFR: Therapies involving anti-EGFR (epidermal growth factor receptor or HER1) monoclonal antibodies include cetuximab, a chimeric IgG1-type, and panitumumab, a humanized IgG2-type antibody. These antibodies reversibly inhibit the tyrosine kinase domain of EGFR by competitive binding of ATP. As a result of antibody binding, the receptor internalizes, complement-mediated cytotoxicity appears, and cell division is stopped. However, the anti-EGFR mechanism may not be effective if there are mutations in the *KRAS* gene. Cetuximab seems to be more effective than panitumumab, as IgG1 receptors are more effective than IgG2[18]. However, its efficiency was not proved in clinical trials (Table 1).

Erlotinib is a small inhibitor of EGFR that increases survival by two weeks *vs* GEM monotherapy[28,52]. However, resistance to erlotinib after an initial response can occur due to EGFR mutations, compensation through hepatocyte growth factor receptor (c-Met), human epidermal growth factor receptor (HER2) or K-ras amplification, EGFR-mediated pathway impairment, and histologic transformation with the addition of a mesenchymal component[53]. Combined with GEM or capecitabine, erlotinib can increase survival approximately one month over conventional monotherapy[54,55], proving its positive role in overall survival and progression disease free[28] Long survival was proved in association with radiotherapy and capecitabine, followed by association with GEM[26]. The dose escalated to rash does not improve the survival rate in gemcitabine refractory patients[56]. As second-line therapy, the erlotinib based-therapy failed to show significant improvement in overall survival compared to other regimens[6]. A phase Ⅲ study found that the wild-type *KRAS* genotype is associated with an improved overall survival (OS) in erlotinib-treated PC[57], but it is more of a prognostic than a predictive factor[58]. Other drugs in this class, such as gefitinib, have not been shown to be effective in PC[59]. Lapatinib caused reduction of cell growth and proliferation, but it has only been tested in PC cell lines[60]. Vatalanib is an oral poly-tyrosine kinase inhibitor with strong affinity for platelet-derived growth factor and vascular endothelial growth factor (VEGF) receptors (VEGFRs). In metastatic disease it provided limited survival gain compared to historic controls[61].

Anti-HER2: Trastuzumab, a humanized direct antibody against HER2 (human epidermal growth factor 2) kinase, was used in combination with GEM, but there was no survival benefit in phase Ⅱ studies[29,30]. As the presence of HER2 is relatively low in PC specimens[62,63], anti-HER2 and anti-EGFR therapies can be combined, producing a synergistic effect in animal models that is independent of EGFR density[64]. The mechanism of this combined action

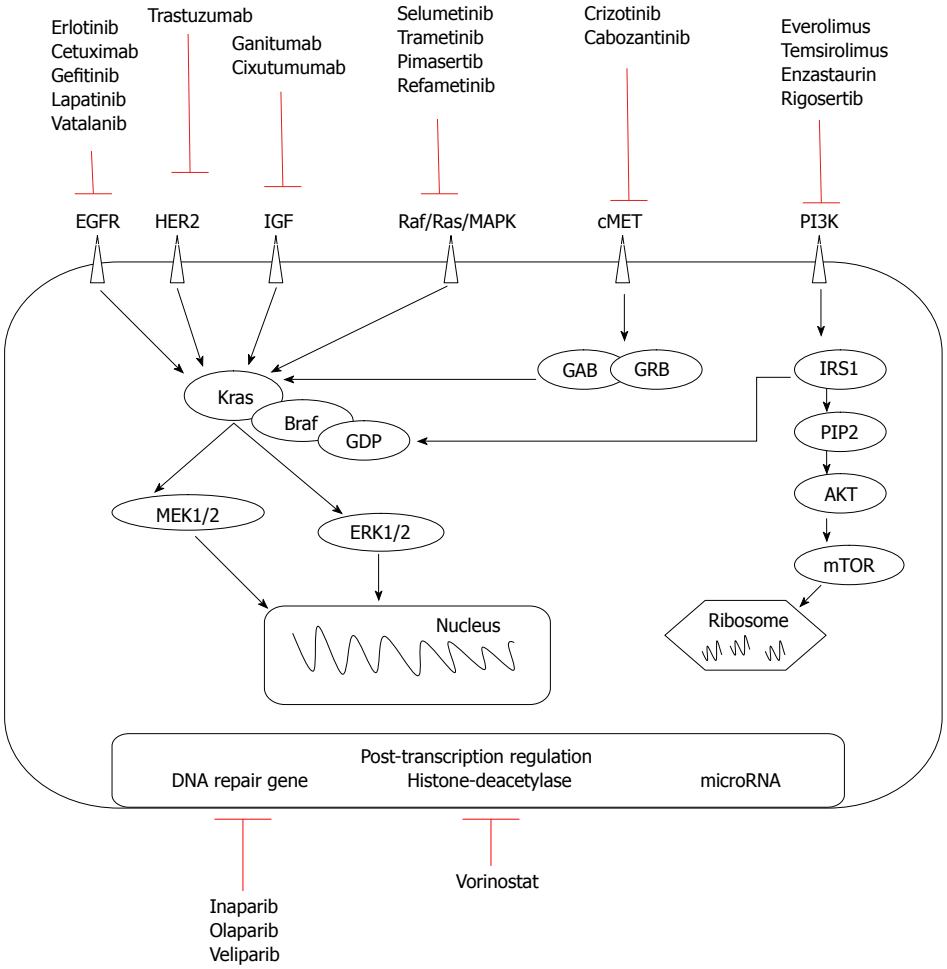

Figure 1 New targeted therapy at the cell surface of the tumor.

is based either on decreased Akt phosphorylation or on disturbance of EGFR/HER2 heterodimerization[65]. The same mechanism of action occurs with vitamin E isoforms, such as tocotrienols, which inhibit cell proliferation and cell survival in studies on PC cell lines[66].

Anti-MAPK: Inhibitors of the Ras/Raf/MAPK cascade, which represents the effect of K-ras activation, are being tested in clinical trials. In GEM failure therapy, selumetinib had the same efficacy as capecitabine[31], though it seems promising in association with erlotinib[67]. Trametinib inhibits the proliferation of PC cell lines with increased efficiency if EGFR/HER2 inhibitors are added, likely because inhibition of the MAPK pathway leads to activation of the tyrosine kinase pathway through feedback mechanisms[68]. Trials with trametinib and other MAPK cascade inhibitors (pimasertib ClinicalTrial.gov NCT01668017, NCT01390818 and refametinib ClinicalTrial.gov NCT01764828, NCT01392521) are still ongoing.

Anti-IGF-1R: IGF-1R is potentially a predictive marker of resectability in PC. A phase Ⅱ study for treatment of metastatic PC with monoclonal antibodies against IGF-1R showed that ganitumab resulted in a 10-mo survival benefit[34].However, a phase Ⅲ study showed no survival improvement[33]. Experimental studies that have associated anti-EGFR therapy with anti-IGF-1R monoclonal antibodies have shown promising results[69], but addition of cixutumumab to erlotinib and GEM did not lead to longer survival in metastatic PC[24].

Anti-c-Met: c-Met and its ligand are overexpressed in PC, but are not sufficient for tumorigenesis in the absence of other pro-oncogenes. Crizotinib is an inhibitor of c-Met that has a role in reducing tumor progression and metastasis, showing efficacy in stimulating apoptosis in combination with GEM[70-73]. Cabozantinib is another inhibitor of c-Met and tumor stem cell markers. Treatment in association with IGF-1R inhibitors may represent a future therapy[74].

Anti-PI3K/Akt/mTOR: The PI3K/Akt/mTOR pathway is one of the major signaling pathways mediating the effect of K-ras. Akt stimulates the phosphorylation of mTOR kinase *via* activation of cyclin D1 and VEGF. mTOR inhibitors, such as everolimus and temsirolimus, have been tested in a phase Ⅱ trial in patients with GEM-refractory PC, but with negative results[75,76].

Table 1 Results of different studies concerning new targeted therapy

Ref.	Patients no./disease stage	Study type	Drugs	OS	PFS	Benefit
Burtness *et al*[19], 2014	87/metastatic	II RCT	Docetaxel + Irinotecan ± Cetuximab	6.5 *vs* 5.4	3.9 *vs* 4.5	Negative
Fensterer *et al*[20], 2014	73/resected	II	GEM + Cetuximab	22.4	NA	Negative
Philip *et al*[21], 2010	743/locally advanced or metastatic	III RCT	GEM ± Cetuximab	5.9 *vs* 6.3	3 *vs* 3.5	Negative
Munter *et al*[22], 2008	66/locally advanced	II RCT	RT + GEM ± Cetuximab	15	-	Negative
Lim *et al*[23], 2014	127/locally advanced	Retrospective	GEM + Capecitabine *vs* GEM + Erlotinib *vs* GEM	21 *vs* 12 *vs* 15	8.9 *vs* 5.2 *vs* 3.9	Negative for Erlotinib
Philip *et al*[24], 2014	10/metastatic	I RCT	GEM + Erlotinib + Cixutumumab *vs* GEM + Erlotinib	7 *vs* 6.7	3.6 *vs* 3.6	Negative
Watkins *et al*[25], 2014	44/advanced	II	GEM + Capecitabine + Erlotinib + Bevacizumab	12.6	8.4	
Herman *et al*[26], 2013	48/metastatic	II	Capecitabine + Erlotinib + RT followed by GEM + Erlotinib	24.4	15.6	
Feliu *et al*[27], 2011	42/advanced	II RCT	GEM + Erlotinib	8	5	Negative
Moore *et al*[28], 2007	569/advanced	III RCT	Gem + Erlotinib *vs* GEM	6.2 *vs* 5.9	3.7 *vs* 3.5	Positive
Harder *et al*[29], 2012	17/metastatic HER2+	II	Capecitabine + Trastuzumab	6.9	12.5	Negative
Safran *et al*[30], 2004	34/metastatic	II	Gemcitabine + Trastuzumab	7		Negative
Bodoky *et al*[31], 2012	70/advanced	II	Capecitabine *vs* Selumetinib	5 *vs* 5.4	88% *vs* 84%	Negative
Infante *et al*[32], 2014	160/metastatic	II RCT	GEM + Trametinib *vs* GEM	8.4 *vs* 6.7	-	Negative
Fuchs *et al*[33], 2015	322/metastatic	III RCT	GEM + Ganitumab *vs* GEM	7.2 *vs* 7	3.7 *vs* 3.6	Negative
McCaffery *et al*[34], 2013	84/metastatic	II RCT	GEM+Ganitumab *vs* GEM	16 *vs* 5.9		Positive
Kindler *et al*[35], 2012	125/metastatic	II RCT	GEM + Ganitumab *vs* GEM + Conatumumab *vs* GEM	8.7 *vs* 7.5 *vs* 5.9	5.1 *vs* 4 *vs* 2	Positive
Bramhall *et al*[36], 2002	239/advanced	RCT	GEM + Marimastat *vs* GEM	165.5 d	92.5 d	Negative
De Jesus-Acosta *et al*[37], 2014	17/metastatic second line therapy	I	GEM+ inhibitor γ secretase	4	1.5	Positive
Goldstein *et al*[38], 2015	861/metastatic	III RCT	GEM + Nab-paclitaxel *vs* GEM	8.7 *vs* 6.6	-	Positive
Hosein *et al*[39], 2013	19/advanced second line therapy	II	GEM + Nab-paclitaxel	7.3	-	Positive
Pant *et al*[40], 2014	30/advanced locally	II	GEM + Capecitabine Bevacizumab	10.4		Negative
Kindler *et al*[41], 2010	535/advanced	III RCT	GEM + Bevacizumab *vs* GEM	5.8 *vs* 5.9	3.8 *vs* 2.9	Negative
Crane *et al*[42], 2009	82/advanced	II	RT + capecitabine+bevacizumab, followed by GEM + bevacizumab	11.9		Negative
Ko *et al*[43], 2010	36/metastatic GEM refractory	II	Bevacizumab + Erlotinib	102 d		Negative
Van Cutsem *et al*[44], 2009	607/metastatic	III RCT	GEM + erlotinib + bevacizumab *vs* GEM + erlotinib	7.1 *vs* 6	4.6 *vs* 3.6	Negative
IokaT *et al*[45], 2015	632/advanced	III RCT	GEM + axitinib *vs* GEM	5.1 *vs* 5.4	-	Negative
Spano *et al*[46], 2008	103/advanced and metastatic	II RCT	GEM + axitinib *vs* GEM	6.9 *vs* 5.6	-	Negative
Kindler *et al*[47], 2011	632/advanced or metastatic	III RCT	GEM + axitinib *vs* GEM	8.5 *vs* 8.3	-	Negative
Rougier *et al*[48], 2013	427/metastatic	III RCT	GEM + Aflibercept *vs* GEM	6.5 *vs* 7.8	3.7 *vs* 3.7	Negative
Chiorean *et al*[49], 2014	27/advanced		GEM + Sorafenib followed by RT + GEM	12.6	10.6	Negative
Cascinu *et al*[50], 2014	144/advanced	II RCT	GEM + Cisplatin + Sorafenib *vs* GEM + Cisplatin	7.5 *vs* 8.3	4.3 *vs* 4.5	Negative
Gonçalves *et al*[51], 2012	104/advanced or metastatic	III RCT	GEM + Sorafenib *vs* GEM	5.7 *vs* 3.8	9.2 *vs* 8	Negative

OS: Overall survival; PFS: Progression free survival; RCT: Randomized control trial; Advanced diseases: Locally advanced and metastatic; RT: Radiotherapy; GEM: Gemcitabine.

Rapamycin, another mTOR inhibitor, has also failed to demonstrate efficacy in the treatment of PC in humans[76]. Everolimus and enzastaurin had no effect on GEM-resistant tumor therapy or on advanced tumors[75,77]. Rigosertib, a small molecular inhibitor of PI3K, added no survival benefit in a phase III trial[78]. Early phase clinical trials of other inhibitors of the P13K/Akt/mTOR pathway or combining these inhibitors with chemotherapy in PC are ongoing parentheses ClinicalTrials.gov; NCT02294006, NCT01087554, NCT01537107 parentheses.

Therapy against DNA repair genes

PC may induce expression of DNA repair genes at post-transcriptional level from BRCA category 1 or 2 in 7%-10% of sporadic tumors[79]. We believe that such

Table 2 Potential therapeutic targets using miRNA

Ref.	miRNA	Oncogene/tumor suppressor	Target genes	Cellular process affected
Moriyama *et al*[99], 2009	miR-21	Oncogene	*CDK6, PDCD4, CDKN1A, FAS, IL6R, SOCS5, APAF1, NFIB, TPM1*	Apoptosis, cell proliferation, cell invasion
Park *et al*[94], 2009	miR-221	Oncogene	*CDKN1B, CDKN1C, KIT*	Cell migration, proliferation
Habbe *et al*[100], 2009	miR-155	Oncogene	*AGTR1, APC, ARID2, BACH1, CEBPB, CYR61, DET1, EDN1, ETS1, FADD, FGF7, FOXO3*	Cell migration
Chen *et al*[101], 2011	miR-196a	Oncogene	*NRAS, HOXB8, HMGA2, ANXA1*	Cell growth and differentiation
Cai *et al*[95], 2013	miR-181b	Oncogene	*BCL2*	Sensitization to gemcitabine
Yan *et al*[96], 2010	miR-20a	Oncogene	*STAT3, CDH1*	Proliferation and invasion
Torrisani *et al*[102], 2009	Let-7	Tumor suppressor	*KRAS, HMGA2, TRIM71, NF2*	Cell proliferation
Ji *et al*[98], 2009	miR-34a	Tumor suppressor	*NOTCH1, BCL2, E2F3, VEGFA, SIRT1, CCND1, CDK6*	Apoptosis, cell proliferation
Zhao *et al*[103], 2010	miR-217	Tumor suppressor	*KRAS, SIRT1, PTEN*	Cell proliferation, invasion
Yu *et al*[97], 2010	miR-96	Tumor suppressor	*KRAS*	Invasion, cell migration, apoptosis
Li *et al*[104], 2010	miR-146a	Tumor suppressor	*EGFR*	Invasion
Hou *et al*[105], 2012	miR-216a	Tumor suppressor	*PTEN, CDC42, CD44, SIRT1*	Tumorigenicity

tumors are more sensitive to the administration of polymerase inhibitors (iniparib), as verified *in vitro*[80] and *in vivo* in a patient who achieved pathologic complete response[81]. Treatments with olaparib or veliparib, in combination with GEM or alone, are currently being assessed in ongoing trials (ClinicalTrials.gov; NCT00515866 and NCT01908478)[82].

Therapy against histone deacetylases

Chromatin is formed by the wrapping of DNA around histones, a process that is regulated by histone acetylation status. Epigenetic regulation of tumor suppressor genes *via* deacetylation of histones is involved in the apoptosis, differentiation and growth of cells, which influence tumor cell survival. Suberoylanilide hydroxamic acid (vorinostat) administered in combination with GEM and bortezomib, a 26S proteasome antagonist, confers a strong apoptotic, especially in association with bortezomib and GEM and radiosensitizing effect through the nuclear factor-κB pathway, which is not activated in normal tissue[83-86].Phase Ⅰ and Ⅱ trials using such substances in association with radiotherapy are ongoing(*e.g.*, Clinical Trial.gov. NCT00983268, NCT00243100 and NCT00948688 for nonmetastatic disease).

Therapy against microRNAs

miRNAs are single-stranded chains of non-coding RNA of 18-24 nucleotides that inhibit gene expression at the post-transcriptional level *via* triggering complete degradation of the proteins or halting translation. miRNAs can influence the proliferation, apoptosis, and susceptibility of tumors to chemotherapeutic agents. miR-21 regulates the expression of the tumor suppressors *CDKN1A, PTEN* and *PDCD4,* and can be stimulated by taking medications that interfere with tyrosine kinase pathways[87]. This miRNA is overexpressed in 79% of evaluated PCs, and represents an unfavorable prognostic factor[88]. miR-21 is also frequently found in chemoresistant pancreatic cells[88-90], and a lower level was associated with better response to GEM[91]. In addition, miR-21 upregulates Bcl-2 and reduces chemosensitivity to GEM, thus increasing cell proliferation[92].

Inhibition of miR-221 in PC cells suppresses proliferation and upregulates the tumor suppressors *PTEN*, and p27, p57 and PUMA[93]. Introducing antisense oligonucleotides targeting miR-221 or miR-21 induces apoptosis and increases cell sensitivity to GEM[94]. Furthermore, miR-181b increases the response of animals and chemoresistant cell lines to chemotherapy[95]. miR-20a targets tumor suppressor gene *CDH1* and reduces proliferation and metastasis[96]. miR-96 regulates the expression of *KRAS*, and shows low expression in PC compared to normal tissues[97]. Administration of a synthetic precursor of this miRNA also decreases cell proliferation and invasion[97]. Therapeutic overexpression of miR-34, which targets the tumor suppressor p53, decreases cell growth, arrests the cell cycle in G1 and G2/M phases, and sensitizes cells to chemotherapy[98]. A summary of cancer-related target genes is presented in Table 2[94-105].

Therapy against stromal compartments

A number of studies have targeted stromal elements of PC, including the extracellular matrix, various intracellular signaling pathways, and immune cells (Figure 2, Table 3)[36,106-114].

Therapies against stromal extracellular matrix: In the past few years, scientists have begun to appreciate the importance of the microenvironment in sustaining pancreatic tumor growth. The microenvironment of PC is characterized by an extensive deposition of extracellular matrix components and hypovascularity. These desmoplastic features are believed to prevent drug delivery and contribute to primary resistance of drug therapy. When targeting the stromal tissue, the difference between local tumor and metastasis microenvironments should be considered. Metastasis is characterized by the ability of tumor cells to escape

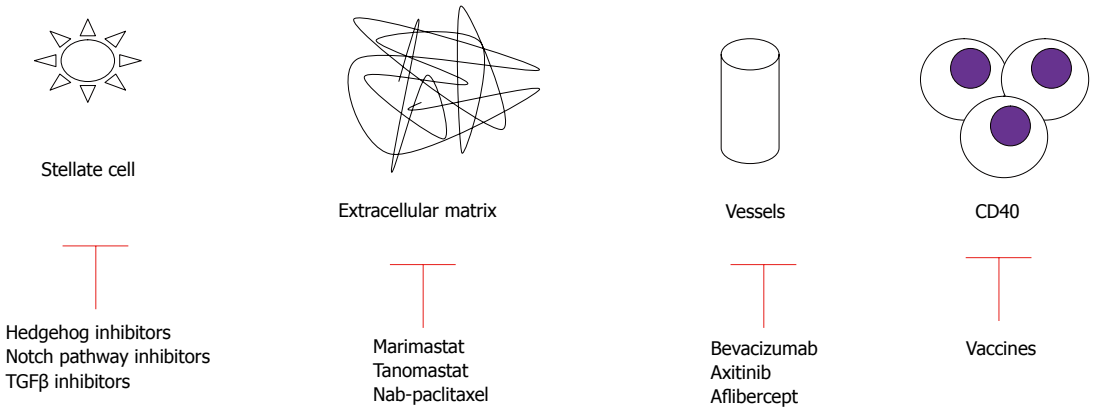

Figure 2 New targeted therapy directed against stromal compartments.

Table 3 Studies with monoclonal antibodies that target the tumor stromal component

Ref.	Stromal component	Therapeutic target	Treatment
Strimpakos *et al*[106], 2013	Extracellular matrix	Hyaluronan	PEGPH20
Bramhall *et al*[36], 2002	Extracellular matrix	Metalloproteinase	Marimastat
Stephenson *et al*[107], 2011	Signaling pathways	Hedgehog	Vismodegib (GDC-0449)
Oettle *et al*[108], 2009	Signaling pathways	Transforming growth factor β receptor	Trabedersen
Yabuuchi *et al*[109], 2013	Signaling pathways	Notch	PF-03084014
Brahmer *et al*[106], 2012	Immune cells	Receiver for programmed cell death	BMS-936559
Le *et al*[111], 2013	Immune cells	Cytotoxic T-lymphocyte antigen 4	Ipilimumab
Beatty *et al*[112], 2013	Immune cells	CD40	CP-870893
Lutz *et al*[113], 2011	Immune cells	CB8	GVAX
Laheru *et al*[114], 2008	Immune cells	CB8	GVAX

GVAX: Granulocyte-macrophage colony-stimulating factor vaccine.

from the primary tumor, survive in circulation, and invade and establish colonies in distant sites, thus warranting special consideration in the design of clinical studies[115].

Matrix metalloproteinases are a family of proteolytic enzymes responsible for the breakdown of connective tissue proteins. These enzymes are crucial in maintaining the growth, differentiation and repair of normal healthy tissue, but aberrant expression is associated with invasive activities of solid tumors[116]. However, inhibition of matrix metalloproteinases by marimastat and tanomastat showed no clinical activity in combination with GEM[36,117]. The extracellular matrix also contains hyaluronan (a nonsulfated glycosaminoglycan), is highly abundant in pancreatic tumors, and has been implicated in angiogenesis, epithelial mesenchymal transition, and chemoresistance[118]. A phase Ib study combining GEM with hyaluronidase demonstrated partial response in 64% of PC patients with high levels of hyaluronan[106]. A phase Ⅱ study of this combination is currently underway ClinicalTrials.gov; NCT01453153.

Therapies against intracellular signaling pathways: Transforming growth factor (TGF)-β signaling has been implicated in cancer cell proliferation, tumor angiogenesis, metastasis, and suppression of antitumor immunity[119,120]. Its overexpression is associated with disease stage, clinical prognosis, and the immunodeficient state of the patients. TGF-β signaling is mediated by SMAD4, for which 50% of human PCs show allelic deletion[121]. The complex TGFβ-SMAD4 translocate to the nucleus, where they interact at the promoter with other transcription factors at DNA sequence-specific binding sites or with transcriptional coactivators. Thus, aberration of TGFβ-SMAD4 signaling is believed to be an important step in pathogenesis of this cancer[122]. *SMAD4* mutation leads to feedback overexpression of TGF-β1. Development of anti-TGF treatment in advanced PC is still in the early clinical stage (ClinicalTrials.gov NCT00844064).

The hedgehog pathway has been shown to be an important signaling system in the microenvironment of PC. The sonic hedgehog ligands are present in the fibroblasts of the PC, but not in the normal pancreatic fibroblasts[123]. Binding of the sonic hedgehog ligand to its patched receptor activates the smoothened and zinc finger proteins, driving the expression of several target genes responsible for desmoplastic reactions and inhibition of pancreatic cell autophagy[124]. Sonic hedgehog is expressed in cancer stem cells (CSCs), rare tumor cells with abilities of self-renewal which are responsible for tumor recurrence and metastasis, as well as resistance to current therapies[125]; thus, this factor represents an attractive target for therapeutic intervention. Saridegib IPI-926 is an inhibitor of this

pathway that elevates intratumoral concentrations of GEM, reduces the dense fibrotic reaction, and increases tumor neo-vascularization in an animal model[126]. However, in a double-blind randomized placebo-controlled phase Ⅱ study, the combination of GEM with Saridegib was associated with shorter survival in PC patients, and the trial was terminated prematurely[127].

The dense fibro-inflammatory microenvironment of PC results in hypoxia, which activates hypoxia-inducible factor-1α and promotes tumor cell secretion of sonic hedgehog. As a result, the epithelial to mesenchymal transition is activated, CSCs are maintained, and resistance to therapy occurs. Moreover, hypoxia-inducible factor-1α activates leptin receptors and influences metastasis and survival[128], and activates actin-related mechanisms as well[129]. Myo-inositol trispyrophosphate can reverse hypoxia and decrease desmoplasia in an animal model, with improved susceptibility to GEM treatment[130,131]. Gene expression of hypoxia-inducible factor 1α was reduced in an animal model by administration of a novel synthetic compound[132], which is currently being tested in an ongoing trial (ClinicalTrials.gov; NCT01248637).

Hypoxic conditions can also trigger Notch signaling, which plays a critical role in organ development and cell differentiation. Notch signaling mediates PC stem cell function, which contributes to chemotherapy resistance, tumor recurrence, and metastasis. Upon receptor activation, Notch is cleaved by a cascade of proteolytic enzymes, including metalloproteinases, tumor necrosis factor-α-converting enzyme, and γ-secretase[133]. The oral γ-secretase inhibitor RO4929097 has completed a phase Ⅰ trial for treatment of metastatic cancer, and results are promising Recently, preliminary results from two phase Ⅰ clinical trials testing anti-Notch antibodies (OMP-59R5 and demcizumab) have been presented[134,135]. A phase Ⅰ study of an oral Notch inhibitor (MK-0752) in combination with GEM is now ongoing (ClinicalTrials.gov; NCT010983440).

Enhanced drug delivery to microenvironment: Inefficient drug delivery might explain the lack of efficacy of systemic treatments. Novel drug delivery vehicles have reformed the clinical use of traditional cytotoxic agents. Nab-paclitaxel is an albumin-bound formulation that increases tumor accumulation of paclitaxel *via* binding of albumin to the surrounding stroma that is enriched in secreted protein acidic and rich in cysteine (SPARC). Nab-paclitaxel was developed to exploit the ability of SPARC to bind to albumin as a means of increasing drug delivery to the tumor[136]. In an animal model, intratumoral concentration of GEM was increased 2.8-fold in mice receiving nab-paclitaxel in combination with GEM, and treatment of patients with nab-paclitaxel alone was also more effective than GEM alone (aggregate tumor regression rates of 55%, 36% and 24% for nab-paclitaxel plus GEM, nab-paclitaxel alone and GEM alone, respectively)[137]. These findings suggest that nab-paclitaxel is able to destroy or alter the characteristics of the tumor stroma and increase vascularization in order to achieve enhanced delivery of cytotoxic chemotherapy to the tumor. Indeed, in two GEM-resistant xenografts, a profuse desmoplastic stroma remained after treatment with vehicle or GEM alone, whereas the administration of nab-paclitaxel resulted in a significant reduction in stromal content[108]. In another study using the GEM-resistant mouse model, treatment with nab-paclitaxel and GEM also resulted in an increase in intratumoral GEM concentration and a reduction in tumor size compared with treatment with either agent alone[138].

In a recently published phase Ⅲ study comparing nab-paclitaxel plus GEM *vs* GEM alone, the addition of nab-paclitaxel significantly prolonged median OS from 6.7 to 8.5 mo, with a corresponding increase in response rate from 7% to 23%[139], and after one year from 22% to 35%[137,140]. The nab-paclitaxel/GEM combination has become the second regimen shown to be superior to GEM alone and has been approved by the FDA for treatment of advanced PC. In a second-line setting, nab-paclitaxel monotherapy demonstrated clinical activity in GEM-refractory advanced PC patients in a phase Ⅱ trial[39]. In this trial, high expression of stromal protein was used to specifically enrich the concentration of a cytotoxic agent in the tumor. Additionally, Alvarez *et al*[141] demonstrated that nab-paclitaxel reduces the stiffness and the number of cancer-associated fibroblasts in human tumors treated with nab-paclitaxel. Its combination with different agents is now one of the most popular areas of clinical research in advanced PC. Another innovative approach to improve drug delivery that is under development is the use of nanotechnology and cancer-specific liposomes[142].

Antiangiogenic therapies

Antiangiogenesis is clinically ineffective in treating PC patients. Although most preclinical models of PC have suggested potential activity of many antiangiogenic agents, they failed to simulate human tumor microenvironments where dense stromal tissue with decreased vascular density is now known to be the main obstacle for effective drug delivery. Moreover, the withdrawal of antiangiogenic agents after therapy may be associated with increased tumor aggressiveness and invasion, offsetting the potential therapeutic benefits offered by antiangiogenic agents. It has also been said that angiogenesis inhibition might alter the natural history of tumors by increasing tumor invasion and metastasis[143].

Overexpression of VEGF in PC has been associated with tumor progression and a worse prognosis. Therefore, similar to other cancer therapies, angiogenesis is considered to be a therapeutic target[144,145]. Humanized monoclonal antibodies such as bevacizumab have affinity for circulating VEGF-A, but phase Ⅱ and Ⅲ studies

showed no survival advantage when bevacizumab was combined with GEM and erlotinib[41-44]. A meta-analysis found that therapy with bevacizumab-GEM was associated with a modest response rate, without survival modifications[52]. Associations of two chemotherapeutic agents (GEM, capecitabine) with two biologic therapies (erlotinib, bevacizumab) provided an additional ten months of survival in metastatic disease[25]. The proposed mechanism for this effect involves the overexpression of platelet-derived growth factor and fibroblast growth factor[146]. The development of bevacizumab-related hypertension is also associated with better survival[147]. Other VEGF inhibitors, such as axitinib and aflibercept, provide no survival advantage[46-48,148]. In addition, sorafenib (an inhibitor of VEGFR and Ras/Raf/MAPK signaling) had no supplementary value for patient survival over GEM[51].

SPECIFIC IMMUNOTHERAPY

In pancreatic adenocarcinoma, interactions between tumor and host cells are mediated by inflammatory cells, fibroblasts and vascular endothelial cells. Intratumoral desmoplastic tissue is less vascularized, and cytotoxic substances cannot easily penetrate the connective matrix. Therefore, inflammatory cells and macrophages represent potential therapeutic targets. These cells can acquire antitumor properties, which is the main purpose for specific immunotherapies, including vaccines and adoptive cell therapy.

Antitumor vaccines are biologic preparations that involve administering an antigen that is specific for a particular tumor type and stimulating the body's natural ability to protect itself. There are a number of ways to deliver these vaccines: whole-cell recombinant vaccines, dendritic cell (DC) vaccines that combine antigen with DCs to present to white cells, DNA vaccines (by inserting viral or bacterial DNA into human or animal cells), or T-cell receptor peptide vaccines (by inserting peptides to modulate cell-mediated immunity).

Whole-cell recombinant vaccines

The advantage of using whole-cell recombinant vaccines is that tumor cells express a wide range of tumor-associated antigens. This rich source of antigens contains epitopes of the two types of T cells (CD8+ and CD4+), compared with peptide-based vaccines that contain only one epitope. Autologous tumor cells are the best source of protein for immunization, but only 10%-15% of patients diagnosed with pancreatic tumors are candidates for surgical treatment. In addition, it is difficult to prepare a sufficient quantity of tumor cells required to achieve the vaccine due to prolonged culture periods and possible contamination with bacteria and fungus. To avoid these difficulties, allogeneic tumor cells can be used, which can be produced in larger quantities and do not require determination of the patient's human leukocyte antigen and cell types. Furthermore, multiple allogeneic tumor antigens can be processed using the mechanism of cross-presentation and simultaneous induction of CD4+ and CD8+ cells[149].

Algenpantucel-L: Algenpantucel-L contains cell lines expressing α-galactosyl epitopes on the surface of proteins and glycolipids. In humans, these epitopes are missing, but there are natural anti-α-gal antibodies that stimulate the immune response, including against tumor cells[150,151]. In a phase Ⅱ study with this type of immunotherapy in combination with GEM and 5-FU/irradiation, algenpantucel-L was injected intradermally (up to 14 vaccinations)[152]. The adverse reactions were local response and peripheral hypereosinophilia. Survival at 1 year was 86%, better than the 81% reported in the RTOG-9704 trial using the same chemoradiotherapy scheme[153]. Interestingly, the patients who received a higher dose of vaccine in the study (300 *vs* 100 million cells/dose) had an increase in 12-mo disease-free (81% *vs* 51%) and overall (96% *vs* 79%) survivals. Additionally, patients in this trial had a higher percentage of lymph node positivity (stage Ⅱb) in comparison with the RTOG-9704 trial (81% *vs* 68%)[152]. Phase Ⅲ studies are ongoing and the results are expected (ClinicalTrials.gov; NCT 01836432).

Granulocyte-macrophage colony-stimulating factor vaccine: Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a potent cytokine that is able to mobilize monocytes, eosinophils and lymphocytes to the tumor sites. GM-CSF vaccine (GVAX) showed tumor-free survival and also caused regression of tumors in mice[154]. In a phase Ⅰ study, 14 patients were vaccinated with a GVAX made from irradiated cancer cell lines (PANC 6.03 and PANC 10.05) that were engineered to express GM-CSF, with an interval of 8 wk after resection of the pancreas and chemoradiotherapy[155]. Patients who developed delayed hypersensitivity reaction were disease-free at 25 mo from diagnosis. Another phase Ⅱ study vaccinated 60 patients with surgical treatment of adenocarcinoma and with radiochemotherapy (5-FU-based regimen) with an allogeneic GVAX[113,114]. A total of five immunotherapy treatments were delivered intradermally and the first treatment was given 8-10 wk after surgical resection resulting in an 85% 1-year survival; the effect was attributed to the induction of CD8+ mesothelin-specific T cells. GVAX immunotherapy induces expression of anti-thyroglobulin antibodies that recognize a unique antigenic repertoire associated with prolonged survival[156]. All these trials demonstrate post-vaccination induction of CD8+ T cells to multiple mesothelin-specific epitopes, which correlates with improved survival[113,114,155]. Mesothelin is a tumor-

associated antigen that is overexpressed in most ductal adenocarcinomas of the pancreas and is thought to be involved in cell adhesion, and, therefore, to play a role in metastasis[157].

Peptide vaccines

Peptide-based anti-tumor vaccines are prepared from fragments of antigenic proteins, which are the minimal immunogenic region of tumor-associated antigens that are simple, safe, stable and economical for this purpose. Multiple peptides related to major histocompatibility complex class Ⅰ have been identified and considered as candidates, and vaccination with synthetic peptides has been studied in clinical trials in combination with chemotherapy sessions in order to produce cytotoxic T lymphocytes[158]. The use of peptide vaccines has some limitations: the existence of a limited number of known antigenic peptides; the presence of suppressive immune cells in tumoral microenvironments; the fact that DCs may have poor functionality in patients with advanced pancreatic tumors; the observation that CD8+ cytotoxic T cells are sometimes ineffective in the reaction with pancreatic tumor cells, which is mediated by production of immunosuppressive cytokines such as interleukin-10 and tumor growth factor.

K-ras vaccine: K-ras is thought to be recognized by helper and cytotoxic T cells, and almost 90% of pancreatic tumors involve mutations in the *KRAS* oncogene. Peptide vaccines against mutated K-ras are safe for administration to humans[159-161], but only one of the nine patients had a cytotoxic T lymphocyte immune response[161]. A study of synthetic vaccine for a K-ras mutation and GM-CSF showed an immune response in 25/48 of the enrolled patients[162]. For these patients, survival was 148 d compared to 61 d for the non-responders. Twenty patients in this study, and another group of 23 patients, have been followed-up for a long time and have shown a median 5-year survival rate of 20% (four patients), while a 29% survival rate was observed in another group of patients with immune response; adverse effects to the vaccine were minimal[163]. Using synthetic K-ras vaccines based on long peptides to induce antigen-specific polyclonal CD8+ and CD4+ T, Weden *et al*[163] reported a 10-year survival rate of 20% in a group of patients after pancreatic tumor resection. Another recent study showed no effect of a 21-mer peptide vaccine based on a *KRAS* mutation in 24 patients vaccinated monthly for 3 mo[164]. Administration of Reolysin, an oncolytic virus that replicates and kill cells with a *KRAS* mutation, was well tolerated by patients with breast tumors[165], but further studies are expected.

Immunotherapy in the form of vaccination against mutant K-ras has been developed as an adjunct to surgical resection and appears as a promising principle of adjuvant therapy. Taking into account that K-ras vaccination is virtually free of side effects, the results should encourage much larger controlled studies.

Telomerase peptide vaccine: Telomerase is a ribonucleotide enzyme that maintains cellular stability and is expressed by almost all cancer cells (85%-90%)[166], including PC[167]. Activation of reverse transcriptase from human telomerase increases cell viability, and is thus an attractive target for an immunotherapy antigen. In a phase Ⅰ-Ⅱ study, the administration of a telomerase peptide vaccine (GV1001) and immunogenic response was found to be correlated with prolonged survival (25% at 1 year) and good tolerability[168]. However, a phase Ⅲ study in unresectable and metastatic pancreatic ductal adenocarcinoma that compared PrimoVax (GV1001 and GVAX) administered sequentially with GEM against GEM alone was closed due to lack of survival (median OS: 5.9 mo *vs* 7.3 mo)[169,170]. A second GV1001 phase Ⅲ trial (TeloVac) in unresectable and metastatic PC compared the association between the vaccination and subsequent or concurrent chemotherapy (GEM and capecitabine) *vs* chemotherapy alone; there were no significant survival differences (median OS: 6.94 and 8.36 mo *vs* 7.89 mo, respectively)[171]. Furthermore, patients in the sequential arm received only 2 mo of chemotherapy before being taken off an active therapy that has a historical median progression-free survival of 4.3 mo[172]. Despite the disappointing phase Ⅲ results, the findings have identified biomarkers that may predict response to this vaccine and new research may indicate benefit in a subgroup of patients[173]. In addition, there is another ongoing study in patients with advanced disease that includes radiochemotherapy (ClinicalTrials.gov; NCT01342224).

Survivin-based vaccine: Survivin is an inhibitor of apoptosis and is found in PC. There have been isolated cases of complete remission with a survivin-based vaccine in patients with metastatic disease[158]. This effect was confirmed only in combination with GEM in an experimental study using a modified vaccinia Ankara in a murine pancreatic model, which showed enhanced survivin-specific CD8 interferon-γ immune responses in the vaccinated mice[174].

Mucin 1 vaccine: Mucin (MUC)1 is highly expressed in PC[175], and phase Ⅰ and Ⅱ studies of MUC1 antigen-pulsed DC vaccines showed hopeful results in advanced PC[176,177]. A phase Ⅰ study in advanced PC showed that the vaccinia virus expressing carcinoembryonic antigen (CEA) and MUC1 and co-stimulatory molecules was well tolerated and provided an OS advantage in immune-responsive patients[178]. However, a phase Ⅲ trial using fowlpox viruses expressing these same molecules failed to show improvement in OS in PC patients when compared to chemotherapy or best supportive care in a palliative setting[179]. Administration of a pox virus-based vaccine targeting

MUC-1 and CEA induced a favorable immune response on T cells, but has not been confirmed as beneficial in a phase Ⅲ study[176,178]. Intratumoral administration of the recombinant fowlpox PANVAC plus subcutaneous recombinant vaccinia and recombinant GM-CSF is currently underway in a phase Ⅰ study. In another study, 16 patients with advanced PC who were vaccinated with DCs pulsed with MUC1 showed an increase in CD8+ cells in peripheral blood; 2/15 patients with resected PC were alive and disease free at 32 and 61 mo[176].

Anti-VEGFR vaccine: An anti-VEGFR vaccine was given in association with GEM to patients with unresectable or metastatic disease, and produced an OS rate of 8.7 mo; phase Ⅱ study results are expected[180].

Personalized peptide vaccination: Personalized peptide vaccination was attempted after preparation of pre-vaccination peripheral blood mononuclear cells and plasma as a first-line therapy in association with GEM in unresectable patients. This attempt showed a 1-year survival rate of 38%[181]; however, further evaluations are needed.

Nanoparticles: Nanoparticles are non-specific and are taken-up in the spleen. They can be safely used as a vaccine platform without the risk of prolonged side effects. In animal models, nanoparticulate delivery of diphtheria toxin DNA effectively kills mesothelin-expressing PC cells[182].

Heat shock proteins: Heat shock proteins also play a role in the stabilization and delivery of peptides, and in inducing immunity against autologous tumors[183]. In one study, 3/10 patients treated with an autologous vaccine prepared from resected tumors showed no tumor recurrence at 2.6, 2.7 and 5.0 years of follow-up, though there was no correlation between stimulating immunity and survival[184].

DC vaccines

DCs are the most potent antigen-presenting cells, and they can cause a high antigenic response *via* stimulation of T and B cells. DC vaccines combine tumor antigens with DCs for presentation to effector T cells. Viral or bacterial DNA is inserted into human cells to modulate cell-mediated immunity by the DNA vaccines. It has been shown that DC vaccine plus lymphokine-activated killer cell treatment and chemotherapy prolonged OS compared to effects observed in patients who received only DC vaccine or chemotherapy[158,185]. In a multi-center study of 255 patients who received chemotherapy plus vaccine, the median survival was 16.5 mo, with erythema reaction after vaccination identified as a factor related to better survival[186]. The effects were considered likely due to the enhancement of tumor cell immunogenicity by treatment with GEM, which increases the efficacy of the vaccine[187]. However, tumor-reactive T cells in peripheral blood were decreased and the cytotoxic T cell-mediated killing was normal[188]. The combination of these vaccines with mRNA encoding CEA produced an effective immunization and survival benefit for three patients with resected pancreatic tumors receiving neoadjuvant therapy, each of who survived 30 mo after diagnosis[189]. The combination of DC vaccines with DNA for MUC1 has been found to be beneficial in a small portion of resected patients[176], and as ineffective in metastatic disease[177]. The combination with telomerase reverse transcriptase mRNA demonstrated encouraging results when administrated after radical surgical treatment[190]. Targeting more than one checkpoint pathway at the same time might be another option for obtaining increased efficacy.

Administration of the anti-cytotoxic lymphocyte antibody (ipilimumab) and GVAX increased the survival of 15/30 previously treated patients with metastatic disease compared to GEM alone (5.5 mo *vs* 3.3 mo), supporting the approach of blocking cytotoxic lymphocytes by promoting the GM-CSF antitumor response[111]. Survival was correlated with CD8+, mesothelin-specific T cell quantity. A phase Ⅱ study of this protocol is under development due to this promising result. Despite the encouraging findings, however, clinical responses have been seen in only a minority of patients, presumably due to insufficient expansion of antigen-specific cytotoxic T lymphocytes capable of eradicating tumor cells. Interestingly, endoscopic ultrasound-guided fine-needle injection of OK432-pulsed DCs into a tumor followed by intravenous infusion of lymphokine-activated killer cells stimulated with anti-CD3 monoclonal antibody was synergistically effective in a phase Ⅰ study[191].

CD40 is a potential immunomodulatory target, because it is a co-stimulatory molecule for antigen-presenting cells. GEM with CD40 agonist-activated T cells reduces tumor burden in advanced PC patients in a phase Ⅰ study[192], by decreasing tumor stroma and increasing infiltration of activated macrophages[112].

Adoptive cell therapy consists of re-transferring autologous cytotoxic T lymphocytes harvested from the patient after *in vitro* activation of K-ras, telomerase, or mesothelin. This method helps the immune system to recover more quickly after chemotherapy and improves responses to other immunotherapies. However, extensive studies have not been performed[193-195].

IMMUNOTHERAPY TARGETING TUMOR STEM CELLS

Most patients with PC who initially respond to standard chemotherapy relapse because of small populations of tumor cells/tumor stem cells (*i.e.*, CSCs). CSCs are

better able than other tumor cells to multiply and to initiate new tumors and sustain tumor growth. It has been shown that pancreatic tumors that are resistant to chemoradiotherapy are rich in CSCs. These tumors are candidates for immunotherapy, and CSC-targeted therapy can be applied to prevent resistance to chemotherapy.

Targeted immunotherapy on tumor stem cells using γδ T cells, natural killer cells, and anti-tumor vaccines based on DCs has been successfully used to activate responses of CSC-specific cytotoxic T lymphocytes, leading to the expression of high levels of interferon-γ and enhanced destruction of CSCs *in vitro*. Transfer of stem cells may have antitumor effects due to decreased activity of Wnt or Akt pathways[196,197]. Antitumor action will be possible only if three conditions are met: direct tumor migration and intratumoral incorporation, release of the antitumor agent, and generation of a specific organ-vector[197].

The use of immunotherapy for treatment of pancreatic ductal adenocarcinoma is promising, though its immunotolerant environment continues to be a major hurdle. Therapeutic vaccines have the ability to activate antitumor immune responses; however, these strategies need to be combined with immune-modulating agents, chemotherapies or radiation, depending on the patient disease status. There is also a great need to optimize vectors, antigens, and patient selection. Additionally, more preclinical and early-phase clinical trials need to be conducted to determine if and which chemotherapies would complement immunotherapies, and determine how to optimally sequence the administration of immunotherapy with chemotherapy and radiation. Combinations of active and passive immunologic treatments, targeted agents and conventional chemotherapies might be important strategies for increasing efficacy.

CONCLUSION

The goal of these new treatments is to obtain faster and more stable tumor response. Passive immuno-therapy may have a role in combination with radio-chemotherapy. Furthermore, vaccines would allow restoration of specific immune responses after adjuvant or palliative treatment, and would continue the fight against residual tumor cells. Knowing the genetic implications in PC, the combination of two or more vaccines would be beneficial.

In the future, treatment will likely include personalized medicine to each patient, tailored for numerous molecular therapeutic targets of multiple pathogenetic pathways in PC, and is expected to occupy a central role in stem cell therapy.

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REVIEW

Androgens and esophageal cancer: What do we know?

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Abstract

Significant disparities exist between genders for the development and progression of several gastrointestinal (GI) diseases including cancer. Differences in incidence between men *vs* women for colon, gastric and hepatocellular cancers suggest a role for steroid sex hormones in regulation of GI carcinogenesis. Involvement of intrinsic gender-linked mechanisms is also possible for esophageal adenocarcinoma as its incidence is disproportionally high among men. However, the cause of the observed gender differences and the potential role of androgens in esophageal carcinogenesis remains unclear, even though the cancer-promoting role of androgen receptors (AR) shown in other cancers such as prostate and bladder suggests this aspect warrants exploration. Several studies have demonstrated expression of ARs in esophageal cancer. However, only one study has suggested a potential link between AR signaling and outcome - poorer prognosis. Two groups have analyzed data from cohorts with prostate cancer and one of these found a decreased incidence of esophageal squamous and adenocarcinoma after androgen deprivation therapy. However, very limited information is available about the effects of androgen and AR-initiated signaling on esophageal cancer cell growth *in vitro* and *in vivo*. Possible mechanisms for androgens/AR involvement in the regulation of esophageal cancer growth are considered, and the potential use of AR as a prognostic factor and clinical target is highlighted, although insufficient evidence is available to support clinical trials of novel therapies. As esophageal adenocarcinoma is a gender linked cancer with a large male predominance further studies are warranted to clarify the role of androgens and ARs in shaping intracellular signaling and genomic responses in esophageal cancer.

Key words: Esophageal cancer; Androgens; Androgen receptor

Core tip: Esophageal cancers, especially adenocarcinoma, are gender-linked malignancies, with a male predominance. Previous studies have demonstrated expression of androgen receptors in both adenocarcinoma and squamous cell esophageal cancer. However, the impact of androgens in development and progression of these cancers is unclear. Androgen-deprivation therapy has not been explored, even though it is successfully used in treatment of prostate cancers. Further studies are warranted to clarify the role of androgens and

androgen receptors in shaping intracellular signaling and genomic responses in esophageal cancer.

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WHY ANDROGEN RECEPTORS?

Gender associated differences in the incidence of various gastrointestinal (GI) cancers are well recognized, with some diseases dominated by the female gender, including gallstone disease and primary biliary cirrhosis, and others by the male gender, including gastroesophageal reflux disease (GERD) and colon cancer[1-4]. Esophageal cancers of both major subtypes show a male predilection, occurring 3-4 times more commonly in men than women globally[5]. This is most marked for esophageal adenocarcinoma (EAC), with reported sex ratios (M:F) ranging from 5:1 in France, 6:1 in Australia and Sweden, 8:1 in the United States to 10:1 in the United Kingdom[6-8]. An analysis of the Surveillance, Epidemiology and End Results (SEER) Registry in the United States confirmed a significant rise in EAC among males and a slower rise in females from 1973 to 2008, with an overall M:F ratio of 7.66[8]. This gender bias towards men is out of proportion to the gender distribution for the underlying risk factors, predominantly obesity and GERD[9]. For the other major subtype, esophageal squamous cell cancer (ESCC), a male propensity also occurs, but is less marked than in EAC, and it appears to occur concomitantly with male dominance of the main risk factors, smoking and alcohol consumption[10]. Reasons for the disproportionate rise in EAC in men have not been investigated in detail, but some studies implicate sex hormones as contributing factors[11].

Sex steroid hormones, predominantly estrogens in women and androgens in men, demonstrate gender specific concentration profiles. These hormones regulate cell growth and behavior *via* a variety of estrogen and androgen receptor subtypes which are distributed widely throughout normal and abnormal human tissues, including cancers. The role of sex hormones in the development of prostate and breast cancers is well known[12,13]. Estrogen receptors (ER) have been shown to have a role in esophageal adenocarcinomas[14,15]. Androgen receptors (AR) are also widely expressed in human tissues and have been identified in esophageal cancer[16-20]. Higher circulating levels of testosterone and dihydrotestosterone (DHT) have been identified in patients who develop Barrett's esophagus, the precursor lesion for EAC, after controlling for age, body mass index (BMI), and GERD symptom profiles[21]. Analysis of the SEER database has shown that patients with a previous diagnosis of prostate cancer are less likely to develop EAC, raising the possibility that the androgen deprivation therapy used for prostate cancer may reduce the risk of EAC[22]. This study also found that the incidence of ESCC was lower in individuals with a previous diagnosis of prostate cancer and suggested that lifestyle modifications could be an additional factor. Nevertheless, the presence of ARs in EAC and the association between testosterone and Barrett's esophagus suggest that androgens might play a role in the development of EAC, and would provide a logical explanation for the male dominance of the disease[21].

However, this hypothesis is contradicted by findings in overweight or obese individuals who develop esophageal adenocarcinoma. As obesity has been unequivocally shown to be a major risk factor for the development of esophageal adenocarcinoma, consideration must also be given to the lower level of circulating androgens in obese individuals[9,23]. Testosterone is converted into estrogens in intra-abdominal adipose tissues by aromatase, and this might further decrease local concentration of androgens. Hence, local conversion of androgens to estrogens might contribute to the development of EAC, and provide an explanation for an interaction between obesity, androgens, and male gender in the genesis of EAC.

CIRCULATING ANDROGENS

Testosterone, dihydrotestosterone (DHT), androstenedione, and dehydroepiandrosterone (DHEA) and its sulfate (DHEAS) comprise the group of circulating human androgens which prevail in men. These are 19-carbon steroid molecules synthesized from cholesterol in the adrenal cortex, testes (in men) and ovaries (in women). Androgens can be converted into 18-carbon estrogens by aromatase enzymes predominantly in the liver, but also in gonadal, adipose and other tissues. An estimated 5% of the serum testosterone produced in men is catalyzed to the more potent androgen DHT by 5α reductase enzymes. AR affinity of DHT is 3-fold greater than the affinity of testosterone and 15- to 30-fold greater than adrenal androgens affinity[24]. High AR affinity of DHT makes this androgen the most potent hormone activator of AR-regulated transcription. The more abundant but less potent adrenal steroids DHEA and DHEAS, are precursors for intracellular production of more active androgens, and also estrogens under 5α reductase and aromatase activity[25].

The vast majority of testosterone circulates bound to plasma proteins: 40%-50% of testosterone is bound to albumin, 50%-60% is strongly bound to sex hormone-binding globulin (SHBG), with only 1%-2%

being free[26]. Both free testosterone and albumin-bound testosterone are bioavailable. However, they show some differences in biological effects[27]. For instance, free testosterone and DHT act *via* ARs in the cell nucleus and regulate AR-dependent gene transcription. Protein bound circulating androgens, however, are biologically active by a different signaling pathway. Testosterone and DHT bound to SHBG has been shown to act *via* a specific cytoplasmic G-protein coupled SHBG receptor in prostate cancer cells, rather than by binding nuclear AR[28]. Thus, the free circulating androgens are a potent minority and act *via* nuclear AR, whereas SHBG-bound testosterone and DHT may exert effects by cytoplasmic SHBG receptors.

The levels of all androgens increase at puberty and peak during adolescence, then gradually decrease with age[29]. Alterations in the ratios of circulating androgens followed by depletion of sex steroid hormones are important consequences of normal aging and are associated with vulnerability to disease in hormone-responsive tissues[30]. The significant decline in male sex steroids, and its clinical consequences, has been dubbed androgen deficiency in aging males (ADAM)[30]. In contrast to menopause in females, androgen deficiency in aging males is rarely accompanied by the loss of reproductive function. Androgen deficiency in aging males associated hormonal changes are gradual, with bioavailable testosterone levels declining 2%-3% annually from approximately 30 years of age[30,31].

In women, testosterone is produced primarily through peripheral conversion of androstendione (50%). Testosterone is also produced in the ovaries (25%) and the adrenal glands (25%)[32]. During pregnancy, the placenta may also serve as a source of the testosterone[33]. There is at least a 10-fold difference in testosterone levels between males and females of reproductive age. The plasma concentration of the hormone in males is between 10 to 35 nmol/L and in females is 0.7-2 nmol/L[34]. This contrast adds support to the hypothesis that estrogens might be protective against development of gastrointestinal cancers, but androgens may facilitate carcinogenesis[15,35]. This hypothesis is compatible with epidemiological data, which show a decline in male dominance in older age groups, *i.e.*, postmenopausal females. The SEER dataset has shown a peak gender ratio of 11:1 in favor of males in the 50-54 years age bracket, but falling to 4:1 in the 75-79 year age group[8].

ANDROGEN RECEPTOR SIGNALING

Androgens regulate protein synthesis and influence tissue remodeling *via* the AR, which along with other steroid hormone receptors are members of the nuclear receptor superfamily[36] (Figure 1). As the *AR* gene is located on the X chromosome (Xq11.2) it exists as a single copy in males. The AR protein is a ligand-inducible zinc finger transcription factor that regulates target gene expression[37]. Androgen receptors possess four structurally and functionally distinct domains: the NH_2-terminal transactivation domain, the DNA-binding domain, a hinge region and the COOH-terminal ligand-binding domain[37]. Two AR isoforms have been found: the predominant isoform B (110 kDa) and the less dominant and shorter isoform A (80 kDa)[38]. Novel AR splice variants designated AR3, AR4 and AR5 in androgen-insensitive prostate cancer cell lines have recently been described[39].

Non-ligand-bound AR is located in the cytoplasm where it associates with heat shock proteins (HSPs). Ligand binding induces a conformational change in the AR, resulting in release of heat-shock proteins, dimerization, phosphorylation, and translocation of the complex to the nucleus (Figure 1). Microtubule proteins, specifically filamin A (FlnA) help to transport ligand-AR complexes to the cell nucleus[40]. Homo-dimerized AR interacts with a constellation of transcriptional co-regulators and transcription factors leading to specific transcriptional activation or repression of target genes[41]. AR-inducible genes coordinate the development of male sexual characteristics (both anatomical and mental), spermatogenesis, and maintain skeletal homeostasis[42].

NONGENOMIC AR SIGNALING PATHWAY

Extranuclear or non-genomic androgen effects are distinct from androgen genomic actions. Extranuclear effects can be detected within in the first seconds or minutes after the application of androgen to cells or tissues[43,44]. Nongenomic androgen activity involves rapid induction of second messenger signal transduction cascades, including calcium and cAMP fluxes, activation of protein kinase A(PKA), protein kinase C (PKC), and mitogen-activated protein (MAP) kinases[45] (Figure 2). These dynamic changes in protein kinase activity shape a specific phosphorylation profile that, in turn, transfers new signals to the cell nucleus to switch on genes[46].

Extranuclear androgen signaling is not only faster than genomic signaling, but might occur through cytoplasmic receptors that are different to the traditional nuclear AR. Recent reports confirm that opposite androgen and AR-mediated effects in prostate cancers might depend not only on cell type and tumor stage, but also on AR type and localization[47]. Several membrane-localized receptor targets for androgens have been proposed[37,48]. Androgens have been shown to rapidly stimulate PKA and induce production of cAMP *via* binding to G-protein coupled membrane receptor for the SHBG-testosterone complex[28,37].

It has also been suggested that testosterone might bind a membrane-associated receptor (mAR) which might be distinct from the classical nuclear AR. Nuclear-localized ARs lack a standard transmembrane

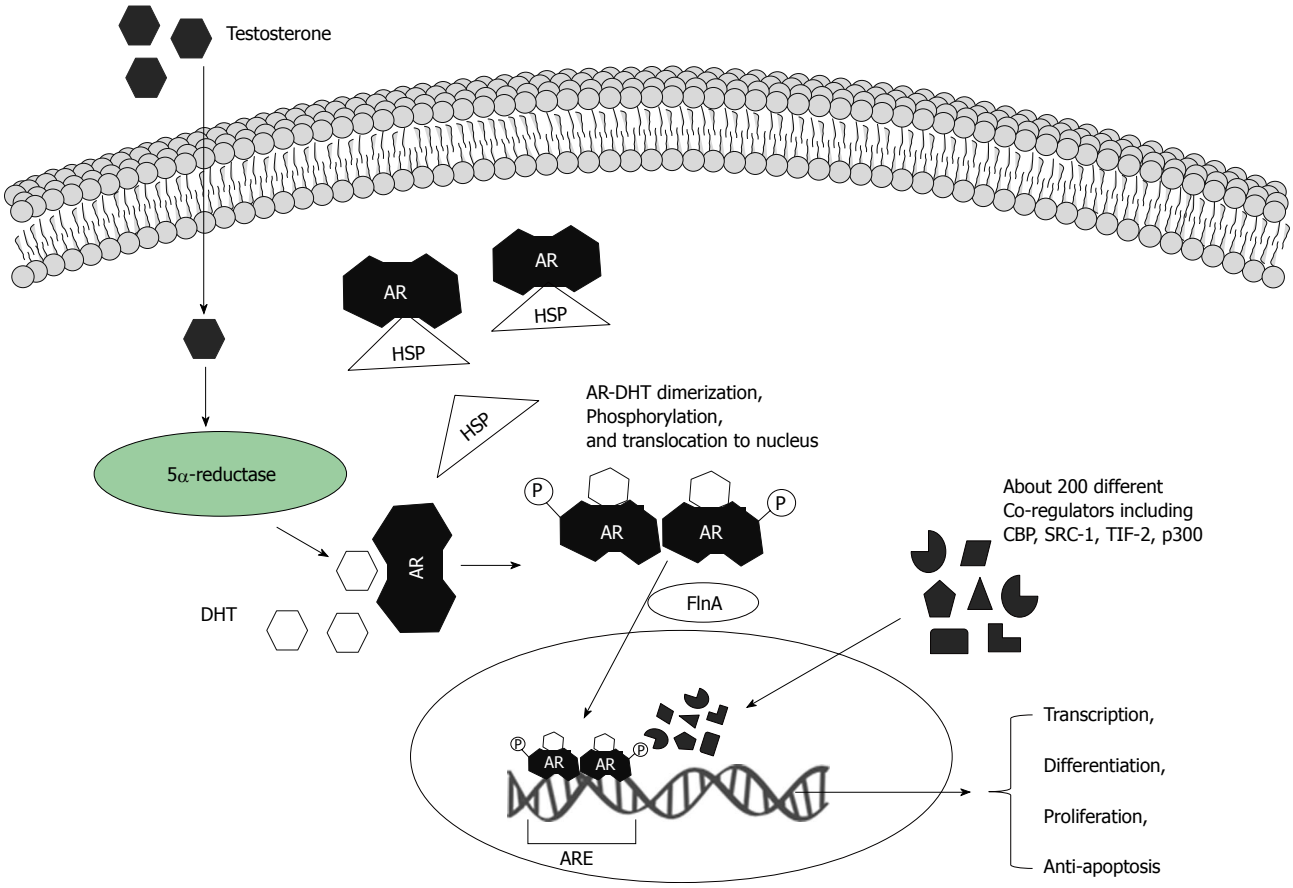

Figure 1 Biological actions of androgens *via* androgen receptors. Testosterone molecules translocate *via* the plasma membrane and are transformed into dihydrotestosterone (DHT) by 5α-reductase. The androgen receptor (AR) is located in cytoplasm and bound to heat shock protein (HSP). DHT binds to the AR and HSP is then released. Ligand-AR complexes can be phosphorylated (and/or are modified by other post-translational mechanisms). Two ligand-AR complexes form homodimers and move into the nucleus. AR nuclear translocation is facilitated by filamin A (FlnA). In the cell nucleus ligand-AR complexes bind to specific DNA elements - androgen-responsive elements (ARE), which are in target gene promoters. These regulate target gene expression at the transcriptional level. A large variety of co-factors and regulators can orchestrate AR-induced gene transcription.

structure and hydrophobicity, and thus it is likely that AR should undergo significant posttranslational modification[49], and/or interact with other proteins that might facilitate AR anchorage to the membranes. Supporting this, Akt1 and lipid raft association with mAR has recently been demonstrated in prostate cancer cells[50]. Several groups have reported specific binding of testosterone to the plasma membrane in different cell types[48,51]. Although mAR has not yet been purified or cloned, using sub-cellular fractionation and immunohistochemistry techniques, membrane association of classical ARs has been shown in Xenopus oocytes, in Chinese Hamster Ovary cells, Sertoli cells, and T cells[49,51-53]. Importantly, mAR activation has been shown to induce profound apoptosis of prostate cancer cells *in vitro* and *in vivo* in mouse xenografts, with suppression not only of cell growth, but also metastatic motility of cancer cells[48]. Activation of these mARs appears also to initiate apoptotic pathways in colon cancers, even in the absence of intracellular AR[54].

Data from these studies indicate that functional mARs trigger strong anti-tumorigenic effects, suggesting mAR as a novel target for the development of selective cancer treatments[55]. However, it remains unclear whether mARs are also expressed in other tumors, including esophageal cancers, and also whether activation of mAR could induce anti-tumorigenic effects similar to those seen in prostate cancer.

ANDROGEN RECEPTORS AS A THERAPEUTIC TARGET

Since Huggins and Hodges[56] demonstrated the effectiveness of surgical castration in men with prostate cancer in 1941, the role of androgens and AR signaling in the carcinogenesis of prostate cancers has been under investigation. Chemical castration with anti-androgen therapy for prostate cancer is standard clinical practice, and has led to substantial gains in survival[57]. Intriguingly, higher serum testosterone levels are not associated with higher rates of prostate cancer. It appears that the prostate is saturated at low levels of circulating androgens and that neither low or high serum testosterone levels are associated with a decrease or increase in the risk of this cancer[58]. When

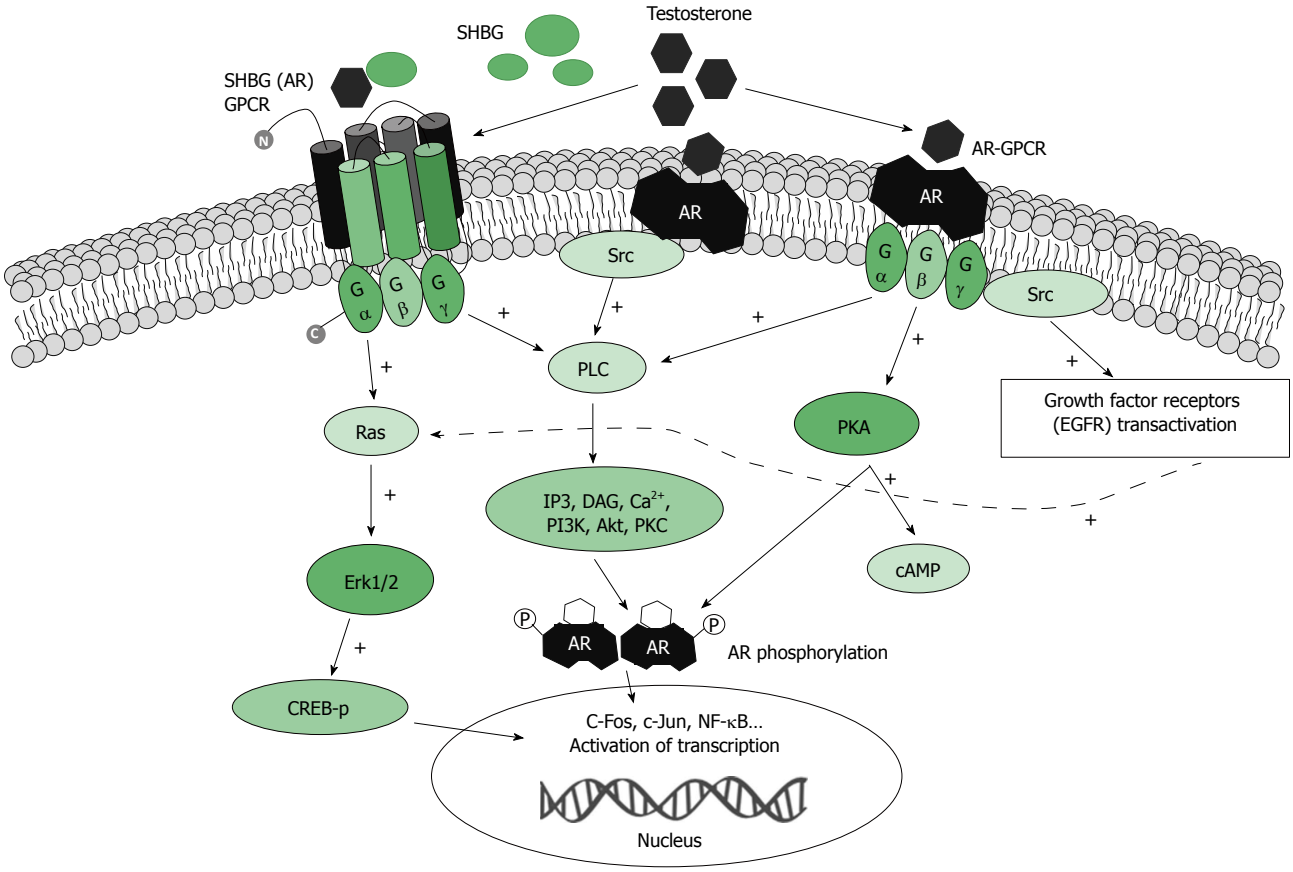

Figure 2 Schematic presentation of testosterone-activated extra-nuclear signaling pathways. Testosterone binds to undefined membrane-associated androgen receptor(s) (mAR) that might transduce signaling downstream to phospholipase c (PLC). Activation of PLC produces several second messengers including Ins(1,4,5)P3 (IP3) and DAG. Ca^{2+} influx then leads to an increase in intracellular Ca^{2+}. Alternatively, in the ERK pathway, Testosterone binds to the membrane-associated receptor, which associates with and activates Src kinase. In a third proposed mechanism SHBG-AR GPCR activates Ras, which in turn activates the cascade of phosphorylation. The ERK pathway phosphorylates CREB to modulate gene expression. AR: Membrane associated androgen receptor; CREB: cAMP response element binding protein; DAG: Diacylglycerol; GPCR: G-protein-coupled receptor; IP3: Inositol trisphosphate; PLC: Phospholipase C; SRC: Src kinase; RAS: RasGTPase protein; +: Indicates positive effect on activation.

considering promotion of prostate cancer growth, the AR is sensitive to basal levels of androgens, and only complete elimination of circulating androgens is effective treatment. In a recent population study a reduced risk of developing esophageal cancer was shown in subset of 343538 patients with prostate cancer who were exposed to androgen deprivation therapy, supporting the involvement of androgens in the development and/or growth of esophageal malignancies[22].

Given the shared embryological origin of prostate and urogenital epithelium, it was also recently proposed that AR signaling might contribute to bladder cancer[59]. This is supported by evidence in murine models that bladder cancer is not induced in AR knockout mice compared with controls[60]. However, clinical studies associate the loss of AR with more advanced stage, indicating that ARs might not be a good therapeutic target[61].

The role of ARs has also been investigated in breast cancer. Emerging evidence suggests that androgen signaling pathways may exert inhibitory effects on the growth of normal mammary epithelial cells and thus play a protective role in the pathogenesis of breast cancer[62]. *In vitro* studies have demonstrated that androgens may counteract the proliferative effect of estrogens in AR-positive breast cancer cells[63]. Up-regulation of AR expression or treatment with AR agonists markedly decreases ER-alpha transcriptional activity[63]. However, the utility of AR ligand therapy has yet to be verified clinically.

While prostate and breast tissue develop and function under direct control of steroid hormones, gastrointestinal tissues are not traditionally considered to be targets of steroid hormones. The mechanisms of AR functioning in these tissues are largely unknown. The expression of ARs in normal and cancerous gastrointestinal tissues also remains controversial. Nevertheless, colonic tissues have been shown to express the majority of functional steroid hormone receptors[54,64]. In a clinical study conducted a decade ago, ARs were expressed in all samples from 35 patients with colon adenocarcinoma, with similar expression in both neoplastic and non-neoplastic surrounding mucosa. However, binding activity for AR exhibited in cancer specimens differed from non-

neoplastic colonic mucosa[65]. Subsequent studies have shown specific AR genotypes to be associated with colorectal cancers[66].

In vivo studies also support a role for ARs in colon cancer. In 1983 Izbicki *et al*[67,68] reported that the administration of androgens promoted colon cancer tumorigenesis in rats, and a year later that the steroid hormones induced tumor growth remission in a mouse xenograft model. More recently, it has been suggested that enhanced susceptibility of male mice to intestinal tumor growth derives from the classical nuclear AR, while in contrast membrane-localized mAR has anti-tumorigenic effects independent of classical AR signaling[56]. mAR consistently induces tumor regression reduced invasiveness, and potent pro-apoptotic responses, supporting potential targeting of mAR for treatment of colon cancer[69].

A few studies have attempted to define the role of ARs in gastric cancers[70-72]. Gan and co-authors evaluated 60 pairs of fresh gastric cancers with matched normal gastric mucosa from patients undergoing gastrectomy[71]. Samples were processed to determine levels of AR mRNA and proteins. Unlike the typical nuclear expression seen in breast and prostate cancer cells, the ARs in the gastric cancers were localized mostly in cytoplasm. Also, the protein level of AR receptors in the gastric cancers was significantly lower than that expressed in normal gastric mucosa, but positive AR expression correlated with a better prognosis[71,72]. However, the authors ultimately concluded that the functional significance of ARs in gastric cancer appeared to be limited.

ANDROGEN RECEPTORS IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

In 1985, Kobayashi[73] investigated the effects of sex hormones on the development of ESCC in a rat model. The highest incidence of ESCC was in male rats, followed by female rats treated with testosterone. This fell to 0% in castrated rats treated with estradiol. Female rats with no hormonal manipulation also had low rates (8%). The authors concluded that ESCC growth is inhibited by estrogen and stimulated by androgens. However, the expression of ARs was not addressed in this study.

AR expression in ESCC is summarized in Table 1. Matsuoka *et al*[74] were the first to detect ARs in a cell line (KSE-1) derived from a male esophageal squamous cell cancer. The KSE-1 cell line had a binding content of 4.2 fmol/mg protein for the ER and 2.2 fmol/mg of protein for the AR in the cytoplasm. Proliferation of this cell line was suppressed by estrogen and accelerated by testosterone. Ueo *et al*[75] characterized ARs and conducted treatment experiments in two ESCC cell lines, KSE-1 and KSE-2. Similar to Matsuoka *et al*[74], the proliferation of KSE-1 was increased by dihydrotestosterone and decreased by estradiol, although sex hormones had no impact on the growth of KSE-2. Receptor analysis found KSE-1 to be positive for both AR and ER, but KSE-2 was negative for both. In a mouse xenograft model, KSE-1 tumor growth was suppressed by estradiol, whereas no effect was seen in KSE-2. Interestingly, no growth-promoting effect was seen with dihydrotestosterone in either cell line *in vivo*, which is consistent with the concept of testosterone saturation in the context of prostate cancer[58]. Tanaka *et al*[76] further investigated KSE-1, and found that the tumor growth stimulation that occurs with AR activation is mediated by fibroblast growth factor 8 (FGF-8) signaling known as androgen-induced growth factor.

Yamashita *et al*[77] identified ARs in ESCC specimens from 21 patients. Two tumors were successfully xenografted into male nude mice and cultured as cell lines, and the growth of the cell lines in the presence of sex hormones was assessed. Similar to the other studies, Testosterone stimulated growth, whereas estrogen had no impact. Tihan *et al*[19] used immunohistochemistry to determine AR expression in ESCC resection specimens. Positive staining for AR was found in 3 of 14 (21%) specimens. No differences in survival were found with respect to AR status, but the study size was small. Yang *et al*[20] detected ARs and ERs in ESCC specimens from 31 patients (26 male, 5 female) using a radio-ligand binding assay method. Compared with the normal esophageal tissues, more ARs were detected in the ESCC tissues (40.56 ± 18.19 fmol/mg *vs* 7.84 ± 3.21 fmol/mg). The expression of AR correlated with gender, tumor differentiation, invasion, and the lymph node metastasis status, but not age of the patient or tumor location[20]. The authors concluded that the expression of AR impacts on the biological behavior and prognosis of ESCC[19,20].

Dietzsch *et al*[78] (2003) investigated androgen pathways in ESCC arising in South African males by targeting AR mutations which were known to occur in prostate cancer. As in prostate cancer, known AR mutations were associated with increased susceptibility to the disease. These authors concluded that this may, in combination with sequencing for other known mutations, be useful in developing a test for genetic susceptibility to the cancer in order to target appropriate investigations. It also suggests that the mutation in question [a short (GGC)n allele] increases AR activity, which would explain the increased incidence of this cancer in men with this mutation, and also implicates AR signaling in the pathogenesis of the disease, at least in some patient populations.

ANDROGEN RECEPTORS IN ESOPHAGEAL ADENOCARCINOMA

Understanding whether androgens influence the development of esophageal cancers is important, as

Table 1 Androgen receptor expression in esophageal cancer

Ref.	No. of patients	Male:female	Histological type	AR	Conclusion
Matsuoka *et al*[74], 1987	NA	NA	SCC cell line	2.2 fmol/mg	Proliferation of KSE-1 cell line is inhibited by estrogen and enhanced by testosterone
Tihan *et al*[19], 2001	25	21:4	AC (n = 11) SCC (n = 14)	7 males and 1females, 5 EAC and 3 SCC were positive	Presence of AR in human esophageal cancer is an impetus for further studies to assess anti-androgen therapy for treatment and or prevention of these tumors.
Yang *et al*[20], 2001	31	26:5	SCC	Cancer tissues: 40.56 ± 18.19 fmol/mg, normal tissues: 7.84 ± 3.21 fmol/mg	AR and estrogen receptors have close relationship with the biologic behavior and prognosis of esophageal SCC
Tiffin *et al*[80], 2003	20	10:10	AC (ND) BE (ND)	Very weakly positive in 1 male with EAC and 1 female with BE	Androgen receptors are not implicated in BE or AC
Awan *et al*[17], 2007	23	20:3	AC (n = 18) SCC (n = 5)	16 samples (EAC = 13, SCC = 3) showed positive nuclear staining. AC occurred in 12 males and 1 female, while in SCC 2 males and 1 female	AR expressed in the stroma of esophageal AC may induce paracrine effects following stimulation by androgens (including tumor-derived), possibly *via* FGFs
Nordenstedt, *et al*[18], 2012	30	20:10	BE (n = 10) Controls (n = 20)	All BE cases were negative. Only 1 male control was found AR expressed in squamous esophageal mucosa	AR were negative in BE warrants further research to find alternative explanations for the male predominance in BE and EAC

AR: Androgen receptor; EAC: Esophageal adenocarcinoma; SCC: Squamous cell cancer; BE: Barrett's esophagus; NA: Not applicable; ND: Not described.

intervention before progression to malignancy offers the possibility of prevention. Barrett's esophagus is recognized to be the precursor for EAC, and offers an opportunity for prevention or early intervention[79]. Case-control studies have tested associations between serum sex steroid hormones and the development of Barrett's esophagus in men, and a strong positive association with free testosterone has been demonstrated[21,79]. This relationship appears to be independent of age or BMI suggesting a direct relationship between testosterone and the progression of Barrett's esophagus to EAC. Contradicting this, however, Nordenstedt *et al*[18] failed to identify ARs in esophageal mucosal biopsies from 10 men with Barrett's esophagus *vs* ARs seen in squamous epithelium from only 1 of 20 controls (10 males and 10 females). They concluded that the absence of ARs in Barrett's esophagus meant that alternative explanations for the male predominance in Barrett's esophagus and EAC should be sought. However, these findings were based on Barrett's esophagus tissues rather than EAC, and the sample size was small.

The first clinical study addressing the role of androgens and AR in EAC was reported by Lagergren and Nyren[35] (1998). This was a population-based, retrospective cohort study of 100215 individuals, including patients diagnosed with prostate cancer and receiving anti-androgen therapy (mainly in the form of estrogen analogues), which attempted to identify whether this treatment altered the risk of EAC. However, analysis by latency intervals after prostate cancer diagnosis revealed no clear trend toward increasing or decreasing risk of esophageal cancer over a 4 year time period[35]. This study did not support either the hypothesis of a tumor-promoting role for androgens, or a protective effect for estrogens. However, the levels of AR expression or serum androgens before and after treatment were not assessed in this study, and the study duration was relatively short. Furthermore, since estrogens were used as anti-androgen therapy, the study actually investigated mixed effects of two different signaling systems rather than AR blockade or deprivation specifically. A more recent study was reported by Cooper and Trudgill[22], who this time identified a reduced risk of developing EAC in the subset of prostate cancer patients undergoing androgen deprivation therapy. This later study suggests further investigation towards potential use of anti-androgens or androgen-deprivation approaches in esophageal cancers should be considered.

Tiffin *et al*[80] analyzed 20 paraffin-embedded specimens of EAC arising in Barrett's esophagus from 10 male and 10 female patients. Both the EAC and Barrett's esophagus regions of the resection specimens were examined. Weak staining for AR was only identified in EAC in one male, and in the Barrett's esophagus segment in one female patient. Mild to moderate staining for estrogen receptors was identified in two EACs from men and in six EACs from women, leading the authors to conclude that androgen pathways were not responsible for gender inequalities in this disease, but that evaluation of ER signaling may be valuable. However, this contrasts with the findings of Tihan *et al*[19] (2001) in which 5 of 11 (45%) EAC specimens were assessed by immunohistochemistry to be AR positive.

Awan *et al*[17] determined AR expression in 18 patients with EAC and 5 with ESCC, with areas of both normal squamous epithelium and cancer examined. Thirteen EAC and three ESCC specimens showed positive stromal staining for AR, but no staining was seen in the cancer epithelium. In normal squamous epithelium from the same patients, ARs were expressed

in 5 patients with EAC and 2 with ESCC. Interestingly, fasting serum testosterone values in male patients were significantly higher in patients with EAC than age-matched controls (median 18.2 nmol/L *vs* 12.5 nmol/L). These levels declined significantly following surgical removal of the cancer, suggesting that paracrine effects of androgens may be implicated in the pathogenesis of the disease[17]. In keeping with the findings of Tanaka *et al*[76] (2001), FGF-8b expression was common in cancer in males but absent in cancer in females and in normal mucosa, further supporting the theory that this is an important growth factor in men.

Paradox of obesity and androgen conversion in esophageal cancers

Androgen as a risk factor for the development of esophageal cancer, in particular EAC, appears at odds with evidence that obesity is a major risk factor for EAC, as obese individuals have lower levels of circulating androgens[7,9,23]. This paradox might be explained by the endocrine actions of visceral fat in males. Male pattern obesity is dominated by the expansion of visceral fat in the abdominal area, which contrasts to predominantly subcutaneous fat deposition in women. Visceral adipose tissue has been shown to act as an endocrine organ and so produces and secretes a wide range of biologically active molecules, including hormones and cytokines[81]. Adipose tissue pathogenicity markedly differs between visceral and subcutaneous fat location. Visceral fat is a highly metabolic tissue marked by higher production of TNFalpha, plasminogen activator inhibitor 1 (PAI1), IL6, and C-reactive protein (CRP), while producing lower amounts of the anti-inflammatory adipokine adiponectin, which correlates more strongly with subcutaneous fat[82-85].

While the accumulation of adipose tissue in abdominal area (visceral fat) is supported by androgens, increased aromatase activity and conversion of androgens to estrogen has been shown to occur in adipose tissues of aging and obese men. Furthermore, adipocytes can secrete estrogens in proportion to total fat mass, and influence physiological processes through paracrine and autocrine actions[85,86]. Active synthesis of estrogens is associated with increased plasma leptin, that, in turn, can stimulate a further reduction of androgen level[30]. In adipocytes, activation of aromatase is self-induced by pro-inflammatory cytokines such as TNF-α which is secreted locally by adipocytes and infiltrating macrophages, and secures conversion of testosterone to estrogen[82,87]. Locally synthesized estrogens may influence cancer cell growth[15]. It is also possible that estrogens may alter the sensitivity of AR to testosterone and thereby impact upon EAC growth, and this might represent a possible therapeutic target in esophageal cancers similar to what has been found in prostate cancers[22,37].

Supporting this, adipose-tissue secreted cytokines and hormones have been shown to exert local paracrine actions in the tissues in which they are formed and on surrounding neighbor cells[81,82,85]. Metabolic conversion of androgen precursors to estrogen and further estrogen metabolites has been observed in several normal tissue and tumors, facilitated by expression of the hormone converting enzyme aromatase as reviewed in detail elsewhere[88]. Notably, the observation that aromatase is often overrepresented in adipose tissue has not yet been verified in visceral fat proximal to esophagus in men.

It has been suggested that increased aromatase activity and conversion of testosterone to estrogens are activated as a feed-back mechanism which regulates adipocyte numbers. This is supported by findings in an aromatase knockout mice where estrogen plays an inhibitory role during adipogenesis to limit adipocyte number[89]. It is possible that in aromatase activation an increased production of estrogen metabolites might result in ineffective, and potentially reversed action of the hormones in aging and obese men, although no work has been reported which addresses this possibility. The potential role of estrogen signaling in esophageal cancers has been recently reviewed, suggesting a role in the genesis of EAC[15]. It remains, however, to confirm that adipose tissue located in close proximity to the esophagus can actively transform androgens to estrogens and influence proliferation of ER-positive esophageal tumors. More detailed investigation of steroid hormone metabolism in adipose tissue located in close proximity to the esophagus is warranted, and an opportunity exists to evaluate abdominal adipose (visceral) tissue samples from individuals with EAC and Barrett's esophagus to see if increased aromatase expression and active conversion of androgens to estrogens is evident.

CONCLUSION

While epidemiological evidence for the male propensity for esophageal cancer is clear, especially the dominance of the male gender in EAC, data evaluating the role of male sex steroids and the androgen receptor in the pathogenesis of this cancer is more difficult to dissect apart. *In vitro* evidence from cell culture work and subsequent testing in murine models suggests a significant influence of sex hormones upon cancer growth, and that this effect is consistent with expression patterns of the receptors. However, the identification of ARs in human tissue specimens has been less straightforward, with expression reported by some authors, but not by others. While androgens and their receptors probably do play a role in the carcinogenesis of esophageal cancer, more work is required to evaluate this role and to understand their

contribution to the genesis of esophageal cancer. Although therapeutic implications and novel therapies are desirable, there is currently insufficient evidence to support a clinical trial of androgen deprivation therapy in this cancer. Further investigation of sex hormone signaling pathways in esophageal cancer appears justified, and in the future this might lead to improved understanding of the genesis of esophageal cancer or even new treatments.

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ORIGINAL ARTICLE

Basic Study

Thrombospondin peptide ABT-898 inhibits inflammation and angiogenesis in a colitis model

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Abstract

AIM: To evaluate the efficacy of the improved thrombospondin mimetic peptide ABT-898 in a murine model of ulcerative colitis.

METHODS: The dextran sodium sulfate (DSS) was used for the induction of colitis in both TSP-1 deficient (TSP-1$^{-/-}$) and wild type (WT) mice during 7 d. While mice were receiving the DSS dissolved in the drinking water, the ABT-898 peptide was dissolved in sterile 5% glucose solution and delivered using mini pumps subcutaneously implanted. Plasma samples were analyzed for interleukin (IL)-6 by ELISA assay and colonic tissues were harvested, fixed and processed for histological evaluation. Immunohistochemistry using antibodies for the detection of CD31 and MECA in endothelial cells was performed. Inflammation was graded in colonic sections and the number of microvessels in each lesion was assessed. Activation of signal transducer and activator of transcription 3 (STAT3) in colonic samples was quantified by immunohistochemistry and Western blotting using antibodies against total STAT3 and phosphorylated STAT3 (pSTAT3) (Ser727).

RESULTS: Treatment with ABT-898 considerably diminished the inflammatory response in WT and TSP-1$^{-/-}$ mice ($P < 0.0001$ in both groups *vs* control). Identification of blood vessels highlighted by CD31/MECA immunohistochemistry, showed significantly reduced vessel counts in colitic lesions of WT and TSP-1$^{-/-}$ mice treated with ABT898 (TSP-1$^{-/-}$ controls/TSP-1$^{-/-}$ treated, $P = 0.0002$; WT controls/WT treated, $P = 0.0005$). Consistently, IL-6 was significantly diminished in plasma samples of TSP-1$^{-/-}$ and WT treated with the peptide when compared to the control mice ($P = 0.0002$ and $P = 0.0148$, respectively). pSTAT3 positive cells were quantified in WT and TSP-1$^{-/-}$ treated with ABT-898. A significant decrease in positive cells for pSTAT3 was observed in treated mice (TSP-1$^{-/-}$ controls/TSP-1$^{-/-}$ treated, $P = 0.0089$; WT/WT treated, $P = 0.0110$). These results were confirmed

by Western blotting analyses showing lower levels of pSTAT3 in colitic lesions from mice treated with the peptide ABT-898.

CONCLUSION: These findings indicate that the new peptide ABT-898 ameliorates inflammation and angiogenesis and might be a therapeutic alternative in IBD and inflammatory diseases.

Key words: Thrombospondin 1; ABT-898; ABT-510; Angiogenesis; Inflammatory bowel disease; Dextran sodium sulfate model; Interleukin-6; STAT-3

Core tip: Inflammatory bowel disease is still incurable and a major burden in the patient's life and health care system. The discovery of new and safe therapeutic alternatives is urgently needed. This study tested the efficacy of a new thrombospondin- derived peptide, ABT-898 in a murine model of colitis. Our results indicate that this peptide was able to ameliorate inflammation and angiogenesis. In addition, mice treated with ABT-898 showed significant decrease of plasmatic Interleukin-6 and lesser activation of signal transducer and activator of transcription 3 in colitic lesions. These findings suggest that ABT-898 may indeed be an alternative treatment for inflammatory bowel disease.

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INTRODUCTION

Thrombospondin 1 (TSP-1) is a well-known anti-angiogenic protein that induces apoptosis in endothelial cells and inhibits their proliferation[1]. This protein regulates inflammation by multiple mechanisms[2] and its expression has been detected in inflammatory diseases such as rheumatoid arthritis and dermatitis[3]. TSP-1 is expressed in kidney diseases including glomerulonephritis[4], suggesting a close association between TSP-1, inflammation and early fibrosis.

TSP-1[5] as well as factors regulating angiogenesis such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are upregulated in inflamed colonic tissues. Angiopoetin-1 and VEGF could activate the innate immune system of the vessel wall, stimulating the production of pro-angiogenic inflammatory cytokines[6]. In addition, these factors have been found altered in the serum of patients with inflammatory bowel disease (IBD)[7]. Mice with a targeted deficiency of TSP-1 (TSP-1$^{-/-}$) show high levels of plasmatic VEGF and bFGF during chronically induced colitis[8].

TSP-1 also modulates vascular leakage and remodeling in acute hypersensitivity[9]. TSP-1$^{-/-}$ mice exhibit a delayed resolution of the inflammation as well as an enhanced vascular remodeling[9]. These mice also display leukocytic infiltrates in the lung, suffer from leukocytosis and augmented colitis[10,11].

As a major activator of transforming growth factor beta 1 (TGFβ1), TSP-1 may play an important role in inflammatory processes[12]. Actually, TGFβ1-deficient mice show enhanced inflammatory phenotype and augmented tumor burden in experimental models of colitis and colorectal carcinogenesis, a phenotype also observed in TSP-1$^{-/-}$ mice[13,14].

The anti-inflammatory and anti-angiogenic properties of TSP-1 are mediated by its interaction with the transmembrane receptors CD36 and CD47[15,16]. TSP-1 induces endothelial cell death upon its binding to CD36. This process upregulates the Fas-Fas ligand system and initiates the apoptotic cascade of caspases[15]. By inducing apoptosis, TSP-1 might also regulate the secretion of cytokines and growth factors implicated in the immune response. TSP-1 also modulates the functions of nitric oxide (NO), a critical molecule involved in a variety of physiological events such as vasodilation and chemotaxis[17].

Peptides corresponding to specific domains of TSP-1, have shown antiangiogenic and anti-inflammatory properties in pre-clinical studies[16,17] and in combined therapies in clinical trials as well[18]. One of these peptides ABT-510 is a nonapeptide peptide simulating the sequence GVITRIR, enclosed within the second type 1 repeat of thrombospondin 1 (TSR). TSP-1 mimetic nonapeptide has the sequence: acetyl-sarcosine-glycine-valine-D-alloisoleucine-threonine-norvaline-isoleucine-arginine-proline-ethylamide (NAcSarGly-Val-D-Ile-T-N-Ile-Arg-ProNHEt). This domain directly interacts with CD36[16] and induces cell death predominantly in endothelial and smooth muscle cells. As a result, this domain has major anti-angiogenic functions. Most recently, a modified and improved TSP mimetic peptide is available, A-428898 (ABT-898; Abbott Laboratories). This peptide is an octapeptide with a sequence that provides more stability and longer half-life (NAcGly-Val-D-Ile-Ser-Gln-Ile-Arg-ProNHEt). Data herein show that mice treated with ABT-898 display reduced inflammation and angiogenesis in colons, lower levels of interleukin (IL)-6 in plasma and decreased signal transducer and activator of transcription 3 (STAT3) activation in colitic lesions. TSP-1 mimetic peptides may ameliorate inflammation and serve as alternative treatment for inflammatory diseases.

MATERIALS AND METHODS

Mice and induction of colitis

All animal procedures were performed with the approval of the Wilkes University Institutional Animal Care and Use Committee, in accordance with the Declaration of Helsinki and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the NIH. DSS (MW: 36000-40000, MP Biomedical, Aurora, OH) was dissolved in the drinking water at a dilution of 2.5% (wt/v) and administered for 7 d to induce acute colitis in WT and TSP-1$^{-/-}$ mice (Jackson Laboratories, Bar Harbor, Maine).

Pump implantation and peptide treatment

WT (n = 16) and TSP-1$^{-/-}$ (n = 13) mice were anesthetized and osmotic mini-pumps (Alzet, Cupertino, CA) were subcutaneously implanted. These mini-pumps contained the peptide ABT-898 dissolved in sterile 5% glucose solution (Abbott Laboratories, Chicago, IL). Pumps delivered this solution at controlled rates (0.5 μL/h). The dose for ABT-898 was 60 mg/kg per day. Pumps containing only sterile 5% glucose were also implanted in WT (n = 15) and TSP-1$^{-/-}$ mice (n = 13) as controls.

Histology and immunohistochemistry

Intestines were removed, opened longitudinally and rinsed with ice-cold phosphate buffer solution (PBS). For morphological studies, tissues were fixed with Histochoice (Electron Microscopy Sciences, Hartfield, PA), processed and cut in serial sections (5 μm). Sections were stained with hematoxylin and eosin for histopathological analysis. Immunohistochemistry (IHC) sections were incubated overnight a purified rat anti-mouse CD31 (BD Pharmingen, San Diego, CA), MECA 32 and STAT3 and phospho-STAT3 (Ser727) (all from BioLegend, San Diego, CA). Sections were immersed in biotinylated goat anti-rat IgG Impress (Vector Laboratories,) diluted in PBS for 30 min. Color was developed using a 3, 3'-diaminobenzidine substrate kit (Vector Laboratories).

Inflammation grade and mean vascular density analyses

Inflammation grading and evaluations of MECA 34/CD31 were performed in colonic sections from mice drinking DSS for 7 d. Sections were screened at low magnification (× 40) to detect areas with colitis. After areas of inflammation were identified, computer digitized images were taken at × 100 or × 400 magnifications using a color digital camera (Olympus Corporation, Tokyo, Japan). Pictures were stored in a memory card and recoded as frame numbers. Frames were blindly analyzed by multiple observers in a monitor in a blindly fashion. A minimum of 5 microphotographs was taken from each colonic section.

Colonic inflammation was graded in hematoxylin-eosin stained sections as follows: 0, no inflammation; 1, modest numbers of infiltrating leukocytes in the lamina propria; 2, infiltration of leukocytes leading to separation of crypts and mild mucosal hyperplasia; 3, massive infiltration of inflammatory cells accompanied by complete disruption of the mucosal architecture and loss of epithelium. The number of pSTAT3 positive cells was recorded for each frame. The number of vessels/field was measured as mean vascular density (MVD). MVD was assessed by counting the vessels in each colitic lesions showing positive stain for MECA/CD31. The number of pSTAT3 positive cells (brown staining) was recorded for each frame/field as well.

Cytokine array and ELISA

Plasma samples were collected from WT and TSP1$^{-/-}$ mice under acute DSS induced colitis after 7 d treated or not with ABT-898. A mouse cytokine Multi-Analyte ELISArray Kit (SABiosciences Frederick, MD) was used to measure the cytokine production of IL1A, IL1B, IL2, IL4, IL5, IL6, IL8, IL10, IL12, IL13, IL17A and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) in mice with or without ABT-898 treatment. The arrays were performed according to the manufacturer's instructions. The absorbance levels of the cytokines were measured on a plate reader (Beckman Coulter DTX 880) at 450 nm. The IL-6 concentrations in the plasma were measured with R&D ELISA (Minn, United States) kit. Briefly, 100 μL of plasma were applied to the immunoplate precoated with anti-human or anti-mouse monoclonal antibody. Secondary detection antibody of each assay was then added to the immobilized IL-6 in the sample. The conjugation of anti-IL-6 with their antigens was visualized using Avidin-HRP substrate. The absorbance levels of proteins were measured on a plate reader at 450 nm. Sampling was performed in triplicates. All the results were representative of multiple and separated experiments. Sampling was performed in triplicates including standards.

Western blotting analyses

Intestinal tissues were collected and briefly rinsed with cold PBS and stored at -80 ℃ until use. Protein was extracted with buffer A (25 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 0.5% NP-40, 0.5% Sodium deoxycholate, 0.05% SDS and 5 mmol/L β-mercaptoethanol) supplemented with protease inhibitors cocktail (BP-477, Boston Bioproducts, Worchester, MA) and phosphatase inhibitors cocktail (BP-480, Boston Bioproducts) prior to use. Tissue was homogenized in buffer A followed by brief sonication (10 s each for 3 times) on ice.

The extract was then centrifuged at 12000 rpm for 10 min at 4 ℃ and the supernatant taken as the total protein lysate for protein analysis. Protein concentration was measured using the Bradford assay (Coomassie Plus, Pierce Rockford, IL). Equal

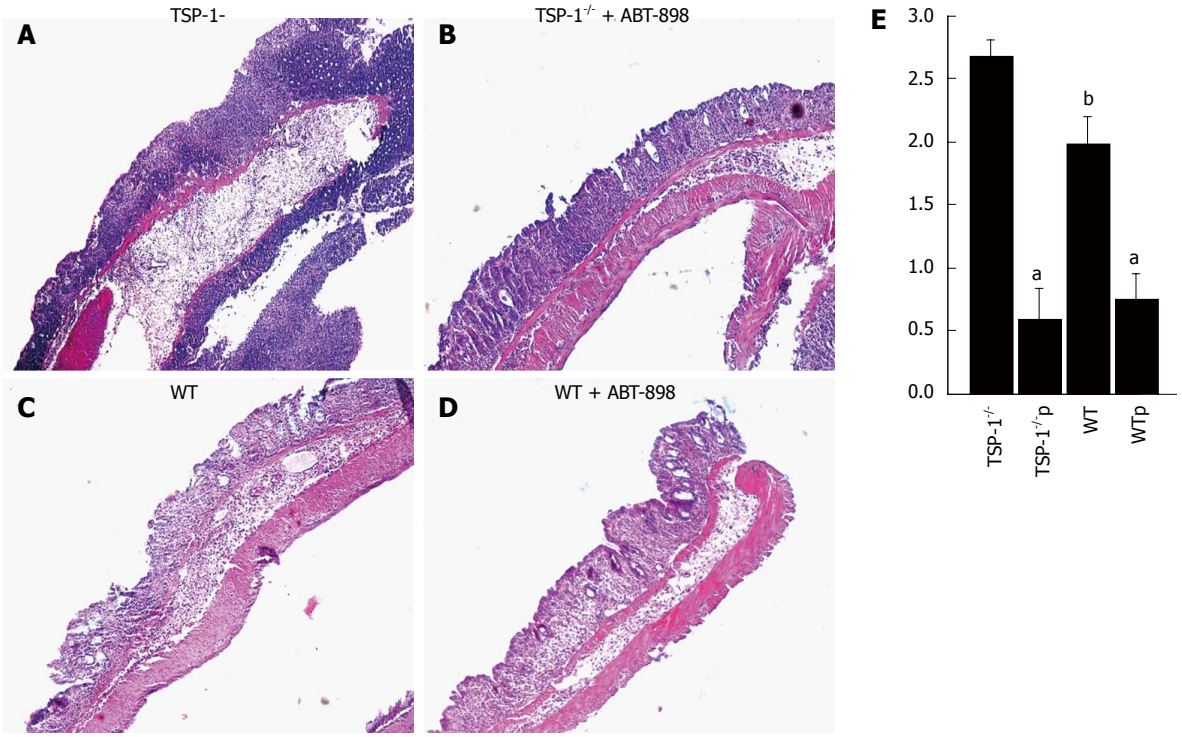

Figure 1 Effects of the TSP-1 mimetic peptide ABT-898 in inflammation. Inflammation was graded by evaluating hematoxylin-eosin stained sections in a blindly fashion (A-D). Sections from colons of TSP-1$^{-/-}$ mice with DSS and treated with 5% glucose (A), sections from colons of TSP-1$^{-/-}$ mice treated with ABT-898 (B), colonic sections from WT mice treated with 5% glucose (C) and with the peptide ABT-898 only (D). The peptide ABT-898 significantly reduced the leukocytic infiltration in both, WT and TSP-1$^{-/-}$ colitic lesions when compared with their controls (E). TSP-1$^{-/-}$ mice controls (A) displayed more inflammation that WT controls (C). $^{a}P < 0.0001$ and $^{b}P < 0.05$ *vs* control. TSP-1$^{-/-}$: TSP-1$^{-/-}$ controls; TSP-1$^{-/-}$p: TSP-1$^{-/-}$treated with the ABT-898 peptide; WT: WT control; WTp: WT treated with the ABT-898 peptide.

amounts of protein were separated by SDS-PAGE and transferred onto the nitrocellulose membrane by a semi-dry transfer procedure (Bio-Rad, Hercules, CA). The quantitative Western blot (WB) was carried out according to the Odyssey protocol (LI-COR, Inc, Lincoln, NE). Briefly, the membrane was blocked for 1 h at room temperature in the blocking buffer. Then, it was hybridized with the primary antibody against total STAT3 or phospho-STAT3 (Ser727) (p-STAT3) at 1:1000 dilution in TBST (50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 0.1% Tween-20) containing 1% bovine serum albumin overnight at 4 ℃. The membrane was then washed with TBST for 3-5 times, followed by hybridization with infrared IRDye®-labeled secondary antibody at a dilution of 1:5000 in TBST/1% BSA for 1 h. The membranes were washed with TBST (× 5) and PBS (× 2) for image acquisition using an Odyssey infrared scanner (Li-cor, United States). Data were analyzed using the Odyssey software 3.0 to quantify of pSTAT3 in three independent Western blotting analyses.

Statistical analysis

Data was analyzed for significance by a one-way ANOVA (analysis of variance). Calculations were performed using StatView system for Macintosh (Abacus Concepts, Berkeley, CA). $P < 0.05$ was considered significant. Where appropriate, values are expressed as the mean ± SE.

RESULTS

ABT-898 decreases inflammation in both WT and TSP-1 deficient mice

Inflammation was graded by evaluating hematoxylin-eosin stained sections in a blindly matter (Figure 1A-D). TSP-1 deficient mice showed enhanced inflammation grade at DSS doses of 2.5% (Figure 1A). The leukocytic infiltrate was usually involving all the layers of the colonic wall with multiple erosions. WT mice treated with DSS only, also showed erosions and heavy inflammation (Figure 1B). However, the inflammatory infiltrate was mostly confined to the mucosa of the colon. Treatment with ABT-898 considerably diminished the inflammatory response in WT and TSP-1$^{-/-}$ mice (Figure 1C and D, respectively; $P < 0.0001$ *vs* control). The peptide ABT-898 significantly reduced the leukocytic infiltration in both WT and TSP-1$^{-/-}$ colitic lesions when compared with their controls (Figure 1E; $P < 0.0001$ *vs* control). TSP-1 deficient mice receiving glucose showed a higher grade of inflammation when compared to WT mice controls (Figure 1A and C). These results were statistically significant (Figure 1E; $P = 0.0157$).

ABT-898 diminishes the microvessel density in colitic lesions in WT and TSP-1 deficient mice

TSP-1 is an angiogenic regulator that induces apoptosis in endothelial and smooth muscle vascular

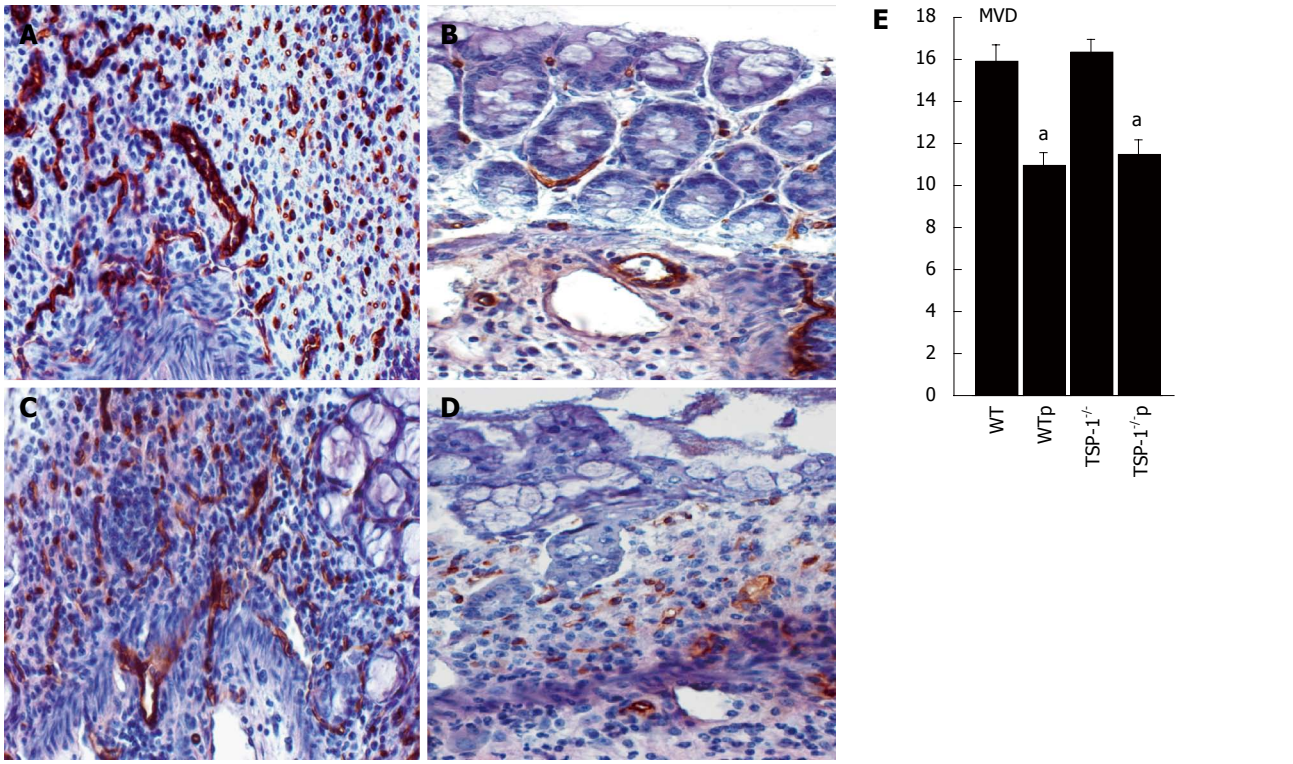

Figure 2 Effects of the TSP-1 mimetic peptide ABT-898 on microvascular density in colons with dextran sodium sulfate-induced colitis. Combined immunohistochemistry against the endothelial markers CD31 and MECA 34 (brown staining) was performed to analyze the vascular density in colitic lesions (A-D). Evaluation of vascular density in TSP-1$^{-/-}$ colonic sections from mice treated with 5% glucose (A), and ABT-898 (B), WT mice controls (C), and the ABT-898 peptide (D). Sections from mice receiving the ABT-898 peptide showed a significant decrease in microvascular density (MVD) *vs* controls (E) ($P < 0.0001$ for TSP-1$^{-/-}$ colons and $P = 0.0048$ for WT). $^{a}P < 0.0001$ *vs* control. TSP-1$^{-/-}$: TSP-1$^{-/-}$ controls; TSP-1$^{-/-}$p: TSP-1$^{-/-}$ treated with ABT898; WT: WT control; WTp: WT treated with ABT-898 peptide.

cells. Colonic tissues of both genotypes TSP-1$^{-/-}$ and WT showed both a high density of microvessels (MVD) in the DSS- induced lesions (Figure 2A and C, respectively). Conversely, CD31/MECA positive blood vessels were significantly reduced in colons from WT and TSP-1$^{-/-}$ mice treated with ABT898, TSP-1$^{-/-}$ controls/TSP-1$^{-/-}$ treated ($P = 0.0002$; Figure 2A, 2B and 2E, respectively). WT controls and WT treated ($P = 0.0005$; Figure 2C-E, respectively). No significant changes were detected in MVC between WT and TSP-1$^{-/-}$mice, indicating that endogenous TSP-1 does not inhibit angiogenesis. Changes in angiogenesis in this model may be related to the concentration of DSS used and the duration of the treatment. TSP-1 deficient mice have shown increased MVC and secretion of pro-angiogenic factors only when using higher concentrations of DSS or when it was delivered during multiple cycles[19,20].

Plasma levels of IL-6 in mice with induced colitis are reduced after the treatment with the ABT-898 peptide

A cytokine ELISA array was used to screen 12 cytokines in plasma of mice treated and untreated with ABT-898. Levels of IL-6 were particularly higher in control mice and very much reduced after the treatment with ABT-898. In order to validate these results plasma levels of IL-6 from treated and untreated mice were measured by using specific sandwich-based ELISA for each cytokine. Consistently, IL-6 was significantly diminished in plasma of TSP-1$^{-/-}$ treated with the peptide ($P = 0.0002$). WT samples under the same treatment showed similar results ($P = 0148$). TSP-1 deficient mice untreated displayed higher levels of IL-6 compared to WT mice treated with saline solutions as well ($P = 0.0114$; Figure 3).

STAT3 is activated in TSP-1 deficient colons treated with DSS and inhibited by the peptide ABT-898

Activation of STAT3 is one of the main signaling mechanisms in response to IL-6 action[20]. The activation of STAT3 is mediated through the phosphorylation at Tyr705 or Ser727 site. This step is required for dimerization, nuclear translocation and DNA binding. Western blot with pSTAT3 (Ser727) antibody showed that pSTAT3 was increased 2-fold in the inflamed TSP-1$^{-/-}$ colon tissues treated with DSS, as compared to the WT. When the colon tissues were not affected by DSS treatment, the levels of pSTAT3 were about the same (Figure 4A). However, both WT and TSP-1$^{-/-}$ showed lower levels of p-STAT3 when treated with the peptide ABT-898 and DSS. pSTAT3 levels were quantified after normalization with total STAT3 in three independent western blotting experiments. These analyses showed that the specific

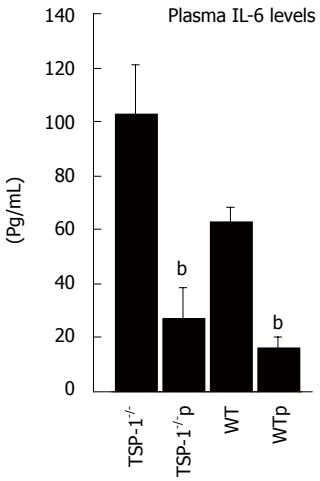

Figure 3 Interleukin-6 protein levels in plasma of mice under dextran sodium sulfate-induced colitis. Interleukin (IL)-6 levels were analyzed using sandwich-based ELISA. Elevated levels of IL-6 were detected in WT and TSP-1$^{-/-}$ plasma samples of mice treated with DSS for 7 d receiving saline injections (P = 0.0114). IL-6 was significantly diminished in plasma of TSP-1$^{-/-}$ treated with the peptide (P = 0.0002) and WT samples under the same treatment (P = 0.0148). $^{b}P < 0.05$ *vs* control. Error bars represent SEM. The results shown are representative of three or more independent experiments. TSP-1$^{-/-}$: TSP-1$^{-/-}$ controls; TSP-1$^{-/-}$p; TSP-1$^{-/-}$ with ABT-898 peptide; WT: WT control; WTp: WT treated with ABT-898 peptide.

pSTAT3 is increased under induced colitis in TSP1$^{-/-}$ and decreased by ABT-898 (Figure 4B).

Counts of pSTAT3 positive cells were significantly reduced in TSP-1$^{-/-}$ colons treated with the peptide ABT-898

IHC with p-STAT3 (Ser727) revealed that active STAT3 was distributed mainly in the nuclei of epithelial cells. Nuclear staining was also observed in the luminal epithelium and crypts of both groups. Nuclei of the endothelial cells in some venules and arterioles were strongly positive. Cytoplasmic staining was observed in cells undergoing mitosis. p-STAT3 was predominantly expressed in the inflamed epithelium and leukocytic infiltrate of TSP-1$^{-/-}$ and WT intestines controls (Figure 5A and C, respectively). When positive cells were quantified in WT and TSP-1$^{-/-}$ treated with ABT-898 mice (Figure 5B and D, respectively) a significant decrease in positive cells for pSTAT3 was observed (Figure 5F, TSP-1$^{-/-}$/TSP-1$^{-/-}$ p, P = 0.0089; WT/WTp, P = 0110).

DISCUSSION

Previous results have shown that TSP-1 significantly diminishes inflammation and angiogenesis in the DSS model of colitis[11]. Secretion of pro-angiogenic factors such as VEGF and basic fibroblast growth factor were significantly higher in TSP-1$^{-/-}$ mice under multiple cycles of DSS[8]. In this study, a second generation TSP-1 derived peptide; ABT-898 significantly ameliorates inflammation and angiogenesis in the

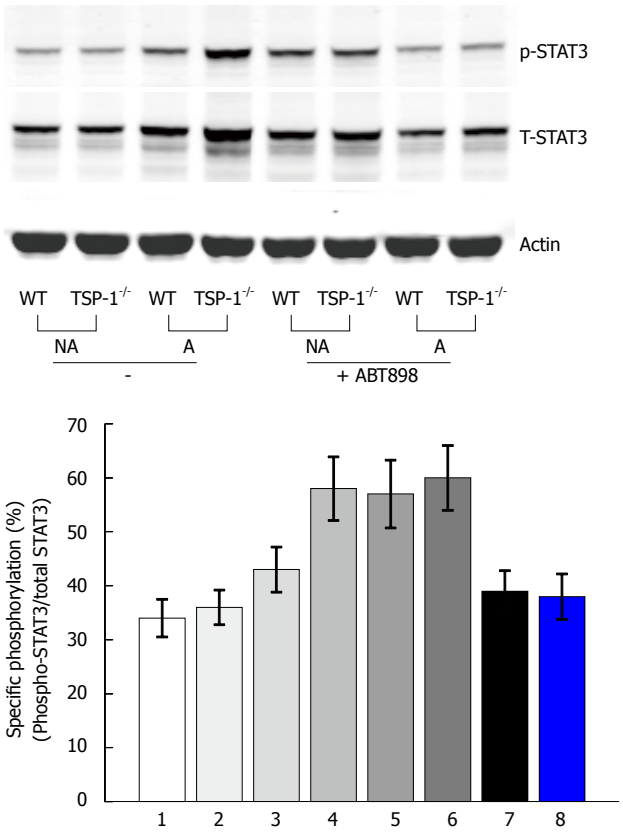

Figure 4 Activation of STAT3 by Western blotting. Tissue lysates were prepared as described in "MATERIALS AND METHODS", equal amounts of protein (80 μg) were resolved by 4%-12% SDS-PAGE, followed by Western blot with antibodies against STAT3, phosphorylated STAT3 (pSTAT3) (Ser727) and actin, respectively (bottom panel). STAT3 activation under acute colitis induced by DSS and the effects of the ABT-898 peptide on STAT3 activation during DSS-induced acute colitis; Top panel shows the specific phosphorylation of STAT3 from three repeated Western blots as represented by the bottom panel. The average values with standard deviations are showed. NA: Not affected colon tissue (no DSS); A: DSS induced colon tissue; TSP-1$^{-/-}$: TSP-1$^{-/-}$ controls; TSP-1$^{-/-}$p: TSP-1$^{-/-}$ treated with ABT-898 peptide; WT: WT control.

DSS mouse model. Data shown herein indicate that ABT-898 significantly reduces the plasmatic levels of IL-6 in these colitic tissues[21].

IL-6 levels have been found elevated in several models of colitis[22,23]. Baseline levels of the anti-inflammatory cytokines IL-10 and TGFβ1 were significantly elevated in IL-6 deficient mice compared with WT mice[23]. IL-6 regulates the secretion of TSP-1 by monocytes[24] and modulates the localization of TSP-1 in the vascular compartment[25]. Several reports have underlined the importance of IL-6 in angiogenesis and inflammation. This cytokine is inhibited when VEGF is silenced using RNAi technology[26]. TSP-1$^{-/-}$ vascular cells could modulate the innate immune system directly and indirectly through production of cytokines such as IL-6. It has been documented that VEGF induces the production of IL-6 in endothelial cells but not in leukocytes[27]. Vascular cells may be a natural source of such production since vascular changes and angiogenesis are critical components of any inflammatory process.

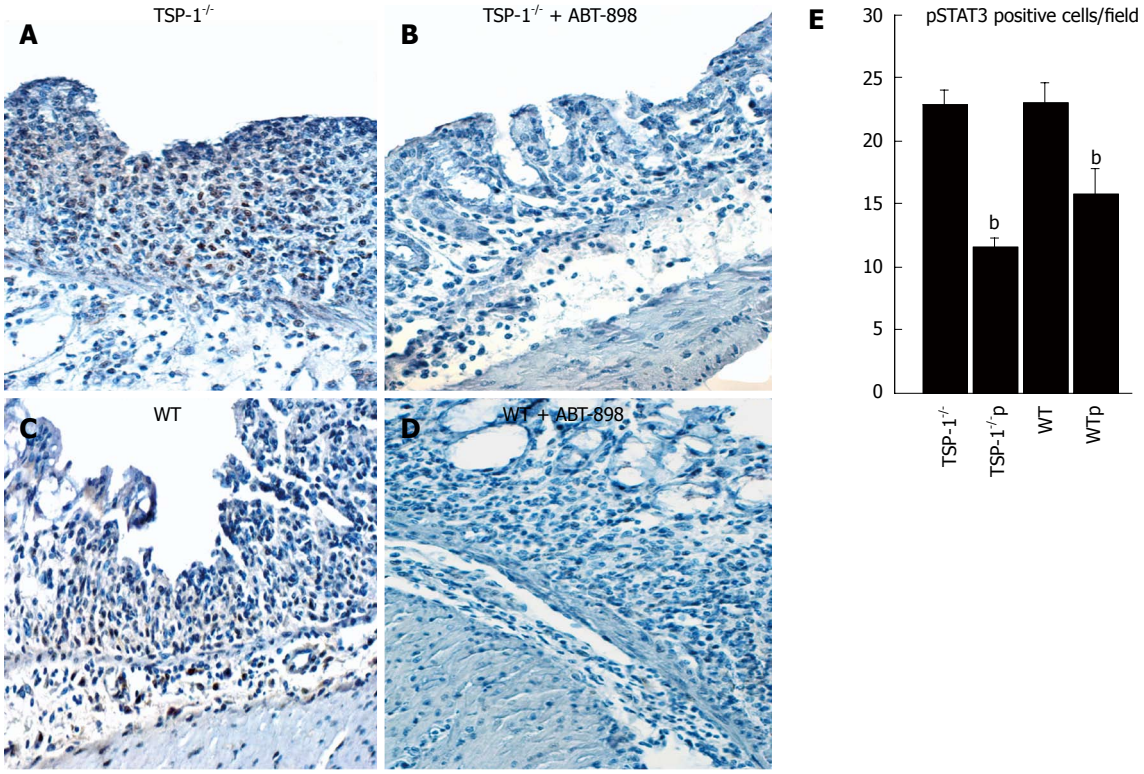

Figure 5 Activation of STAT3 by immunohistochemistry. Immunohistochemistry for pSTAT3, in colitic tissues of TSP-1$^{-/-}$ controls (A), TSP-1$^{-/-}$ colonic tissues treated with ABT-898 (B), colons from WT mice (C) and from WT mice treated with the ABT-898 peptide (D), pSTAT3 positive brown staining was detected in the nucleus of epithelial cells and infiltrating leukocytes. Quantification of pSTAT3 positive cells was performed in colonic sections (E). Colons from WT and TSP-1$^{-/-}$ mice used as control showed higher numbers of positive cells when compared to mice treated with ABT-898. $^{b}P < 0.05$ *vs* control. NS: Not significant; TSP-1$^{-/-}$: TSP-1$^{-/-}$ controls; TSP-1$^{-/-}$p: TSP-1$^{-/-}$ treated with ABT-898 peptide; WT: WT control; WTp: WT treated with ABT-898 peptide.

IL-6 interacts with TGFβ1[28,29] and plays an important role in the development of IBD. Inhibition of IL-6 signaling decreases IL-6 secretion in an inflammatory colon cancer model[28] and high levels of IL-6 have been found in patients suffering of IBD[30]. The functions of IL-6 are regulated by a series of events, starting with the binding to its receptor (IL-6R). Two subunits of the IL-6R have been identified: an 80 kDa ligand-binding subunit, known as IL-6 receptor alpha (IL-6Rα), and a 130 kDa signal-transducing subunit, gp130 (IL-6Rβ). The gp130 is associated with the IL-6/IL-6Rα, initiating the phosphorylation of the C-terminal domain of gp130 by JAK1/2 and the recruitment of STAT3 and its subsequent phosphorylation. STAT3 is considered a potent anti-apoptotic factor, directly involved in IBD and cancer[31].

STAT3 is recruited to the receptor subunit gp130 and activated through phosphorylation by JAK1/2. The phosphorylation then renders the dimerization of STAT3 and its translocation into the nucleus to regulate the transcription of responsive genes, including cytokines and growth factors[19,20]. IL-6 and activated STAT3 have been reported as crucial for intestinal carcinogenesis in a model of colitis-associated cancer[32].

The activity of IL-6 through the STAT3 pathway was evaluated in this study and the status of pSTAT3 was determined. The phosphorylated form of STAT3 is expressed in both WT and TSP-1$^{-/-}$ colons, and almost abolishes in mice treated with the antiangiogenic peptide ABT-898. This peptide inhibits angiogenesis and chemotaxis to the colitic areas, as was evidenced by the diminished inflammation and MVD in both genotypes after the treatment with ABT-898. Treatment of mice under colitis with this peptide decreases MVD in both genotypes even at lower doses of DSS as we show herein. TSP-1 induces apoptosis in endothelial cells through its interaction with CD36. CD36 and IL-6 regulate oxidative stress and both are associated with metabolic and inflammatory diseases[33]. In addition, soluble CD36 has been associated with high levels of IL-6 in men with impaired glucose tolerance[34]. Our results here indicate that ABT-898 is able to inhibit the activation of STAT3 in colitic tissues. These data also confirm the importance of regulating the secretion of IL-6 for the resolution of the inflammatory response. IL-6 thus represents an alternative mechanism by which TSP-1 and its derived peptides could be a protective factor against chronic inflammation and carcinogenesis.

Recent studies have shown that the activation of the axis IL-6/STAT3 enhances the secretion of TSP-1[35,36]. STAT-3 as TSP-1 both exhibit paradoxical roles in inflammation and cancer. In addition, the functions of TSP-1 are dose and tissue depending and may vary due the presence of specific receptors with

which TSP-1 may interact[37]. While the expression of TSP-1 in the normal colon is not significant, in DSS colitic lesions is highly upregulated[5,11]. TSP-1 is intensely expressed during the acute phase of inflammation in the several models[38]. The releasing of TSP-1 upon injury suggests a protective role in intestinal homeostasis. In addition, lacking of endogenous TSP-1 enhances angiogenesis and inflammation in colitis[11].

Data herein indicate that TSP-1 could diminish the level of pSTAT-3. These results could be solely a consequence of the severe inflammation observed in TSP-1 deficient mice. However, results from our gene microarray data might provide some insights. S100A9 is an important marker for inflammation that can activate STAT3[39,40]. TSP-1 derived peptides upregulated S100A9 at the transcriptional level in the same model of colitis[41]. However, the TSP-1 peptide containing the activating domain of TGFβ1 showed the lowest expression levels of S100A9 among the treated groups. These results suggest that TSP-1 could reduce pSTAT3 in the colon by TGFβ1 related mechanisms.

Our data demonstrate that ABT-898 is effective in controlling colonic inflammation and angiogenesis. Treatment of cancers with ABT-898 has shown to be quite effective in pre-clinical studies as well[42,43]. In addition the treatment with this peptide significantly reduces the secretion of IL-6 and inhibits the activation of the STAT3 system. As clinical trials are testing the efficacy of IL-6 antibodies in cancers, the possibility of using peptides such as ABT-898 warrants further investigation.

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COMMENTS

Background

Inflammatory bowel disease (IBD) consists of two major forms of chronic intestinal inflammation, ulcerative colitis and Crohn's disease. Its etiology remains unknown and no cure is yet available. Strong evidence suggests that IBD is the result of an immune deregulation of the intestinal mucosa. Vascular changes and enhanced angiogenesis (formation of new blood vessels from pre-existent ones) indicate that an abnormal angiogenic response may be also implicated, enhancing the recruitment of inflammatory cells and contributing to the mucosal damage. Therapeutic approaches targeting angiogenesis may be safe alternatives for treating inflammatory diseases such as IBD.

Research frontiers

Thrombospondin 1 (TSP-1) is natural antiangiogenic that has a role in inflammation and cancer. This protein has multifunctional domains that interact with a variety of growth factors and extracellular proteins. During the last decade, peptides derived from specific domains of TSP-1 have been developed. One of these peptides ABT-510 mimicked the anti-angiogenic properties of TSP-1 and it was evaluated in pre-clinical experiments and proved safe in clinical trials. However, this peptide seemed to be effective only when combined with cytotoxic therapies. This study evaluates the efficacy of a second-generation peptide ABT-898, designed to be more effective by providing a longer half-life and better solubility.

Innovations and breakthroughs

This study is using a model of colitis to test the therapeutic effects of a new and improved TSP-derived peptide. ABT-510 was effective not only inhibiting angiogenesis but also ameliorating the inflammation and mucosal damage. These results link an antiangiogenic peptide with the signal transducer and activator of transcription 3 (STAT3), a key factor in inflammation and cancer. These data suggest an alternative mechanism by which TSP-1 might decrease inflammation warranting further investigation.

Applications

The study suggests that the TSP-1 peptide ABT-510 might be effective as a therapeutic agent for inflammatory bowel disease and other inflammatory conditions.

Terminology

Thrombospondin 1 is a disulfide-linked homotrimeric protein. This protein is an adhesive glycoprotein secreted by a variety of cells but it is storage in the extracellular matrix. TSP-1 has the following major domains: an amino-terminal heparin-binding domain, a procollagen domain, a properdin-like type I repeats, and a globular carboxy-terminal domain. The protein also contains type II repeats with epidermal growth factor-like homology and type III repeats that contain an RGD sequence. ABT-510 (Abbott Laboratories) was formulated based on the sequence GVITRIR within the type I repeats: Ac-Sar-GV-Dallolle-T-Nva-IRP-ethylamide. ABT-898: Ac-GV-Dallolle-SQIRP-ethylamide.

Peer-review

This is an interesting study evaluating the efficacy of second generation TSP-1 derived peptide in a colitis model. These data suggest that ABT-898 could be an effective therapeutic tool for inhibiting inflammation and angiogenesis in inflammatory bowel disease.

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ORIGINAL ARTICLE

Basic Study

Insights into glycan biosynthesis in chemically-induced hepatocellular carcinoma in rats: A glycomic analysis

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Author contributions: Amin A designed the study, supervised the experimental work, interpreted the data and helped to write the manuscript; Bashir A helped in data interpretation, writing the manuscript and in the presentation of some figures; Bashir A performed the *t*-test statistical analysis; Zaki N helped with data interpretation; McCarthy D performed the glycomic experiment, helped in writing the manuscript and in the presentation of some figures; Lotfy M shared in the statistical analysis; all authors read and approved the final manuscript.
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Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at (a.amin@uaeu.ac.ae).

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Abstract

AIM: To evaluate the qualitative and quantitative changes in N-linked glycosylation, which occurred in association with diethyl nitrosamine-induced hepatocellular carcinoma (HCC) in rodents.

METHODS: Liver tissues of (1) normal (non-tumor-bearing) rats; and (2) tumor-bearing rats; were collected and were used for histological and GlycanMap® analyses. Briefly, GlycanMap® analysis is a high-throughput assay that provides a structural and quantitative readout of protein-associated glycans using a unique, automated 96-well assay technology coupled to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and custom bioinformatics. Histopathological studies were carried out to ensure the development of HCC in the tested animals.

RESULTS: The N-glycomic analysis revealed 5 glycans; $Glc_1Man_9GlcNAc_2$, $Gal_2Man_3GlcNac_4Fuc_1Neu_1$, $Man_4GlcNac_2$, $Gal_2Man_3GlcNac_4Neu_3OAc_3$, and $Man_3GlcNac_5Fuc_1$, which showed significant changes in rat HCC tissues when compared with normal liver tissues. Four glycans were increased ($P < 0.05$) and $Glc_1Man_9GlcNAc_2$ was decreased (5.89 ± 0.45 *vs* 3.54 ± 0.21, $P < 0.01$) in HCC tissues compared to normal

liver tissues. An increase (66.5 ± 1.05 *vs* 62.7 ± 1.1, $P < 0.05$) in high-mannose structures in HCC rats was observed compared to normal rats. Importantly, HCC rats showed an increase ($P < 0.05$) in both tumor-associated carbohydrates and in branched glycans. The changes in glycans correlated well with glycan flow changes reported in the glycan biosynthetic pathway, which indicates the importance of enzyme activities involved in glycan synthesis at different subcellular localizations.

CONCLUSION: The reported HCC-associated changes in glycan flow and subcellular localization explain the increase in high mannose glycans and siayl Lewis glycans common in HCC liver tissues.

Key words: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; Hepatocellular carcinoma; Glycomics; Biosynthetic pathways

Core tip: Hepatocellular carcinoma (HCC) is a leading cause of cancer death worldwide, yet it is still poorly diagnosed. Glycans are emerging as sensitive and simple biomarkers of various malignant diseases. Utilizing the cutting-edge N-glycomic analysis, we identified 5 glycans that were significantly different between normal and tumor-bearing rats. An increase in high-mannose structures in HCC rats was observed compared to normal rats. HCC rats showed an increase in both tumor-associated carbohydrates and branched glycans. The changes in glycans correlated with glycan flow changes reported in the glycan biosynthetic pathway, which indicates the importance of enzyme activities involved in glycan synthesis at different subcellular localizations.

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INTRODUCTION

Glycosylation is an important post-transcriptional modification with over 50% of total proteins being glycosylated[1]. Glycosylation is crucial for cellular interaction; therefore, cancer involves noticeable changes in glycan biosynthesis. Thus, protein glycosylation (N- and O- glycosylation) is sensitive to environmental changes and is commonly disrupted in various diseases such as cancer[2-4]. Altered N-glycans and O-glycans may result from changes in the expression levels of glycan synthetic enzymes or from disruption of the biosynthetic pathway such as the ER-Golgi chain[5]. These changes can provide valuable insights into cancer development and its progression, and these altered glycosyl epitopes are classified as tumor-associated carbohydrate (TAC) antigens. Some of those TAC antigens are already in clinical use for the diagnosis and monitoring of cancers[6-9]. Recent technological advancements in the area of glycan isolation and characterization has turned glycomics into a new potential tool for cancer prognosis and treatment.

Since the beginning of the glycomics era, liver diseases such as fibrosis, cirrhosis and hepatocellular carcinoma (HCC) have been the prime targets in many investigations[10-13]. That trend was mainly driven by the lack of reliable diagnostic biomarkers of HCC. Many studies have shown that the serum N-glycan profile is a non-invasive marker for liver diseases such as HCC[12,14-17]. Serum N-linked glycoproteins are mainly produced in the liver and by B-lymphocytes, thus changes in the serum N-glycan profile reflect the physiology of liver and/or B-cells[12]. Various studies have shown significant changes in total N-glycan levels in the serum of HCC patients, thereby demonstrating the potential of glycans and glycome profiles as efficient biomarkers of HCC[18,19]. Alpha fetoprotein (AFP) is the only known biomarker used for HCC. AFP levels remain unchanged during the onset of HCC, which makes its application as a differential diagnostic marker of other liver diseases unreliable[20,21]. The glycosylated form of AFP, AFP-L3, was proposed as new tumor marker and was approved by the FDA in 2006, for the early detection of primary HCC[22]. Studies also showed an increase in the fucosylated glycans in HCC patients compared to patients with chronic liver diseases[23,24]. However, there was no such increase in fucosylation in the liver tissues of HCC patients when compared with normal tissues, signifying the importance of glycan analyses in cancer tissues along with serum[25]. The serum profile of diethyl nitrosamine (DEN)-induced HCC has been investigated for new biomarkers[26]. The present study focuses on the analysis of N-glycan changes in DEN-induced HCC liver tissues. The glycan profiles of liver lysates from tumor-bearing rats and normal rats were analyzed using the Ezose Glycan*Map*® platform to identify changes in the *N*-glycans of liver tissues in a HCC rodent model. Changes in N-glycan levels in the liver tissues could identify novel alterations in N-glycan profiles that are highly specific to HCC and its progression. A glycomic marker in rat livers may also be useful for screening drugs in this DEN-HCC rat model.

MATERIALS AND METHODS

Animal care and use statement

Adult male albino rats, Wistar strain, (150-200 g) were obtained from the Animal House, UAE University, UAE.

They were maintained on a standard pellet diet and tap water *ad libitum* and were kept in polycarbonate cages with wood chip bedding under a 12 h light/dark cycle at room temperature (22 ℃-24 ℃). The rats were acclimatized to the environment for two-weeks prior to experimental use. This study was approved by the Animal Research Ethics Committee, UAE University.

Hepatocarcinogenesis model

Hepatocarcinogenesis was initiated by DEN and promoted by 2-Acetylaminofluorene (AAF) as described by Espandiari *et al*[27] and modified by Amin *et al*[28]. Briefly, as a mitotic proliferative stimuli, 4-d fasted rats were re-fed and the following day the rats were injected once intraperitoneally with DEN at 200 mg/kg b.wt., dissolved in saline. Two weeks post-DEN treatment, the rats received 6 daily intragastric doses of 2-AAF (30 mg/kg in 1% Tween 80) to promote liver cancer.

Treatment regime

The rats were divided into 2 groups (7 animals per group) as follows: Group 1 (normal): Rats were administered water at 5 mL/kg b.wt throughout the experimental period and were injected with one dose of saline. In Group 2 (tumor-bearing or HCC): Hepatocarcinogenesis was developed as detailed earlier. Group 1 was treated with an equal volume of vehicle. After 22 wk of DEN administration, all animals were anesthetized 24 h after the last treatment. Following anesthesia, liver samples were dissected out.

Histology and immunohistochemistry

Diethylether-anesthetized rats were sacrificed and the livers excised. Samples of the right, left and caudate liver lobes were immediately fixed in 10% buffered formalin for histopathological examination. The remaining liver was frozen in liquid nitrogen and stored at -80 ℃. Histological sections were embedded in paraffin after being dehydrated in ethanol. Five-micrometer sections were mounted onto slides, stained with Hematoxylin and Eosin and then examined under an Olympus DP71-light microscope.

For the immunohistochemistry assay, mounted sections were immersed in sodium citrate buffer (0.1 mol/L, pH 6) and placed in a water bath for 15 min to unmask antigen epitopes. To prevent nonspecific binding to endogenous peroxidase, the sections were incubated with 0.3% H_2O_2 (Sigma Chemical Co., United States) in methanol. Anti-GST-p (Medical and Biological Laboratories Co., Tokyo, Japan) was incubated with the slides overnight at 4 ℃. The slides were then washed with PBS and incubated with secondary antibody, polyvalent biotinylated goat-anti-rabbit antibody, for 10 min at room temperature (1:200 dilution). The universal LSAB kit and DAB plus substrate kit were both used to perform a standard staining protocol and additional counter-staining was performed using hematoxylin. Slides were mounted and observed under an optical microscope (Olympus DP71), and tissue micrographs were obtained. In individual samples, ten fields were randomly selected to quantify positive cells (400 ×). A color image processor was used to count GST-p foci for more than 15 cells.

Glycomic analysis

Sample preparation: Liver tissue was collected from normal and tumor-bearing rats. The liver tissues were lysed in 50 mmol/L Tris-acetate, pH 7.4, containing 1% sodium dodecyl sulfate, 5 mmol/L EDTA, and 0.15 mmol/L NaCl followed by homogenization with a Polytron homogenizer. Homogenates were centrifuged to extract soluble materials, and 1/10 vol of 20% Triton X-100 was added. The extracts were dialyzed against 20 mmol/L ammonium bicarbonate for 48 h at 4 ℃. After the dialysis, the recovered solution was lyophilized using a SpeedVac concentrator. Residual materials were reconstituted in 50 mmol/L ammonium bicarbonate and frozen until used.

N-linked glycan isolation and purification: The protein concentrations were normalized to 1 mg/mL and each sample was analyzed in duplicate to quantitate N-linked glycans using Ezose Sciences' proprietary GlycanMap® methodology reported by Nishimura, Furukawa and Miura[29-31]. Aliquots of each sample were spiked with internal standards to aid quantification. The aliquots were denatured and then trypsinized, followed by heat-inactivation. The N-glycans were then enzymatically released from the peptides by treating with PNGase F (New England Biolabs) and the released glycans were subjected to solid-phase processing using chemo-selective beads. After being captured on the beads, the sialic acid residues were methyl esterified to stabilize them in the mass spectrometer. The glycans were simultaneously released from the beads and labeled, and aliquots of the recovered materials were spotted onto a MALDI target plate. Utilizing a fully automated, 96-well format, robotic technology, steps from initial aliquoting to spotting on the MALDI plate were performed.

Mass spectrometric analysis: MALDI-TOF MS analysis was performed on an Ultraflex Ⅲ mass spectrometer (Bruker Daltonics) in the positive-ion, reflectron mode using a proprietary matrix composition. From the bead-based processing step, samples were spotted in quadruplicate, and spectra were obtained in an automated manner using the AutoXecute feature in flexControl software (Bruker Daltonics). Mass spectra were analyzed using Ezose's proprietary bioinformatics programs (Figure 1A).

Quantification using internal standard: Raw mass spectra were smoothed and baseline subtracted

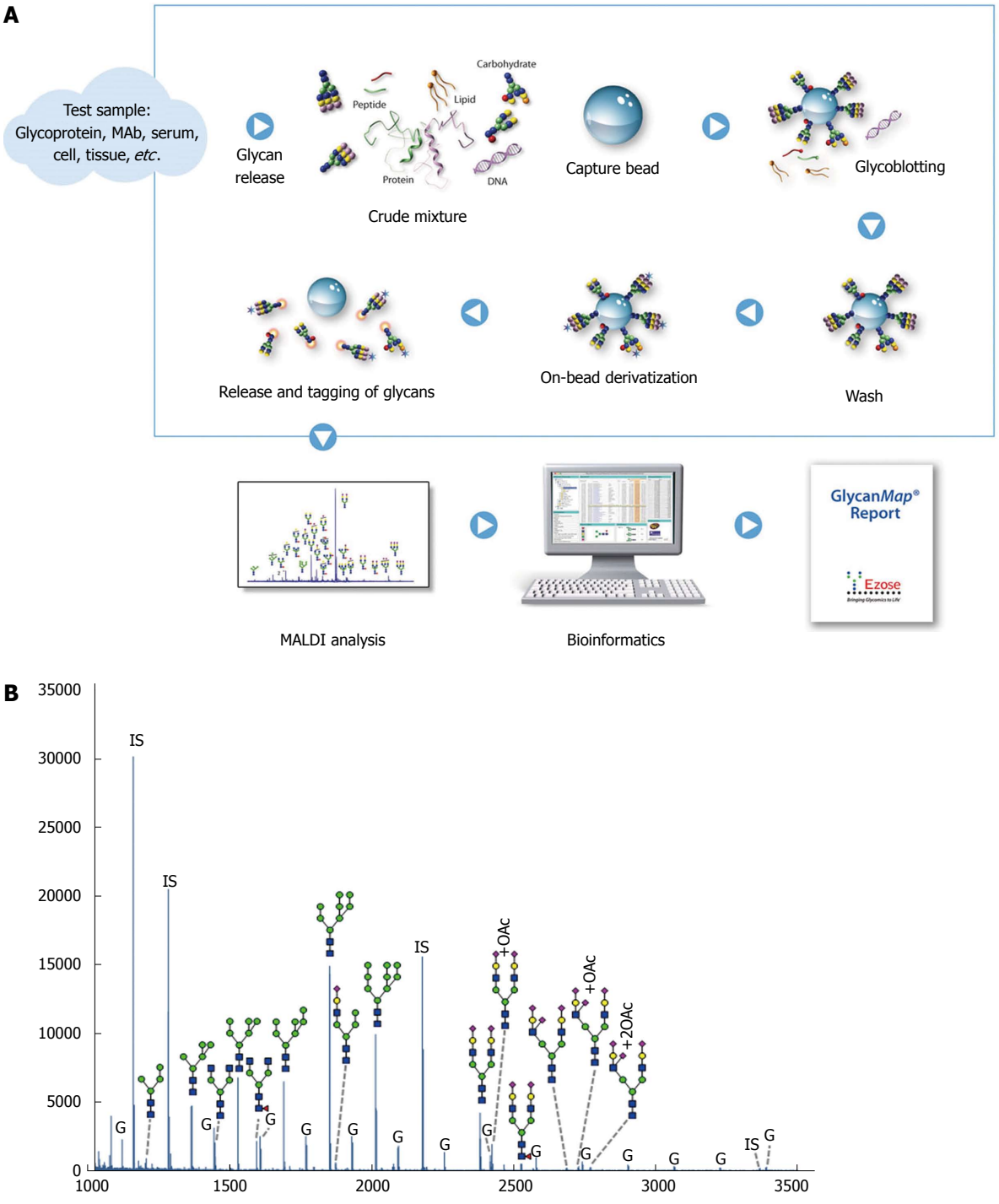


Figure 1 GlycanMap workflow. A: Workflow; B: Glycan profile in rat liver lysates. The spectrum was generated from sample N5 and is representative of the N-glycan profile in rat liver lysates. Taking advantage of the symbol nomenclature of the Consortium for Functional Glycomics, selected peaks were labeled with the proposed glycan structures. All spectra were collected on a MALDI-TOF mass spectrometer in reflection mode, which yields high mass accuracy and isotopic resolution for more accurate glycan identification. Glycans were quantified by comparing the peak intensity of each glycan-derived peak to those of the internal standards (IS). The liver lysates also contained varying amounts of glycogen (G).

prior to peak detection. Glycan compositions were assigned to each peak based on m/z. The intensities were normalized to that of an internal standard with known concentration. The normalized intensities from each of the quadruplicate spectra were averaged. Data were also corrected for the inherent decrease in peak intensity as m/z increases in mass spectrometric measurements, yielding absolute concentrations (in nmol/μg protein) that are equivalent to those created by HPLC/fluorescent glycan analysis methods. Glycan amounts were calculated by comparing peak heights to those of the internal standards. Glycan amounts were compared between treatment groups.

Statistical analysis

Data was analyzed by GraphPad Prism 6. Analysis of

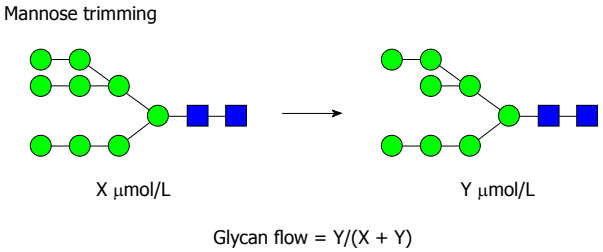


Figure 2 Example of a glycomics analysis of an individual biosynthetic step in the mannose trimming pathway.

differences between the means of the tumor-bearing and control groups was performed using the Student's *t*-test.

The glycan flow was calculated by comparing the concentration of product glycan (Y) to the total concentration of product and substrate glycan (X + Y) as described below. An example of a glycomics analysis of an individual biosynthetic step in the mannose trimming pathways is shown below (Figure 2).

The statistical significance of differences in the glycan flow was evaluated using the Student *t*-test. Glycan changes that yielded P values < 0.05 in this analysis were considered significant. The statistical analysis was carried out by Asma Bashir from UAE University.

RESULTS

DEN-induced foci of altered hepatocyte formation and GST-p expression

HCC was induced by DEN-2AAF, and HCC induction in the rat model was verified by assessing serum AFP (data not shown) as reported previously[28]. The histological examination was carried out to assess HCC induction. Hepatic nodules were evident only in animals treated with DEN-2AAF, but not in normal rats (Figure 3). These nodules represent the classical foci of altered hepatocytes and are made up of big and irregularly shaped hepatocytes with large hyperchromatic nuclei. As induced GST-p is normally considered an early biomarker of hepatocarcinogenesis, GST-p foci larger than 15 cells were examined using a color image processor. The GST-p positive foci were significantly increased in animals treated with DEN-2AAF. Histological examination provided evidence of successful tumor development in DEN-induced HCC rats.

Glycan analysis

The Ezose Science proprietary Glycan*Map*® methodology (Figure 1A) was utilized to quantify *N*-linked glycans. This technique was previously developed by Nishimura, Furukawa and Miura[29-31]. Repeatability of the assay was evaluated using a standard human serum sample. Five aliquots of the standard were analyzed in parallel with the individual rat liver lysate samples and used to evaluate repeatability. Coefficients of variation (CVs) for individual glycans ranged from 7.5% to 24.8%, with a pooled CV of 14.8%. An overview of the *N*-glycan profile in rat liver is shown in Figure 1B. In total, 29 glycans were detected in our study, Figure 3A represents the overall profile of N-glycans analyzed. The glycan structure and the code used are shown in Figure 4.

HCC is associated with glycan level changes

Twenty nine glycans were detected and the changes in these glycans were analyzed. Glycans; $Glc_1Man_9GlcNAc_2$ (code: 102000), $Gal_2Man_3GlcNac_4Fuc_1Neu_1$ (code: 54110), $Man_4GlcNac_2$ (code: 42000), $Gal_2Man_3GlcNac_4Neu_3OAc_3$ (code: 54033), and $Man_3GlcNac_5Fuc_1$, (code: 35000) showed significant changes in rat HCC liver tissues when compared with normal liver tissues. Four were increased and one was decreased in HCC rats compared to normal rats (Figure 5).

Differential effects of HCC on glycans

The glycans detected can be classified into three categories including high mannose (simple mannose chains), hybrid (with fucosylation and sialylation) and complex (with complex branching structures) (Figure 6A). High mannose glycans, with the exception of Man_8 (Man_6, Man_7, Man_9 and Man_{10}) were elevated (P < 0.05) in tumor-bearing rats compared to normal rats (Figure 6B). A non-significant decrease in fucosylated and sialylated glycans in HCC liver tissues was seen. However, a significant increase (P < 0.05) in sialyl-Lewis glycans (*i.e.*, with fucose, galactose and sialyl groups) and O-acetylated glycans was seen in HCC liver tissues.

Glycan fluctuations are correlated with their subcellular localization

Analysis of core glycans based on the subcellular compartment/s in which they are synthesized, was useful. Glycans synthesized in ER, cis- and trans-Golgi were all increased in HCC tissues compared to normal liver tissues. Although not all were significantly increased, the overall trend was a higher level in HCC tissues. The levels of glycans synthesized in medial-Golgi did not appear to follow a trend (Figure 7C).

HCC alters the glycan flow

To correlate the alterations in N-glycans with the enzymes involved in the biosynthetic pathway, the glycan flow was analyzed based on the ratio of adjacent glycans in the synthetic pathway. N-Glycans are synthesized from Glc_3Man_9 in ER and Golgi *via* long chain glycosyltransferases (GT) and glycosylhydrolases (GH), and glycan flow is directly related to enzyme activity involved in these reactions[32]. In our study, the glycan flow was significantly decreased from Glc_1Man_9 to Man_9 (P < 0.005) (Figure 8A) explaining the increase in glycan Glc_1Man_9 levels (Figure 5A). A

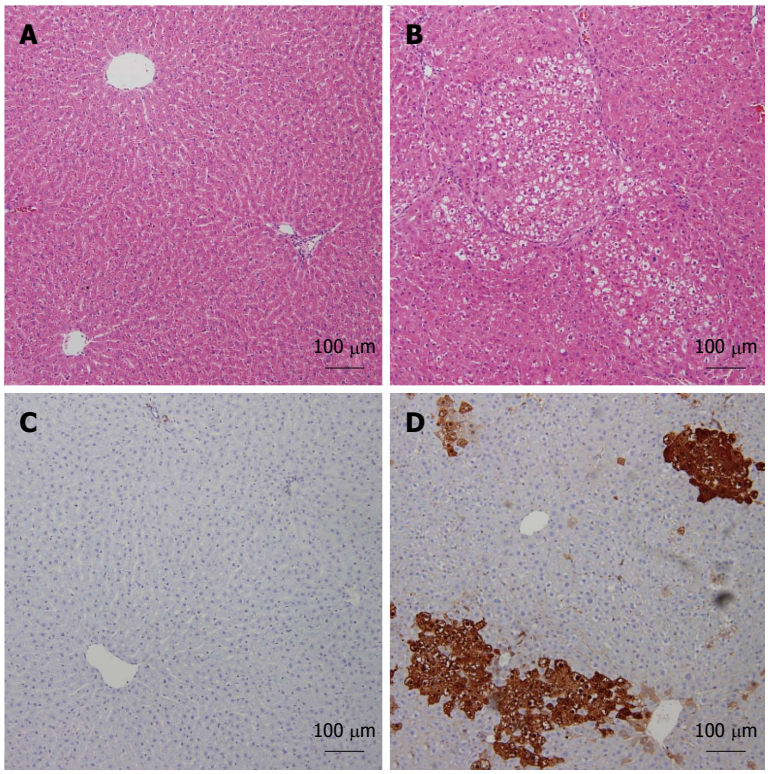


Figure 3 Diethyl nitrosamine-induced increase of foci in altered hepatocytes and induction of GST-p expression in liver. Representative images of Hematoxylin and Eosin-stained liver sections from both studied groups: Normal (A), hepatocellular carcinoma (HCC) (B). Immunohistochemistry analysis of both normal (C) and HCC (D) livers labeled with GST-p are shown.

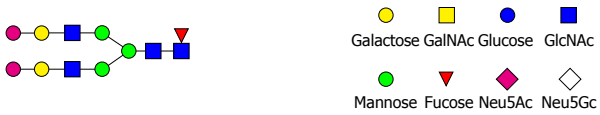
decrease (P = 0.057) in glycan flow in other mannose trimming reactions in the ER (from Man_9 to Man_8) was also observed. Glycan flow in cis-Golgi increased in HCC rats compared to normal rats with Man_8 to Man_7 flow increasing significantly ($P < 0.05$) (Figure 8B). The complex glycans synthesized in trans-Golgi showed an increase in flow from $Man_3GlcNac_4Fuc_1$ to $Gal_2Man_3GlcNac_4Fuc_1Neu_2$ in trans-Golgi (Figure 8D). This explains the increase in $Gal_2Man_3GlcNac_4Fuc_1Neu_1$ (Figure 5) in HCC liver tissues, which is an intermediate glycan in the reaction of $Man_3GlcNac_4Fuc_1$ to $Gal_2Man_3GlcNac_4Fuc_1Neu_2$.

DISCUSSION

Glycosylation is sensitive to microenvironment alterations and thus is involved directly in metastatic diseases such as cancer. These changes in glycosylation lead to the formation of tumor-specific glycans which are now believed to play an important role as biomarkers[33,34]. The relatively high stability of glycans compared to other molecules (RNA and proteins) along with the development of new techniques such as MALDI-TOF and ESI profile have revolutionized the field of glycan research. Interestingly, aberrant glycosylation has been shown to develop before any changes in apoptosis and cell differentiation associated with cancer[35].

High mannose glycans have been shown to be prevalent in HCC liver tissues compared to normal liver tissues. One high mannose, $Glc_1Man_9GlcNAc_2$, was significantly elevated in HCC rats compared to normal rats in the present study (Figure 5). A similar increase in high mannose glycans was reported in other cancers[36,37]. High mannose glycans have also been shown to increase in invasive and non-invasive breast cancer cells compared to normal epithelial cells[36,37]. Another study showed up-regulation of high mannose oligosaccharides on cell surface glycoproteins in cancer cells compared to normal cells[38]. N-linked glycans are constructed by a series of glycosyl transferases both in the ER and Golgi stacks. The chain starts with high mannose type glycans and then sequential mannose removal and addition of N-acetylglucosamine and glucose residues leads to the formation of complex and hybrid glycans. Thus, high mannose glycans may be increased (accumulated) if either the mannose trimming enzyme is altered or downstream processing events are terminated or abberated. ER mannosidase Ⅰ (ERManI), an ER-based mannosidase, is responsible for a step-wise trimming of Man residues transforming Man_9 to Man_5[39]. ERManI expression has been shown to be aberrant in liver tissues of HCC patients and in hepatoma cells lines[40], which explains the increase in glycan $Glc_1Man_9GlcNAc_2$ (code: 102000) in the present study (Figure 5). Studies have shown that ERManI knockdown leads to accumulation of Man_9 and Gal_1Man_9, and a decrease in Man_5 and Man_6

Codes used	Glycan name	Proposed structure
34000	$Man_3GlcNac_4$	
34100	$Man_3GlcNac_4Fuc_1$	
35000	$Man_3GlcNac_5$	
35100	$Man_3GlcNac_5Fuc_1$	
42000	$Man_4GlcNac_2$	
43010	$Gal_1Man_3GlcNac_3Neu_1$	
44010	$Gal_1Man_3GlcNac_4Neu_1$	
44100	$Gal_1Man_3GlcNac_4Fuc_1$	
45100	$Gal_1Man_3GlcNac_5Fuc_1$	
52000	$Man_5GlcNac_2$	
53010	$Gal_1Man_4GlcNac_3Neu_1$	
54010	$Gal_2Man_3GlcNac_4Neu_1$	
54020	$Gal_2Man_3GlcNac_4Neu_2$	
54021	$Gal_2Man_3GlcNac_4Neu_2OAc_1$	+ OAc
54030	$Gal_2Man_3GlcNac_4Neu_3$	
54032	$Gal_2Man_3GlcNac_4Neu_3OAc_2$	+ 2OAc:
54033	$Gal_2Man_3GlcNac_4Neu_3OAc_3$	+ 3OAc:
54110	$Gal_2Man_3GlcNac_4Fuc_1Neu_1$	
54120	$Gal_2Man_3GlcNac_4Fuc_1Neu_2$	



Glycan compositions are expressed as a five-digit code, which represents the number of hexoses (Gal, Man, or Glc), N-acetylhexosamines (GlcNAc or GalNAc), deoxyhexoses (Fucose), N-acetylneuraminic (Neu5Ac) or O-acetates (OAc). Proposed glycan structures were assigned based on molecular weight and literature precedent

Figure 4 This figure lists the glycan codes and proposed structures for all 29 glycans detected in this study.

compared to normal tissues[39].

In liver cancer, induction of both α-1,6- and α-1,3-linked fucosylation has been reported as the most prominent serum N-glycosylation change associated with that disease[41-43]. In the present investigation, fucosylated or sialylated glycans did not increase in HCC liver tissues compared to normal liver tissues, consistent with a study performed on human liver tissues of HCC patients[25]. However, there was an increase in the relative abundance of sialyl Lewis glycans (fucose, galactose and NeuAc groups) in DEN-induced HCC. The sialyl-Lewis glycans are commonly

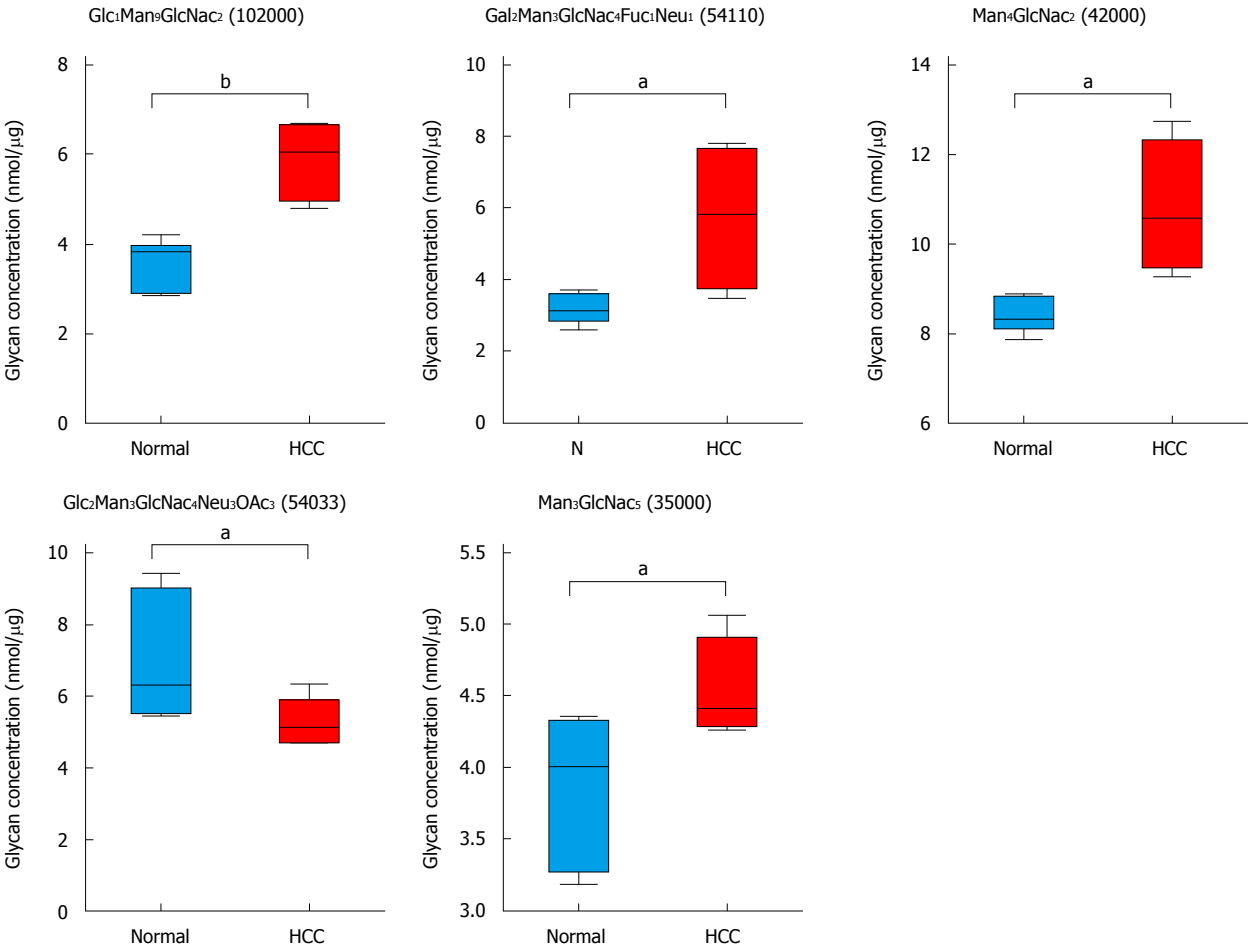


Figure 5 Hepatocellular carcinoma-related alterations in glycans in tumor-bearing animals. Five glycans changed in tumor-bearing rats compared to normal rats. A combined scatter and box-and-whisker plot is shown for each glycan, along with an indication of the statistical significance ($^aP < 0.05$, $^bP < 0.01$ *vs* control, $n = 7$).

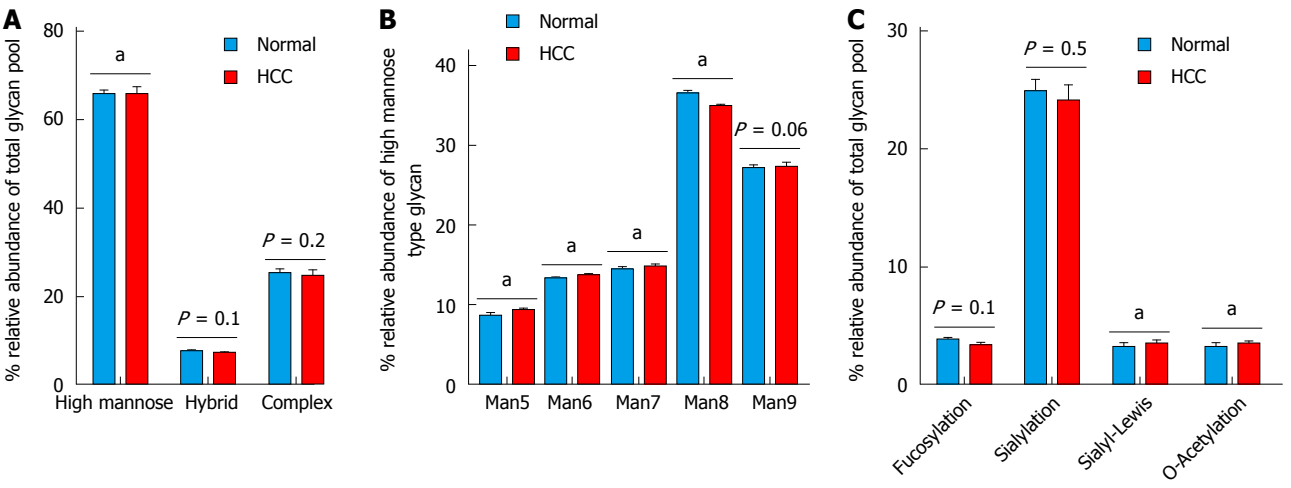
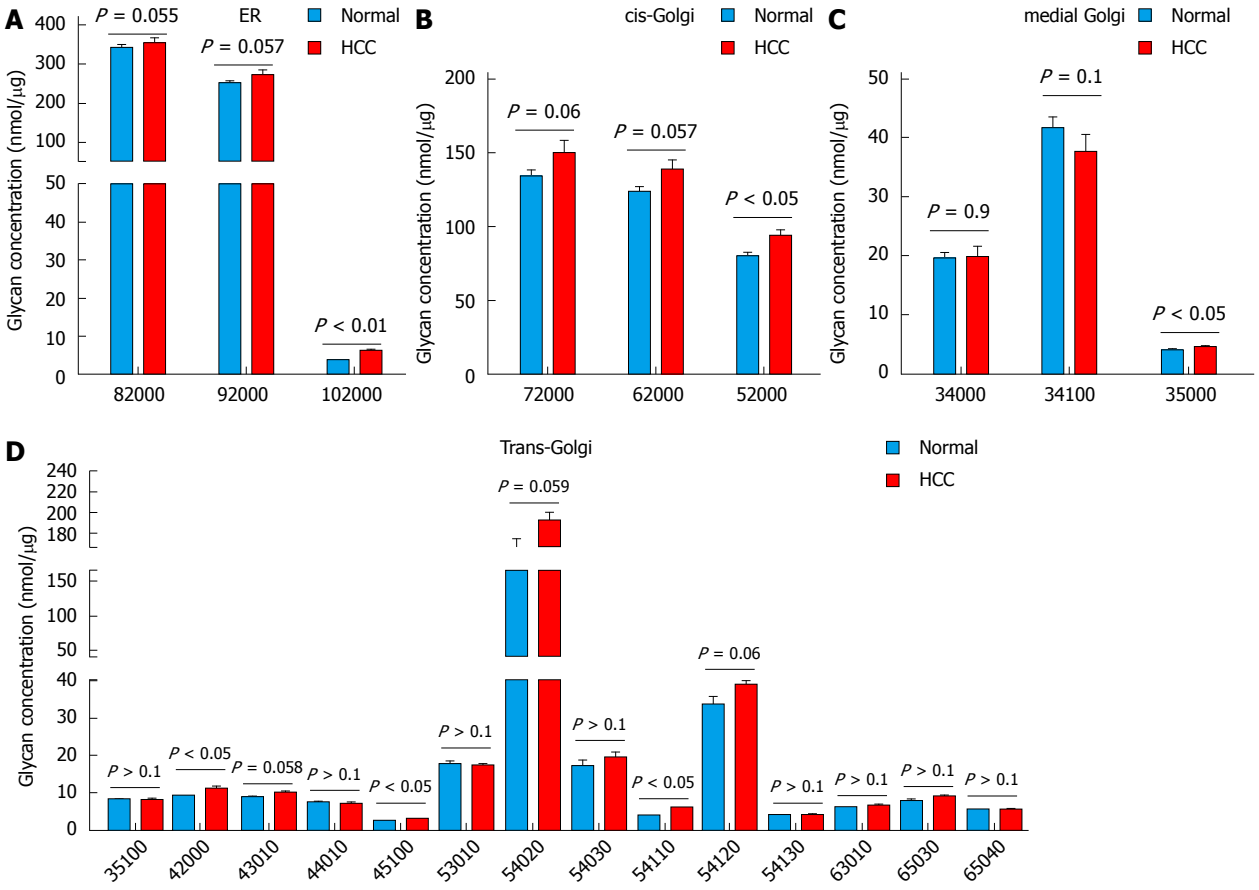


Figure 6 Differential expression of N-glycans structures and determinants. A: N-Glycan structures *i.e.*, high mannose, hybrid and complex; B: Distribution of the different types of high mannose structures (Man5 to Man9); C: Distribution of terminal glycan determinants: fucosylation, sialylation, sialyl-Lewis and O-acetylation. The groups were compared by *t*-test and $^aP < 0.05$ *vs* control $n = 7$.

abberated TAC in cancers[44,45]. One such glycan, Gal2Man3GlcNac4Fuc1Neu1 (code: 54110) was increased in HCC rats compared to normal rats (Figure 5). TACs are expressed both by tumor and host cells and are involved in the key pathophysiological processes during the various steps of tumor progression, including tumor growth, cell migration, invasion, metastasis, angiogenesis, and evasion of innate immunity[8,46-50]. TACs are studied extensively due to their potential as specific tumor biomarkers.



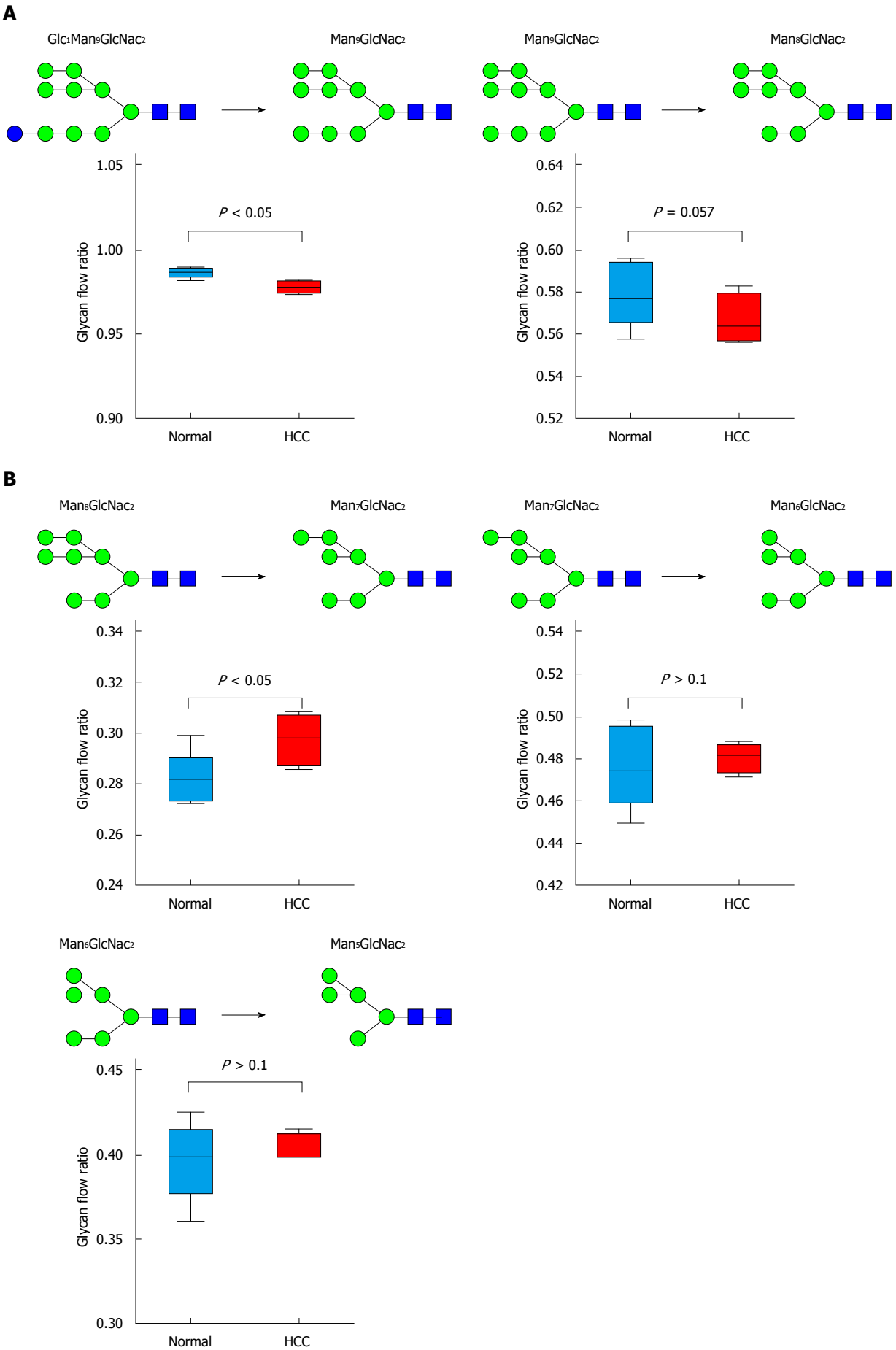
34000: $Man_3GlcNac_4$, **34100**: $Man_3GlcNac_4Fuc_1$, **35000**: $Man_3GlcNac_5$, **35100**: $Man_3GlcNac_5Fuc_1$, **43010**: $Gal_1Man_3GlcNac_3Neu_1$, **44010**: $Gal_1Man_3GlcNac_4Neu_1$, **45100**: $Gal_1Man_3GlcNac_5Fuc_1$, **53010**: $Gal_1Man_4GlcNac_3Neu_1$, **54020**: $Gal_2Man_3GlcNac_4Neu_2$, **54030**: $Gal_2Man_3GlcNac_4Neu_3$, **54120**: $Gal_2Man_3GlcNac_4Fuc_1Neu_2$, **54130**: $Gal_2Man_3GlcNac_4Fuc_1Neu_3$, **62000**: $Man_6GlcNac_2$, **63010**: $Gal_1Man_5GlcNac_3Neu_1$, **65030**: $Gal_3Man_3GlcNac_5Neu_3$, **72000**: $Man_7GlcNac_2$, 82000: $Man_8GlcNac_2$, **92000**: $Man_9GlcNac_2$, **102000**: $Gal_1Man_9GlcNac_2$

Figure 7 Analysis of glycan biosynthesis based on the compartments in which they are synthesized. The glycans are synthesized in ER-Golgi by a chain of enzymes. Glycans synthesized. A: ER; B: Cis-Golgi; C: Medial-Golgi; D: Trans-Golgi. The groups were compared by *t*-test, $P < 0.05$, $P < 0.01$ *vs* control, $n = 7$.

Branched glycans are synthesized by adding antennae branching structures by glycotransferases (GnT) such as N-acetylglucosaminyltransferase V (GnT-V). GnT-V is the key enzyme that catalyzes the formation of 1,6 N-acetylglucosamine (GlcNAc) and adding it to a common core structure of $Man_3GlcNAc_2$ in the medial-Golgi apparatus. One product of this reaction is $Man_3GlcNac_5$ (code: 35000), which was observed to be significantly increased in HCC rats compared to normal rats (Figure 5). $Man_3GlcNac_5$ is a branched glycan synthesized from $Man_3GlcNAc_2$ by GnTs. Overexpression of GnTs is associated with metastasis and their implication in HCC have already been reported[51-53].

Sialylation, the addition of sialic acid moieties, is an important modification of N-glycans that may alter the physical properties of molecules in the plasma membrane and may regulate immune cell function, as well as serve as specific ligands for certain toxins and lectins[54,55]. Sialic acids *e.g.*, N-acetylneuraminic acid (Neu5Ac) are capping structures on the glycans and may be further modified by various functional groups including acetyl, lactyl, methyl, sulfate, or phosphate groups. One such glycan, $Gal_2Man_3GlcNac_4Neu_3OAc_3$ (54033) with three o-acetyl groups was shown to be significantly decreased in HCC rats compared to normal rats (Figure 4). Changes in the O-acetyl group are seen as irrelevant in terms of their role in disease prognosis. Recently, however, O-acetylated Neu5Ac has been shown to contribute to drug resistance in acute lymphoblastic leukemia cells and its removal made the cells vulnerable to cytotoxic drugs[56].

Subcellular localization and flow changes in N-linked glycans showed that the processing starts in the ER by sequential removal of glucose and mannose from $Glc_3Man_9GlcNAc_2$ which continues in the Golgi complex. Mannose trimming is carried out by a series of mannosidases and extension is performed in Golgi by glycotransferases producing the complex-type and hybrid type. This study showed that changes in N-linked glycans followed a pattern depending on subcellular localization (Figure 7), which can be explained by the changes seen in glycan flow in the N-glycan biosynthesis pathway (Figure 8). An increase


A
Glc1Man9GlcNac2
Man9GlcNac2
Man9GlcNac2
Man8GlcNac2
Glycan flow ratio
1.05
1.00
0.95
0.90
P < 0.05
Normal
HCC
Glycan flow ratio
0.64
0.62
0.60
0.58
0.56
0.54
0.52
P = 0.057
Normal
HCC
B
Man8GlcNac2
Man7GlcNac2
Man7GlcNac2
Man6GlcNac2
Glycan flow ratio
0.34
0.32
0.30
0.28
0.26
0.24
P < 0.05
Normal
HCC
Glycan flow ratio
0.54
0.52
0.50
0.48
0.46
0.44
0.42
P > 0.1
Normal
HCC
Man6GlcNac2
Man5GlcNac2
Glycan flow ratio
0.45
0.40
0.35
0.30
P > 0.1
Normal
HCC

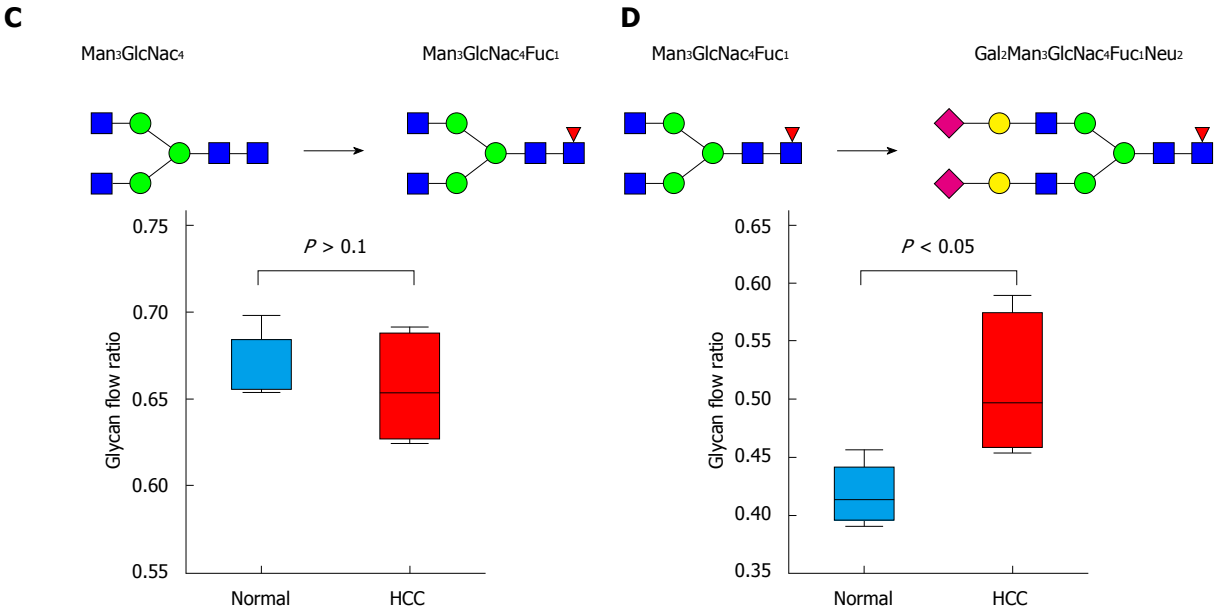


Figure 8 Glycan flow. Changes in glycan flow in the N-glycan biosynthesis pathway. The ratio was compared by *t*-test and *P* value < 0.05 was considered significant. A: ER; B: Cis-Golgi; C: Medial-Golgi; D: Trans-Golgi.

in high mannose glycans (Figure 6A) correlates with decreased glycan flow, showing significant ER stress in HCC liver tissues. An increase in sialyl-Lewis compared to fucosylated glycans (Figure 6C) can also be explained by the increased glycan flow in trans-Golgi which is the normal location for processing complex/hybrid glycans. The glycan levels may not give a clear indication of the disturbance in the synthesis pathway, but it is clearly depicted by glycan flow. The glycan flow clearly depicts the changes in enzymes involved in N-glycan biosynthesis.

In conclusion, we conducted a detailed analysis of N-glycans in HCC tissues compared to normal liver tissues. Five glycans were significantly altered. Together, the changes in glycan flow and subcellular localization provide an explanation for the increase in high mannose glycans and siayl Lewis glycans reported in HCC tissues.

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COMMENTS

Background

Glycosylation is a crucial post-transcriptional modification of many proteins. Protein glycosylation (N- and O- glycosylation) may result from disruption of the biosynthetic pathway such as the ER-Golgi chain and is commonly disrupted in various diseases such as cancer. Some tumor-associated carbohydrate (TAC) antigens are already in clinical use for the diagnosis and monitoring of cancers. Glycomics is a fast-growing recent technology that has been utilized as a new potential tool for cancer prognosis and treatment. The lack of reliable diagnostic biomarkers has turned hepatocellular carcinoma (HCC) into the prime target of many glycomic-based analyses. Numerous investigations have shown significant changes in total N-glycan levels, including Alpha Fetoprotein (AFP), in the serum of HCC patients, thereby demonstrating the potential of glycans and the glycome profile as efficient biomarkers of HCC. As the only known biomarker used for HCC, levels of AFP remain unchanged during the onset of HCC, which makes its application as a differential diagnosis marker of other liver diseases unreliable. Although fucosylated glycans have been shown to increase in HCC patients compared to patients with chronic liver diseases, no such increase was reported in the liver tissues of HCC patients when compared with normal tissues. Therefore, glycan analyses of cancer tissues have become a priority.

Research frontiers

In an attempt to utilize recent technology such as glycomics, the present study focuses on the analysis of the N-glycan changes in chemically-induced HCC liver tissues. Tissue glycan profiles were analyzed using the Ezose Glycan*Map*® platform to identify changes in the *N*-glycans of liver tissues in a HCC rodent model.

Innovations and breakthroughs

The fact that defective glycosylation normally develops prior to any changes in apoptosis and cell differentiation associated with cancer has driven us to study how glycans are synthesized in HCC livers. This study showed a significant increase in the high mannose, $Glc_1Man_9GlcNAc_2$, in HCC rats compared to normal rats. As the increase of such high mannose glycans may result from alterations in the mannose trimming enzyme, ER mannosidase I (ERManI), ERManI was also studied here. Similar to the livers of HCC patients, ERManI expression was shown to be aberrant as reflected in the increase in glycan $Glc_1Man_9GlcNAc_2$ in the present study. TACs are studied extensively due to their potential as specific tumor biomarkers. Thus, as in different types of cancers, the sialyl-Lewis glycans (fucose, galactose and NeuAc groups) are commonly abberated, the present study showed an increase in $Gal_2Man_3GlcNac_4Fuc_1Neu_1$ in HCC rats compared to normal rats. This study also showed a significant decrease in acylated $Gal_2Man_3GlcNac_4Neu_3OAc_3$ in HCC rats compared to normal rats. Recently, O-acetylated Neu5Ac (sialic acid) has been shown to contribute to drug resistance in acute lymphoblastic leukemia cells and its removal made the cells vulnerable to cytotoxic drugs. This investigation also showed a patterned change in N-linked glycans that depended on their subcellular localization. The increased high mannose glycans (decreased glycan flow) reflects a major ER stress in HCC tissues.

Applications

The present detailed glycomic analysis of the N-glycans in HCC tissues

compared to normal liver tissues has led to a better understanding of how the changes in glycan flow and subcellular localization can provide an explanation of the increase in high mannose glycans and siayl Lewis glycans reported in HCC tissues. This may potentially lead to the identification of new and more reliable biomarkers of HCC.

Terminology

Liver cancer was chemically induced in rats. Liver tissues were collected from both cancer-induced and normal animals for further studies. An HCC-specific antigen (GST-p) was targeted with an antibody (Anti- GST-p) to confirm tumor formation. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis was performed and mass spectra were analyzed using Ezose's proprietary bioinformatics programs. Glycan amounts were compared between the different animal groups.

Peer-review

These findings provide new index to diagnose the HCC, which has potential applications in clinic. This study did good work and used modern techniques to illustrate their conclusion. The authors have identified specific glycans from HCC tissue.

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ORIGINAL ARTICLE

Basic Study

SGK1 inhibits cellular apoptosis and promotes proliferation *via* the MEK/ERK/p53 pathway in colitis

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Author contributions: Bai JA and Xu GF contributed equally to this study; Bai JA, Xu GF and Tang QY designed the research; Bai JA and Xu GF performed the research; Yan LJ, Ji QQ, Zeng WW and Wu JD contributed to the model establishment or analytic tools; Bai JA analyzed the data; Bai JA, Xu GF and Tang QY wrote the paper.

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Abstract

AIM: To investigate the role of serum-and-glucocorticoid-inducible-kinase-1 (SGK1) in colitis and its potential pathological mechanisms.

METHODS: SGK1 expression in mucosal biopsies from patients with active Crohn's disease (CD) and normal controls was detected by immunohistochemistry. We established an acute colitis model in mice induced by 2,4,6-trinitrobenzene sulfonicacid, and demonstrated the presence of colitis using the disease activity index, the histologic activity index and hematoxylin and eosin staining. The cellular events and potential mechanisms were implemented with small interference RNA and an inhibitor of signaling molecule (*i.e.*, U0126) in intestinal epithelial cells (IECs). The interaction between SGK1 and the signaling molecule was assessed by co-immunoprecipitation.

RESULTS: SGK1 expression was significantly increased in the inflamed epithelia of patients with active CD and TNBS-induced colitis model (0.58 ± 0.055 *vs* 0.85 ± 0.06, $P < 0.01$). At the cellular level, silencing of SGK1 by small interference RNA (siSGK1) significantly inhibited the phosphorylation of mitogen-activated protein kinase kinase 1 (MEK1) and the downstream molecule extracellular signal regulated protein kinase (ERK) 1/2, which induced the upregulation of p53 and Bcl-2-associated X protein, mediating the subsequent cellular apoptosis and proliferation in IECs. Cells treated with MEK1 inhibitor (*i.e.*, U0126) before siSGK1 transfection showed a reversal of the siSGK1-induced cellular apoptosis.

CONCLUSION: Our data suggested that SGK1 may

protect IECs in colitis from tumor necrosis factor-α-induced apoptosis partly by triggering MEK/ERK activation.

Key words: Colitis; Serum-and-glucocorticoid-inducible-kinase-1; MEK/ERK; Apoptosis; p53

Core tip: This study showed that serum-and-glucocorticoid-inducible-kinase-1 (SGK1) expression was significantly increased in the inflamed epithelia of patients with active Crohn's disease (CD) in a TNBS-induced colitis model. At the cellular level, silencing of SGK1 inhibited the phosphorylation of mitogen-activated protein kinase kinase 1 (MEK1) and the downstream molecule ERK1/2, which induced the upregulation of p53 and Bcl-2-associated X protein, triggering subsequent cellular apoptosis and inhibition of proliferation in intestinal epithelial cells. A MEK1 inhibitor (*i.e.*, U0126) was used to show that this was a MEK/ERK-dependent process. Co-immunoprecipitation analysis uncovered the mechanism of the interaction between SGK1 and MEK1. Our results provide a new therapeutic approach to CD therapy.

Bai JA, Xu GF, Yan LJ, Zeng WW, Ji QQ, Wu JD, Tang QY. SGK1 inhibits cellular apoptosis and promotes proliferation *via* the MEK/ERK/p53 pathway in colitis. *World J Gastroenterol* 2015; 21(20): 6180-6193 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6180.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6180

INTRODUCTION

Crohn's disease (CD) is a chronic, relapsing and debilitating colitis. It is characterized as transmural inflammation with the clinical features of bowel obstruction, stricture, and diarrhea with blood or mucus, or both[1]. In the last decade, substantial advances aimed at uncovering the molecular pathogenesis of CD have been made, and 71 distinct loci for CD on 17 chromosomes have been identified in genome-wide association studies (GWAS)[2]. Nevertheless, the precise pathogenesis of CD remains poorly elucidated. Various components are involved in CD, such as intestinal epithelial cells (IECs), environmental and microbial factors, and innate and adaptive immunity[3]. IECs are the most important component of the epithelial barrier and play a pivotal role in intestinal immune homeostasis[4]. Accordingly, a sequence of events, such as apoptosis due to the stimulation of inflammation, disrupts homeostasis and mucosal integrity. Meanwhile, IEC proliferation and differentiation repair the barrier and sustain homeostasis. In some cases, the balance of disruption and repair is disrupted, followed by chronic gut inflammation, also known as inflammatory bowel disease (IBD), which occurs in CD and ulcerative colitis (UC)[5]. Further investigation of targeted therapies aimed at alleviating apoptosis and boosting IEC proliferation may lead to new developments in CD treatment.

Serum-and-glucocorticoid-inducible-kinase-1 (SGK1) was first named according to its gene upregulation by serum and glucocorticoids in rat mammary tumor cells[6]. The human SGK1 gene is located on chromosome 6q23. It is ubiquitously expressed in almost all tissues of the digestive tract, such as the esophagus, stomach, liver, intestine, and pancreas. SGK1 is also a gene that can encode serine/threonine protein kinase and may be involved in cell signaling pathways related to cellular survival[7]. Human SGK1 expression is stimulated by cell shrinkage and cytokines[8]; intestinal SGK1 is also regulated by saline ingestion[9]. SGK1 disorder contributes to the regulation of cell apoptosis, migration, proliferation, and epithelial transport. In addition, several studies have indicated a fatal role of SGK1 in the pathophysiology of various diseases, including autoimmune disease, inflammation, and tumor growth[10]. Therefore, we deduced that SGK1 inhibition may be a potential therapeutic option in the treatment of these disorders, especially CD.

The mitogen-activated protein kinase (MAPK) cascade is well known as a vital regulator of diverse cellular functions, such as cell proliferation, apoptosis, migration and differentiation[11]. The MAPK cascade acts as a critical mediator that can transduce cellular signaling from the cell surface to the cytosol and nucleus, and can even regulate cellular responses to endogenous or exogenous stimuli[12]. Tumor necrosis factor (TNF)-α has the capacity to trigger many elements of the inflammatory response in the gastrointestinal mucosa, and is an efficient stimulus of the extracellular signal regulated protein kinase αα(ERK)1/2pathway[13]. Three MAPK cascades have been elucidated in mammals: mitogen-activated protein kinase kinase kinase (Raf or MAP3K), mitogen-activated protein kinase kinase (MEK or MAP2K), and extracellular signal regulated protein kinase (ERK or MAPK). The MEK/ERK signaling cascade possesses serine/threonine kinase activities and critically mediates cellular proliferation, apoptosis, cycle progression, and migration in various cell types[14]. One of the critical mechanisms of MEK/ERK cascade-induced apoptosis is p53-related Bax dysregulation. Previous studies have reported that the MEK/ERK cascade mediates gene expression by the phosphorylation of several transcription factors, including p53[15]. In addition, the MEK/ERK pathway participates in cellular apoptosis by regulating gene expression of the pro-survival B-cell lymphoma-2 (Bcl-2) family proteins, such as Bax (a conserved key regulator of apoptosis), and by inducing anti-apoptotic proteins for proteasomal degradation[16].

P53, which has been referred to as the "guardian of the genome", is a tumor suppressor[17]. Numerous studies have indicated that p53-induced apoptosis activates the multi-domain pro-apoptotic protein Bax, thereby triggering caspase activation and cellular apoptosis[18].

It has remained poorly understood whether and how SGK1 is involved in the initiation and development of colitis. In our paper, for the first time, we validated that SGK1 plays a critical role in the protection of IECs from apoptosis in a colitis model and even in CD *via* the interaction with MEK1, which activates the MEK/ERK pathway. Our results provide new potential therapeutic targets for CD therapy.

MATERIALS AND METHODS

Animal care and use statement

The animal protocol was designed to minimize pain or discomfort in the animals. The animals were acclimatized to laboratory conditions (23 ℃, 12 h/12 h light/dark, 50% humidity, *ad libitum* access to food and water) for 2 wk prior to experimentation. Intragastric gavage was performed on conscious animals, using straight gavage needles appropriate for the animal size (15-17 g body weight: 22 gauge, 1 inch length, 1.25 mm ball diameter). All animals were euthanazed by barbiturate overdose (150 mg/kg pentobarbital sodium *iv*) and tissue samples subsequently collected.

Reagents, animals and cell lines

Anti-SGK1, anti-MEK1, and anti-phosphorylated-MEK1 (p-MEK1) antibodies were purchased from SAB, United States. The anti-ERK1/2, anti-phosphorylated-ERK1/2 (p-ERK1/2), anti-Bax, anti-p53, and anti-GAPDH antibodies were purchased from CST, United States. The anti-IgG antibodies, the immunofluorescence staining kit, cell counting Kit-8 (CCK-8), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit and inhibitors of MEK1 (U0126) were purchased from Beyotime Institute of Biotechnology. The immunostaining streptavidin-peroxidase (SP) kit was purchased from the Maixin Institute of Biotechnology, China. TNBS was purchased from Sigma Chemical Co., St. Louis, MO, United States. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640 medium, and fetal bovine serum (FBS) were purchased from Gibco, United States. Female BALB/c mice (6-8-wk-old, 19-22 g body weight) were purchased from the Animal Center of Nanjing Medical College, China. The human colorectal cancer cell line HCT-116 and normal rabbit intestinal endothelial cells 6 (IEC-6) were obtained from Nanjing Medical University, China. All the experiments were approved by Nanjing Medical College Animal Care and Use Committee.

Mucosal biopsy specimens

Biopsy specimens were obtained from inflamed mucosal areas of patients with active CD (n = 8) who were diagnosed according to clinical and macroscopic criteria. Control samples were collected from healthy subjects (n = 8). Tissue samples were immediately fixed in formalin and embedded in paraffin for an immunohistochemistry assay. The study was approved by the Institutional Review Board of Nanjing Medical University.

TNBS-induced colitis and assessment

All mice were randomly distributed into 2 groups and housed at room temperature of 22-24 ℃ with a 12-h light/dark cycle, and access to unrestricted tap water and standard rodent food. Mice were weighed and an acute CD model was induced by intra-rectal instillation of a solution consisting of 2.5% TNBS in 50% absolute ethanol (v/v) *via* a 3.5F catheter under deep anesthesia induced by 3% pentobarbital i.p., as previously described[19]. The mice were held in a vertical position for 1 min to ensure sufficient contact of the TNBS with the entire colon wall and were then returned to their cages. Intra-rectal administration of 100 L 50% ethanol in a similar manner served as the control treatment. Mice were killed by cervical dislocation on days 1, 2, 3, 5, 7, and 10 (n = 4 per day). To evaluate the degree of colitis, the mice were weighed daily, and fecal consistency and presence of bloody stool were recorded for 10 d after TNBS treatment. The colon length was recorded as a parameter of inflammation. The disease activity index (DAI) consisted of weight loss, stool consistency, and the degree of occult blood, as previously described[20]. The histologic activity index (HAI) was determined based on previous criteria[21]. Two personnel blinded to the source of the samples independently performed all evaluations.

Immunohistochemistry

All the tissues that remained were sliced as frozen samples of 5 μm thickness *via* a microtome. Antigen retrieval was performed by incubating the samples in sodium citrate buffer (0.01 mol/L, pH 6.0) for 3 min with heat applied. To block endogenous peroxidase, the samples were incubated with liquid A (endogenous peroxidase blockers) for 10 min, washed in PBS 3 times, then blocked with liquid B for 10 min. Samples were incubated with the primary antibody at room temperature for 2 h. The samples were then washed in PBS 3 times and incubated with liquid C as the secondary antibody for 10 min at room temperature. After washing 3 times with PBS, the samples were incubated with liquid D (streptavidin-peroxidase) for 10 min at room temperature. Finally, all samples were dyed with 0.05% freshly prepared diaminobenzedine (Beyotime Institute of Biotechnology, China) solution for 5 min, monitored under a microscope, rinsed with tap water, counterstained with hematoxylin for 1 min, dehydrated with graded concentrations of ethanol,

hyalinized with dimethylbenzene, and covered with neutral gum. The ethanol enema samples were prepared as negative controls. These slices were visualized with a microscope (IX71; Olympus Corp., Tokyo, Japan). The images were analyzed with Image Pro Plus (version 6.0).

Cell culture and treatment

The HCT-116 cells were cultured in RPMI 1640 medium, and IEC-6 cells were cultured in DMEM (4.5 g/L glucose). All media were supplemented with 10% FBS. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37 ℃. At a density of 80%-85%, cells were cultured with TNF-α (5 ng/mL, respectively) for 0, 1, 6, 12, 18 or 24 h as time-dependent groups. The other three 60-mm cell culture dishes were supplemented with TNF-α at different concentrations of 5, 10, or 20 ng as density-dependent groups. In transfection experiments, 5 μL of Lipofectamine 2000 (Invitrogen, California, United States) was mixed with 95 μL of Opti-MEM (Invitrogen, California, United States), and 2 μL of 50 nmol/L SGK1 siRNA (Genepharma, Shanghai, China) was mixed with 98 μL of Opti-MEM medium. Subsequently, all solutions were mixed and incubated for 20 min at room temperature. After incubation for 6 h with Opti-MEM, each dish was changed to complete medium for 48 h. An equal volume of Lipofectamine 2000 was used as the negative control. The cells were harvested for immunoblotting.

Western blot analysis

Colonic tissues and cells were split, and equal protein amounts were collected for SDS-polyacrylamide gel electrophoresis. The proteins were then transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA) using a transblot. The membranes were then kept in a solution of PBST and 5% non-fat milk for 2 h to terminate the nonspecific sites and were subsequently incubated with prepared primary antibodies at 4 ℃ overnight. After incubation with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG, 1:10000) for 1 h at room temperature, the bands were developed using a chemiluminescence kit (ECL; Pierce Supersignal, United States). GAPDH was used as an internal control. For quantitative analysis, the bands were analyzed using Quantity One software (Bio-Rad Laboratories, CA, United States).The difference between the control and treatment groups was recorded and analyzed using ImageJ software.

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed according to the manufacturer's protocol. Briefly, we washed the adherent cells with ice-cold PBS, added RIPA buffer, scraped the cells and centrifuged the lysate. The supernatant was pre-cleared with the addition of protein A/G-agarose beads (Abmart, Shanghai, China) for 10 min at 4 ℃. After a brief spin, the pre-cleared supernatant was incubated with primary antibodies (1:100) for 2 h at 4 ℃ on a rotator, and the cell lysate-protein-antibody complexes were recovered using recombinant protein A/G-Agarose beads. Empty vector (Input) and purified rabbit IgG-transfected cells were used as negative and positive controls, respectively. Samples were detected with immunoblotting as described above.

TUNEL and proliferation assays

A one-step TUNEL kit was used to perform TUNEL assays according to the manufacturer's instructions. Briefly, the tissue samples and pre-treated cells were immobilized with 4% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 2 min at 4 ℃, followed by TUNEL for 1 h at 37 ℃. The fluorescein isothiocyanate (FITC)-labeled TUNEL-positive IECs were examined using a microscope. The IECs with green fluorescence were defined as apoptotic cells.

A CCK-8 assay was performed following the manufacturer's protocol to evaluate cell proliferation. Pre-treated cells were seeded into a 96-well plate at an approximate density of 2×10^3 cells per well with 100 μL medium. The blank control wells contained medium alone (*i.e.*, no cells or drugs). After culturing for 0, 6, 12, 24 and 48 h, each well was supplemented with 10 μL tetrazolium substrate and incubated at 37 ℃ for 1 h. The optical density (OD) was then read at 450 nm in a Synergy HT microtiter plate reader (Bio-Tek, United States). This experiment was repeated 3 times independently.

Statistical analysis

All data are expressed as mean ± SD, and the statistical significance of differences between 2 groups was analyzed using the Student two-tailed *t* test. The significance levels were set at either $P < 0.05$ or $P < 0.01$. All statistical analyses were performed using SPSS software (version 14.0; SPSS Inc., Chicago, IL, United States).

RESULTS

SGK1 expression was increased in IECs of patients with CD

To evaluate the aberrant expression and localization of SGK1 in CD, we performed immunohistochemistry assays on samples from patients with active CD and normal controls. Weak SGK1 staining was observed in the normal control group, while strong SGK1 staining was observed in the samples from patients with active CD and was mainly located in the cytoplasm of IECs (Figure 1).

TNBS-induced colitis damage was more severe

To evaluate the severity of TNBS-induced colitis in mice, we recorded the appearance of the colon, weight

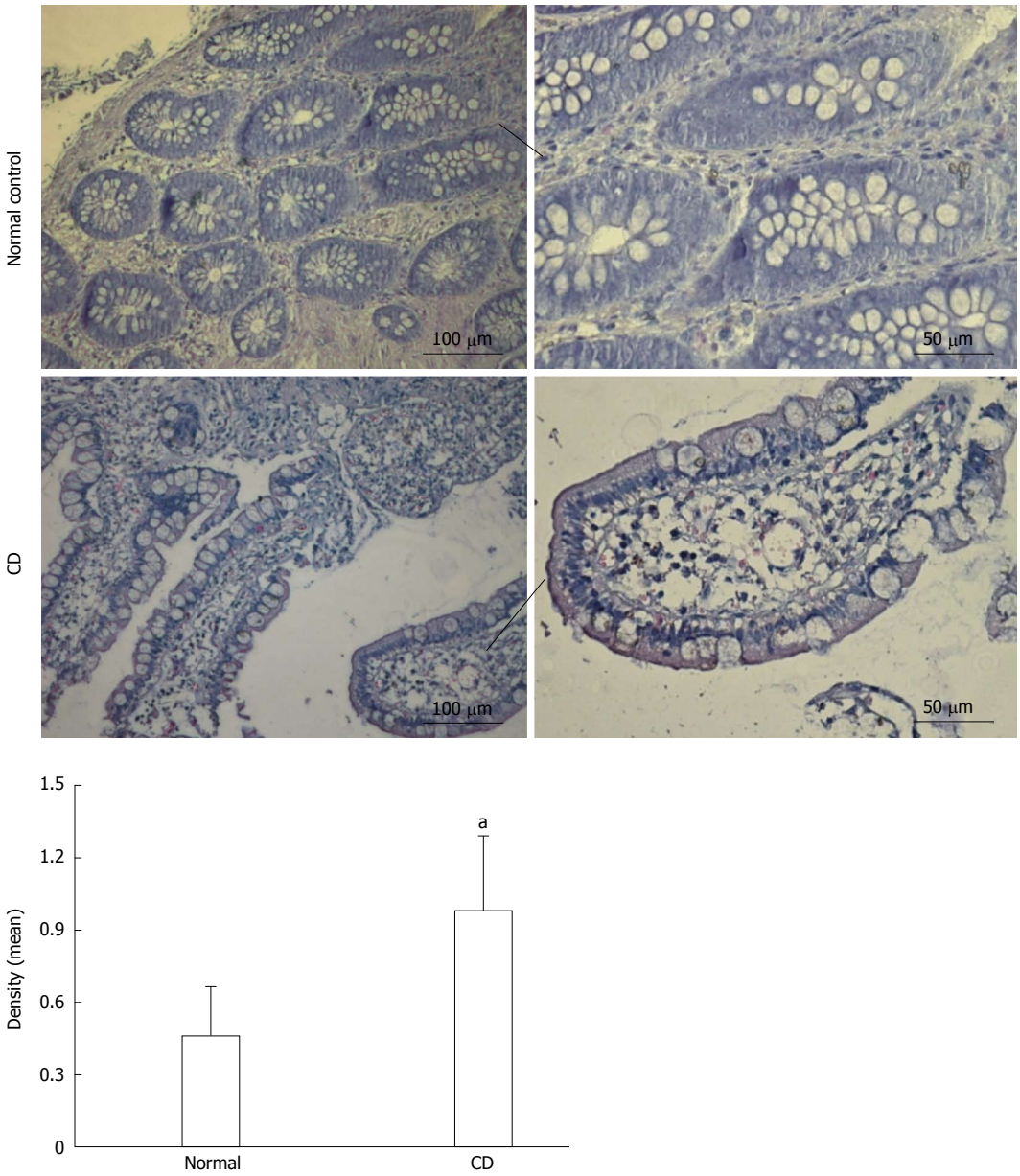

Figure 1 Serum-and-glucocorticoid-inducible-kinase-1 expression was increased in intestinal epithelial cells of patients with Crohn's disease. Immunohistochemistry of serum-and-glucocorticoid-inducible-kinase-1 (SGK1) in samples from patients with active Crohn's disease (CD) and normal controls. Weak staining of SGK1 was detected in the samples from normal controls, while strong staining of SGK1 was observed in samples from patients with active CD. The CD group (n = 8) had 4 males and 4 females. The normal control group (n = 8) had 4 males and 4 females. $^{a}P < 0.05$ *vs* normal controls. SGK1: Serum-and-glucocorticoid-inducible-kinase-1.

loss, length of the colon, stool character, and presence of blood in the stool. The TNBS-induced colitis group showed more severe damage (Figure 2A, upper line) in the colon compared with controls (50% ethanol, Figure 2A, lower line). In addition, the TNBS-induced colitis group showed more obvious weight loss. The mean body weight loss was 6.8% over 24 h and reached a peak of 13.9% at 72 h compared with the baseline body weight at the time of TNBS administration (Figure 2B). In addition, there were more loose stools and apparently bloody stools in the TNBS-induced mice than in the ethanol-treated mice. The length of the colon was significantly reduced in the TNBS group compared with controls (Figure 2C). The DAI and HAI demonstrated maxima of 9.2 and 8.0, respectively, at 72 h (Figure 2D and E). The pathology assay also showed the most severe colitis at day 3 (Figure 3A).

SGK1 is upregulated in TNBS-induced colitis and mostly expressed in the cytoplasm of IECs

Immunohistochemical staining showed that SGK1 was expressed mainly in the cytoplasm of IECs and at higher levels in TNBS-induced colon tissue at day 3 compared with control (Figure 3B). To further confirm the apoptosis of IECs induced by TNBS, we performed a TUNEL assay on the tissue samples and noted a significant increase in IEC apoptosis in TNBS mice (Figure 3C). Western blotting showed that SGK1 expression was markedly increased in mice with TNBS-induced colitis (Figure 3D).

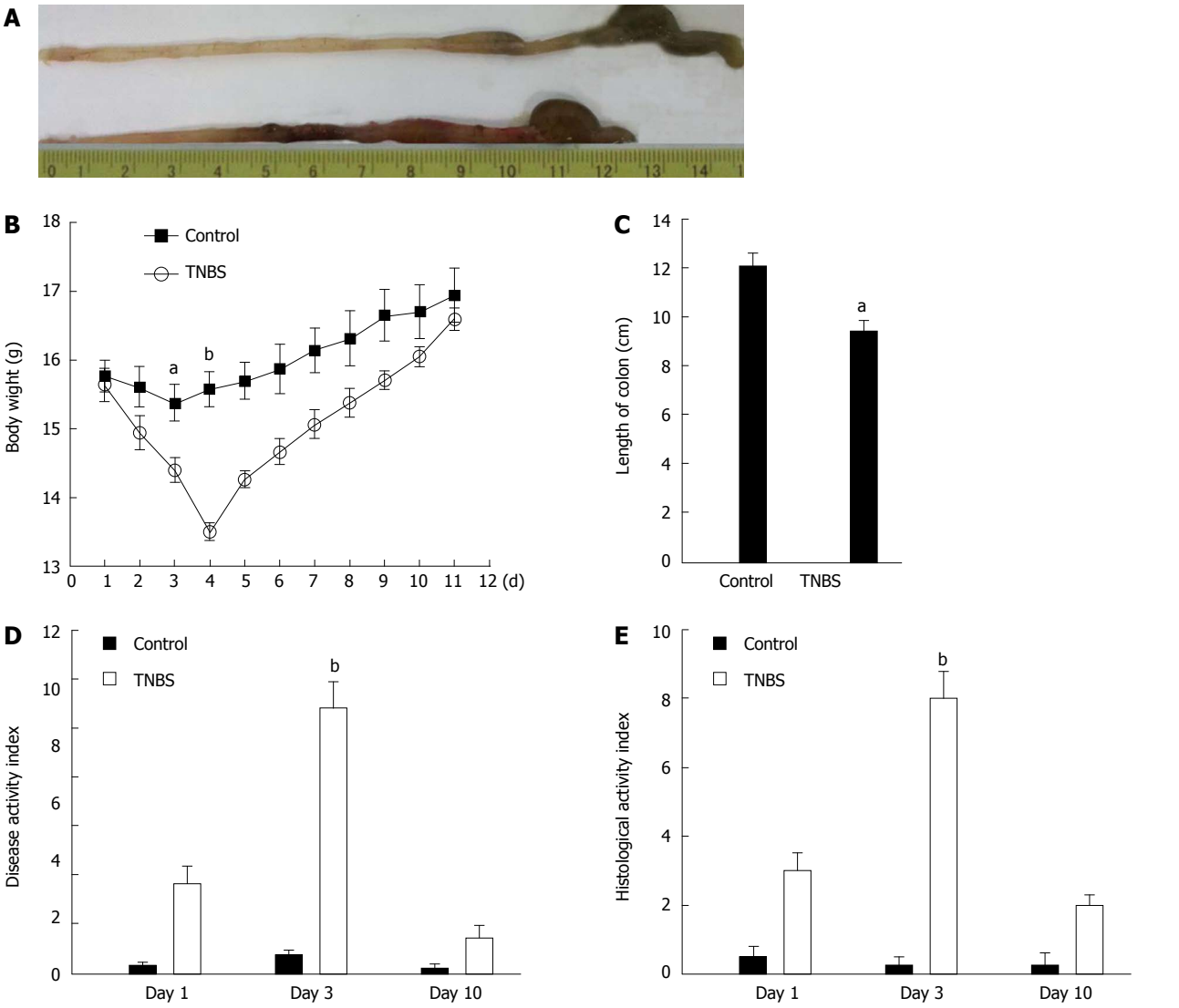

Figure 2 2,4,6-trinitrobenzene sulfonic acid and ethanol colitis model was successfully established. The macroscopic appearance of the colon in TNBS-treated BALB/c mice. A: The colons were obtained 72 h after intra-rectal TNBS or ethanol treatment and were then photographed; B: TNBS or ethanol (the control group) administration caused variation in bodyweight in the murine model; C: Mean colon length; D, E: The DAI and HAI of mice treated with TNBS or ethanol were assessed. Data are presented as the mean ± SD (n = 4). One of three representative experiments is shown. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the ethanol treatment group. TNBS: 2,4,6-trinitrobenzene sulfonic acid.

TNF-α induces SGK1 expression in a time- and dose-dependent manner in IECs

We next examined the biological behavior of SGK1 *in vitro* in HCT-116 cells and IEC-6 cells to elucidate the mechanism. We determined SGK1 expression by western blotting after TNF-α treatment and found that the TNF-induced SGK1 expression occurred in a time- and dose-dependent manner. Specifically, the peak of SGK1 expression after TNF-α treatment appeared at 24 h in HCT-116 cells (Figure 4A and B) and IEC-6 cells (Figure 4C and D) at a dose of 5 ng/mL (Figure 4).

Knockdown of SGK1 induces apoptosis and suppresses proliferation in IECs

Next, we determined whether knockdown of SGK1 by SGK1 siRNA could affect cellular events in IECs. TUNEL assays were used to determine the effect of SGK1 siRNA on apoptosis by DNA fragmentation in IECs. As shown in Figure 5A and B, SGK1 siRNA treatment caused a significant increase in the number of TUNEL-positive cells (n = 6, $P < 0.01$ *vs* the control group). However, pre-treatment with an inhibitor of the MEK1 pathway (U0126, 10 μmol/L) before SGK1 siRNA transfection abolished the increase in TUNEL-positive cells.

Further investigation of cellular proliferation (*via* theCCK8 assay) was performed following the above protocol. The effect of SGK1 silencing on HCT-116 and IEC-6 cell proliferation is shown in Figure 5C and D, respectively. We found that the SGK1 siRNA-transfected HCT-116 and IEC-6 cells showed significantly lower proliferation potential compared with the negative control (NC) siRNA-transfected cells at 0-48 h post-transfection.

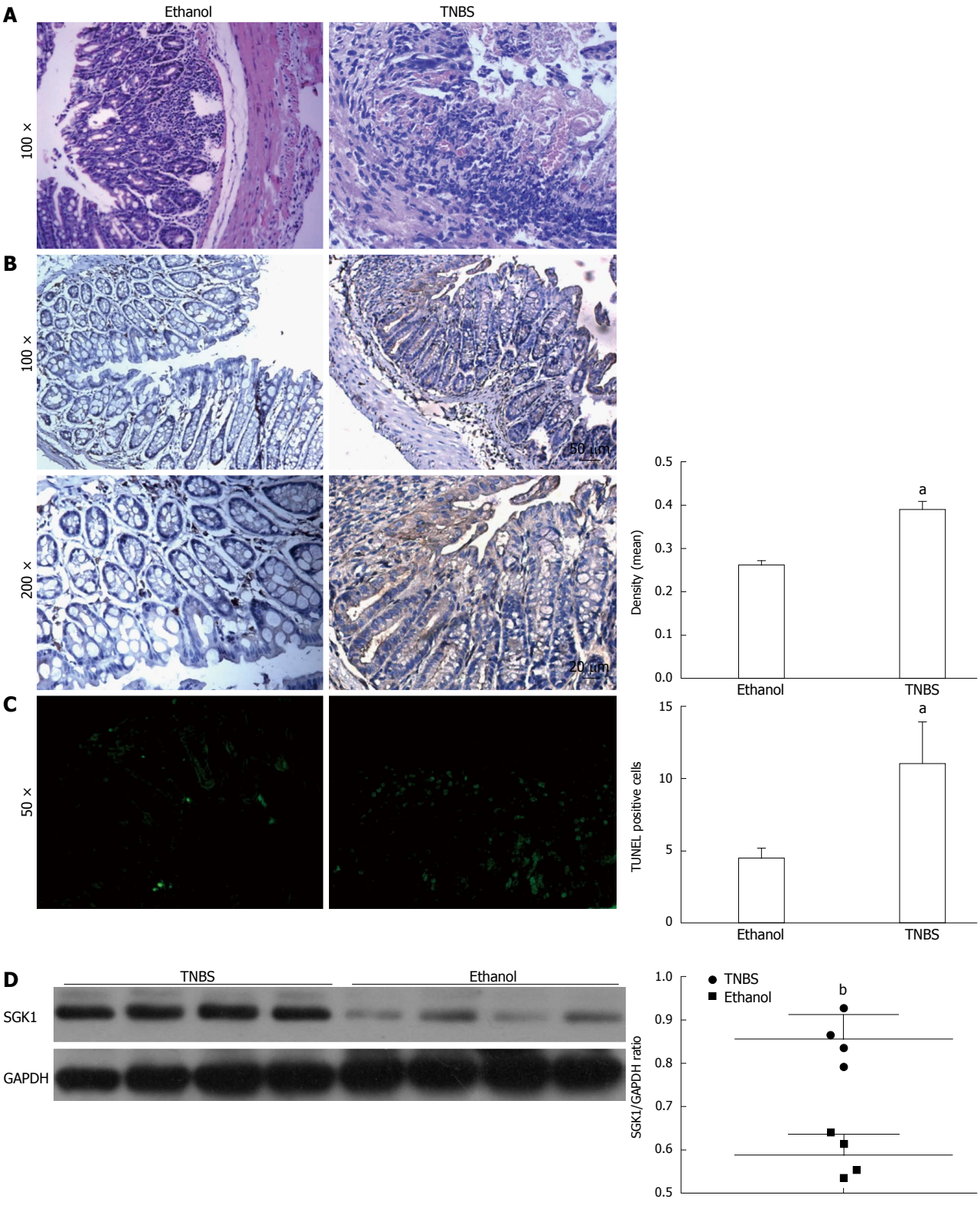

Figure 3 Serum-and-glucocorticoid-inducible-kinase-1 expression was increased in the colonic tissue of a 2,4,6-trinitrobenzene sulfonic acid-induced murine model. A: Histological damage of colonic tissues induced by ethanol or 50% TNBS in mice (HE staining). Colonic tissues were obtained from the mice killed 3 d post-enema with ethanol or 50% TNBS; B: Immunohistochemical localization and upregulation of SGK1 expression in the colitis model. SGK1-dependent staining is indicated by brown areas. The density of positive areas was analyzed with Image Pro Plus; C: The TNBS group showed an increase in TUNEL positive cells. The positive cells were counted, and the results are presented as mean ± SD from six sample fields; D: Western blot analysis was performed to study the SGK1 level in TNBS-induced colitis compared with ethanol-treated mice. The ethanol-treated group served as controls in these experiments. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* the ethanol treatment group. GAPDH served as the internal control.

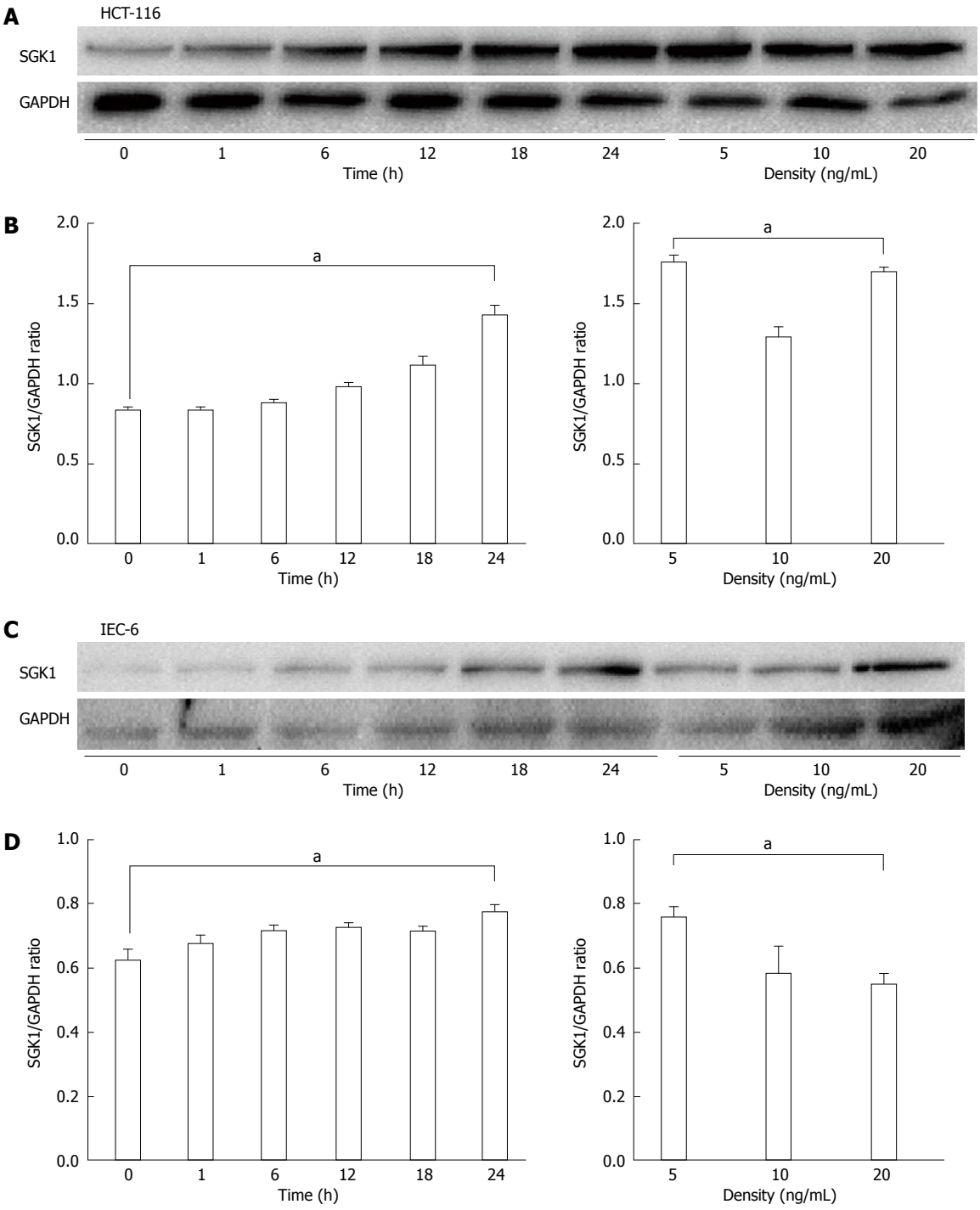

Figure 4 Serum-and-glucocorticoid-inducible-kinase-1 expression levels in tumor necrosis factor-treated intestinal epithelial cells assessed with western blotting. A, B: SGK1 expression levels at different times and doses of TNF in HCT-116 cells; C, D: SGK1 expression levels at different times and doses of TNF in IEC-6 cells. $^{a}P < 0.05$ and $^{b}P < 0.01$ represented 24 h *vs* 0 h after TNF-treatment or 10 ng *vs* 5 ng TNF. GAPDH served as the internal control. SGK1: Serum-and-glucocorticoid-inducible-kinase-1; TNF: Tumor necrosis factor; IECs: Intestinal epithelial cells.

Potential interaction between SGK1 and the MEK1/ERK1/2 pathway in IECs

We further investigated the potential mechanisms involved in SGK1 siRNA-induced cellular events. The efficiency of siRNA knockdown on IECs (assessed by SGK1/GAPDH) was detected by western blotting. As shown in Figure 6A and C, SGK1 expression was significantly downregulated compared with cells transfected with NC siRNA after 48 h. In addition, the ERK1/2 phosphorylation level (assessed by p-ERK/ERK1/2) was significantly decreased after SGK1 knockdown. The quantities were measured with ImageJ and are shown in Figure 6B and D. To identify and characterize the possible interacting proteins of SGK1, co-IP was performed to investigate any potential association (direct or indirect) with IECs. SGK1 antibody co-immunoprecipitated with MEK1 but failed to co-immunoprecipitate with purified rabbit IgG (Figure 5E). In addition, MEK1 antibody co-immunoprecipitated with SGK1 but failed to co-immunoprecipitate with purified

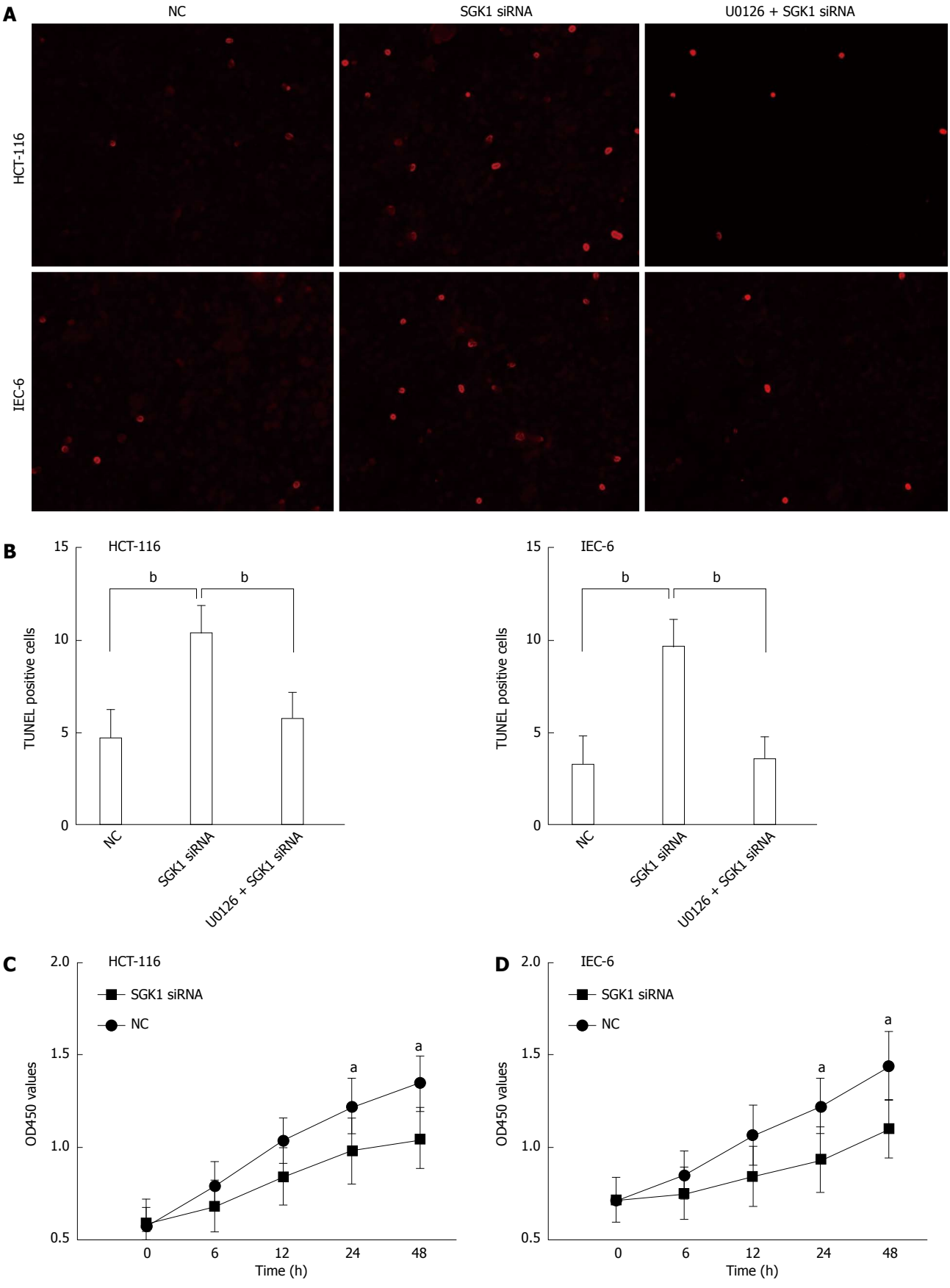

Figure 5 Intestinal epithelial cell apoptosis and proliferation analyzed by TUNEL and CCK-8 assays. A: After the indicated treatment for 48 h, the cells were TdT-UTP nick end labeled and photographed with a fluorescent microscope. Scale bar = 200 μm. The TUNEL-positive cells are colored green; B: Quantitation of TUNEL-positive intestinal epithelial cells (IECs) was performed on all groups. All values are mean ± SD from 6 independent images in each group; C: For the CCK-8 assay, IECs were transfected with SGK1 siRNA or negative control siRNA (NC) as described above. OD450 values indicate the absorbance of IECs post-transfected at a wavelength of 450 nm. The OD450 values of IEC-6 and HCT-116 cells are shown in C and D, respectively. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* negative controls.

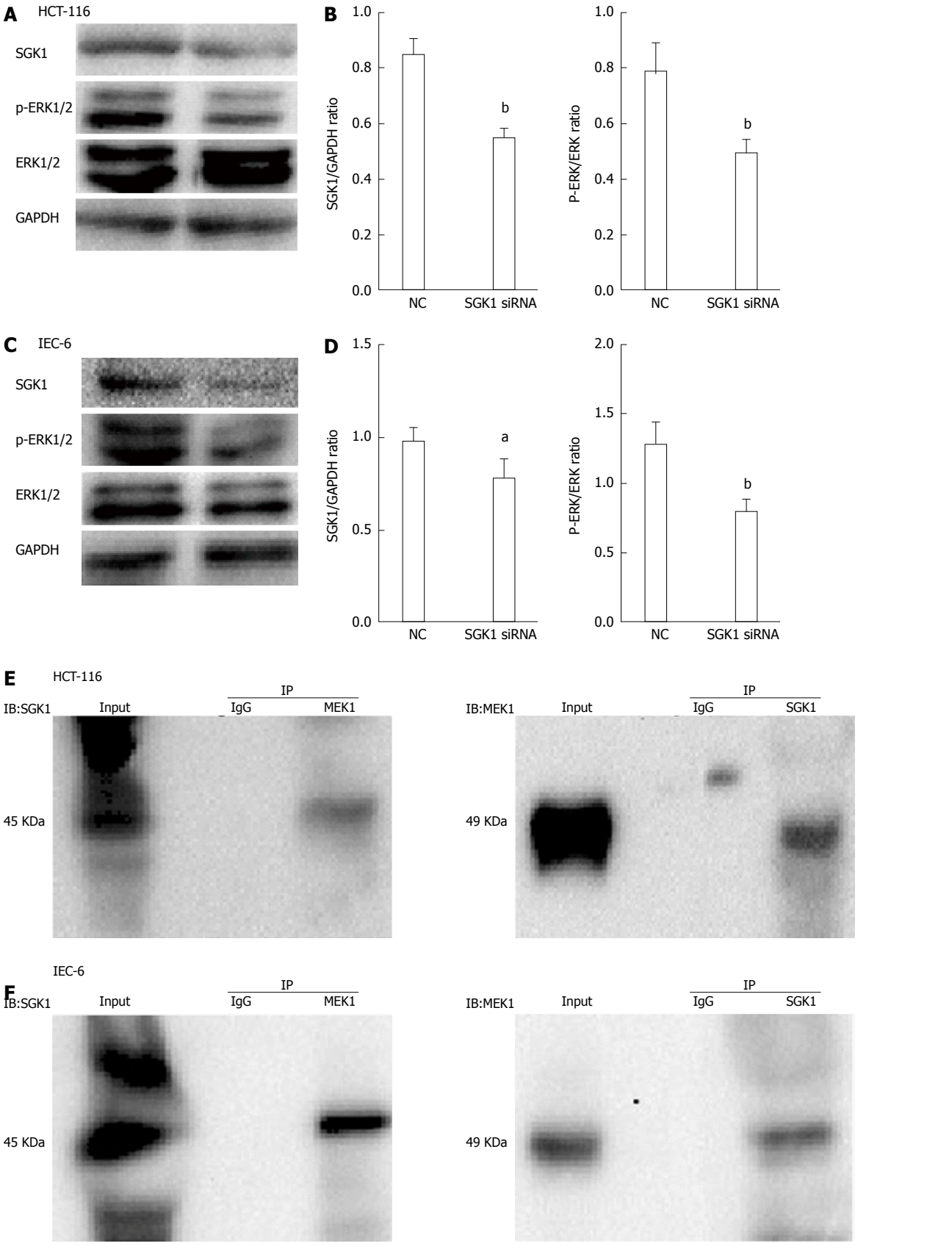

Figure 6 Efficiency of serum-and-glucocorticoid-inducible-kinase-1 silencing after serum-and-glucocorticoid-inducible-kinase-1 siRNA transfection and co-immunoprecipitation of serum-and-glucocorticoid-inducible-kinase-1 and mitogen-activated protein kinase kinase 1. A, B: The efficiency of SGK1 silencing the subsequent dysregulation of p-ERK and ERK in HCT-116 cells. The efficiency of SGK1 silencing was assessed by the ratio of SGK1/GAPDH. The level of ERK phosphorylation was assessed by the ratio of p-ERK/ERK; C, D: The efficiency of SGK1 silencing and the following dysregulation of p-ERK and ERK in IEC-6 cells. The NC group served as controls. GAPDH served as the internal control; E, F: SGK1 was co-immunoprecipitated with MEK1 on IECs. HCT-116 and IEC-6 cell lysates were subjected to co-immunoprecipitation with anti-SGK1 or control IgG and were detected with anti-MEK1 antibodies. MEK1 was co-immunoprecipitated with SGK1. $^{a}P < 0.05$ and $^{b}P < 0.01$ *vs* negative controls.

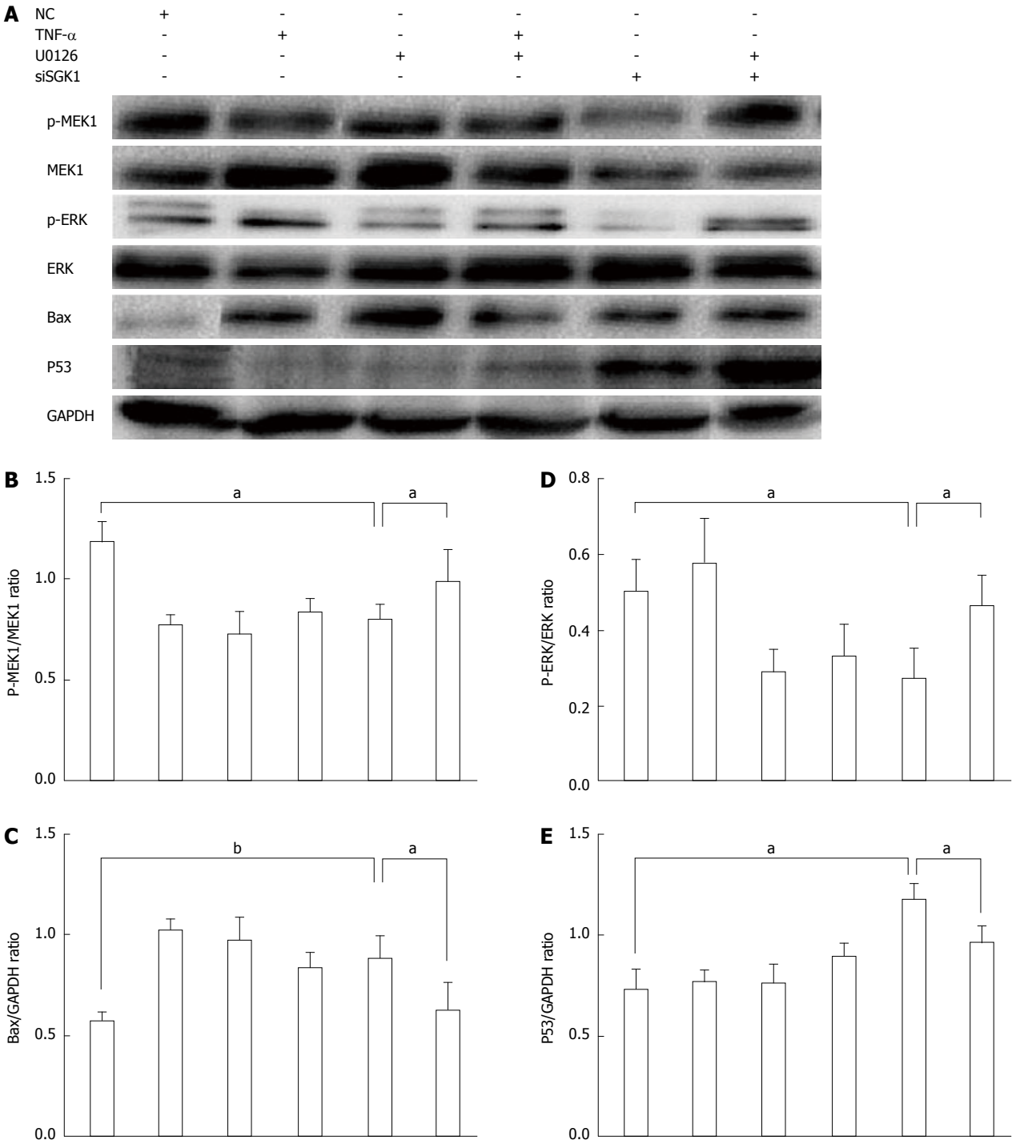

Figure 7 P53 and Bax are downstream of the mitogen-activated protein kinase kinase 1/extracellular signal regulated protein kinase pathway. A: IECs in culture were pretreated with the MEK1 inhibitor (U0126) followed by stimulation with TNF-α and NC or SGK1 siRNA. Protein expression levels were then detected by western blotting; B: The level of MEK1 phosphorylation was assessed by the ratio of p-MEK1/MEK1; D: The level of ERK1/2 phosphorylation was assessed by the ratio of p-ERK/ERK; C, E: p53 and Bax expression levels were assessed by the ratios of p53/GAPDH and Bax/GAPDH, respectively. $^{a}P < 0.05$ and $^{b}P < 0.01$, siSGK1-treated group *vs* negative controls or the U0126-treated group *vs* the non-U0126-treated group before siSGK1 transfection. SGK1: Serum-and-glucocorticoid-inducible-kinase-1; TNF: Tumor necrosis factor; IECs: Intestinal epithelial cells; MEK: Mitogen-activated protein kinase kinase 1; ERK: Extracellular signal regulated protein kinase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

rabbit IgG (Figure 5F).

SGK1 silencing induces apoptos mainly via the MEK1/ERK/p53 pathway

To further explore whether SGK1 silencing-induced apoptosis occurred mainly *via* the MEK1/ERK pathway, we determined the MEK1 phosphorylation level (assessed by p-MEK1/MEK1) in IEC-6 cells and found a significant decrease after SGK1 silencing (Figure 7A and B). In addition, we found a significant inhibition of ERK1/2 phosphorylation after SGK1 silencing, assessed by p-ERK/ERK (Figure 7D). We also found that SGK1

silencing triggered p53 and Bax upregulation, which indicates activation of apoptosis (Figure 7C and E). Furthermore, we treated IECs with U0126 (10 μmol/L) for 1 h before TNF-α (5 ng/mL) treatment in the absence or presence of SGK1 knockdown. U0126 obviously and significantly inhibited MEK1 and ERK1/2 phosphorylation. In addition, inhibition of the MEK/ERK pathway attenuated the upregulation of p53 and Bax induced by SGK1 silencing (Figure 7A, C and E).

DISCUSSION

CD is characterized by inflammation of the gastrointestinal tract, resulting from a range of factors, including immunogens that mediate colitis onset. Numerous studies have focused on effective therapeutic targets aiming to attenuate or even cure CD. SGK1 is well known as a serine-threonine kinase involved in intracellular signal transduction pathways and is induced by serum and glucocorticoids. SGK1 is involved in several cellular functions, including activation of ion channels (*e.g.*, epithelial Na^+ channel, Ca^{2+} channel, and K^+ channel), and the regulation of several enzymes (*e.g.*, glycogen synthase kinase-3, phosphatidylinositol-3-kinase, and Akt) and transcription factors (*e.g.*, β-catenin and nuclear factor-κB)[10,22,23]. Moreover, excessive SGK1 expression is involved in the pathophysiology of several disorders, including inflammatory disease, fibrosing disease, tumor growth, and neurodegeneration[24]. Our study demonstrated for the first time that SGK1 expression was upregulated in both CD patients and in a TNBS-induced mouse model of colitis.

Previous studies have shown that SGK1 can regulate the interaction of membrane receptors that mediate cellular apoptosis in prostate and colon cancers[25,26]. Three pathways are involved in SGK1-related cellular apoptosis. In the first pathway, SGK1 phosphorylates and inhibits pro-apoptotic transcription factors, such as Forkheadbox O3a (FOXO3a) and GSK3β[27]. In the second pathway, SGK1 facilitates cell survival by phosphorylating mouse double minute 2 (MDM2) by enhanced p53 ubiquitylation and degradation[28]. Intracellular MDM2 is critical in regulating the expression of p53. In the third pathway, SGK1 regulates apoptosis by regulating ion channels and transporters, such as $Ca^{2+}$and several K^+ channels[29,30]. In consideration of the roles of SGK1 in the inflammatory process and immunoreaction, together with CD pathogenesis, we suggest that SGK1 may mediate CD initiation by triggering aberrant cellular events, including proliferation and apoptosis, in parallel with the clinical manifestations of hyperplasia and ulcers. Consistent with previous studies, knockdown of SGK1 in our study induced cellular apoptosis and inhibited IEC proliferation.

Recent reports have shown that SGK1 can down-regulate or upregulate ERK2 activity and MEK/ERK complex formation. Additionally, SGK1 has been shown to inhibit ERK1/2 activity in liver HepG2 cells and db/db mice[31,32]. We observed that activation of the ERK1/2 pathway was significantly inhibited after SGK1 silencing. In addition, knockdown of SGK1 induced p53 and Bax upregulation. An increasing number of therapeutic strategies targeting the MEK/ERK pathway have been evaluated in clinical trials. MEK acts as a choke point in the initiation of the MEK/ERK pathway and related oncogenesis[33]. ERK1/2 is a potential kinase that reacts with numerous substrates in both the nucleus and the cytoplasm and plays a pivotal role in cell growth and differentiation. MEK1/2, which exhibits sole substrate specificity for ERK1/2, regulates the MEK/ERK cascade. Dysregulation of the MEK/ERK cascade by cytokines or other stimuli has been shown to be involved in numerous human malignancies, contributing to the initiation of oncogenesis. MEK phosphorylates both serine/threonine and tyrosine residues of ERK1/2, triggering its activation and the subsequent cytosolic and nuclear activity. In addition, this pathway participates in cellular events, such as abnormal proliferation, apoptosis and mobility[34-36]. It has been well known for years that ERK1/2 activation can lead to cellular events through p53 accumulation[37]. Accordingly, wild-type p53 is well known as a critical tumor suppressor and has been called the "guardian of the genome" in mammalian cells. Rubbi and Milner have reported that ultraviolet-induced DNA damage can result in p53 activation and cell cycle arrest[38]. The primary mechanism of DNA damage-related p53 dysregulation is associated with MDM2, which negatively regulates p53 by ubiquitin-mediated proteasomal degradation. To date, numerous p53-inducible gene products have been identified, including pro-apoptotic Bax and p53-upregulated modulator of apoptosis[39]. A diverse range of studies has indicated the role of ERK1/2 in the regulation of anti-apoptotic responses. The MEK/ERK pathway also plays a vital role in the regulation of apoptosis by triggering the expression of Bcl-2 family proteins[16]. The anti-apoptotic effect of ERK is mediated mainly *via* members of the Bcl-2 family, such as Bcl-2 and Bcl-xL. In addition, activated ERK has been reported to interfere with apoptotic factor caspase-8 cleavage and Bax translocation[40]. Our study indicated, *via* co-IP analysis, that SGK1 interacts with MEK1, which promotes MEK1 and ERK1/2 phosphorylation. A MEK1 inhibitor (U0126, 10 μmol/L) showed that SGK acts in a MEK/ERK-dependent manner. Furthermore, U0126 significantly inhibited MEK1 and ERK1/2 activation, which attenuated the upregulation of p53 and Bax, and cellular apoptosis induced by SGK1 silencing.

Taken together, our research showed that SGK1 expression plays a critical role in the initiation and development of TNF-α-induced apoptosis of IECs in colitis *via* modulation of the ERK1/2 cascade. We uncovered its mechanism for the first time by elucidating an interaction between SGK1 and MEK1, which activated the MEK/ERK pathway and mediated

cellular apoptosis and proliferation *via* p53 and Bax dysregulation. Further studies are needed to determine the interactions and the potential clinical value. Our study indicates that SGK1 may protect IECs from apoptosis and provide a previously unrecognized therapeutic approach for the treatment of colitis and even CD.

COMMENTS

Background

Crohn's disease (CD) is a chronic, relapsing and debilitating colitis. Until now, many therapies have been tested in clinical patients with active CD but have shown no marked effects. Serum-and-glucocorticoid-inducible-kinase-1 (SGK1), a potential immunomodulatory factor, is involved in cell signaling pathways and mediates cellular apoptosis, migration, proliferation, and epithelial transport. It is well recognized that impairment of the epithelial barrier is one of the most important factors in the origin of CD. Intestinal epithelial cells (IECs) are the most important component of the epithelial barrier. IEC apoptosis and proliferation regulate the homeostasis of the intestinal mucosa.

Research frontiers

Many studies have shown that SGK1 plays a vital role in the pathophysiology of autoimmune disease and inflammatory disease. In liver HepG2 cells and db/db mice, SGK1 inhibits ERK1/2 activity and mediates cell survival. However, the function of SGK1 in CD remains unclear.

Innovations and breakthroughs

In this study, for the first time, the authors found that SGK1 expression was upregulated in both patients with active CD and in a TNBS-induced mouse model. Silencing of SGK1 inhibited the phosphorylation of mitogen-activated protein kinase kinase 1 (MEK1) and a downstream molecule, extracellular signal regulated protein kinase 1/2 (ERK1/2), which triggered the upregulation of p53 and Bcl-2-associated X protein (Bax), accompanied by cellular apoptosis and IEC proliferation.

Applications

This research demonstrated a function for SGK1 in the protection of IECs from apoptosis *via* activation of the MEK/ERK pathway and may provide a previously unappreciated approach to CD therapy.

Terminology

SGK1 is a ubiquitously expressed gene that encodes a serine/threonine protein kinase and is involved in cell signaling pathways related to cell apoptosis, migration, proliferation, epithelial transport and inflammation-immune functions.

Peer-review

The authors discovered for the first time that SGK1 expression was increased in the tissues of patients with active CD. They established a mouse model of TBNS-induced CD and investigated its relationship with SGK1. SGK1 expression was up-regulated in the TNBS-induced mouse model. Silencing of SGK1 inhibited the phosphorylation of MEK1 and the downstream molecule ERK1/2, which triggered p53 and Bax up-regulation, which, in turn, mediated cellular apoptosis and IEC-6 and HCT-116 cell proliferation. Taken together, this manuscript is highly relevant and interesting.

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ORIGINAL ARTICLE

Basic Study

Gambogic acid induces apoptosis and inhibits colorectal tumor growth *via* mitochondrial pathways

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Author contributions: Huang GM and Sun Y contributed equally to this work; Huang GM, Sun Y and Ge X designed the research; Huang GM, Wan X and Li CB performed the research; Sun Y analyzed the data; Huang GM, Sun Y and Ge X wrote the manuscript.

Ethics approval: The study was reviewed and approved by the Heilongjiang Provincial Hospital Institutional Review Board.

Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Ethics Committee of Heilongjiang Province's Hospital (IACUC protocol number: 2008-010).

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Data sharing: No additional data are available.

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Abstract

AIM: To investigate the effect of gambogic acid (GA) on apoptosis in the HT-29 human colon cancer cell line.

METHODS: H-29 cells were used for *in vitro* experiments in this study. Relative cell viability was assessed using MTT assays. Cell apoptosis was detected by terminal deoxynucleotidyl transferase dUTP nick end labeling and Hoechst 33342 staining, and quantified by flow cytometry. Cellular ultrastructure was observed by transmission electron microscopy. Real-time PCR and Western blot analyses were used to evaluate gene and protein expression levels. For *in vivo* experiments, BALB/c nude mice received subcutaneous injections of HT-29 cells in the right armpit. When well-established xenografts were palpable with a tumor size of 75 mm^3, mice were randomly assigned to a vehicle (negative) control, positive control or GA treatment group (n = 6 each). The animals in the treatment group received one of three dosages of GA (in saline; 5, 10 or 20 mg/kg) *via* the caudal vein twice weekly, whereas animals in the negative and positive control groups were given equal volumes of 0.9% saline or 10 mg/kg docetaxel, respectively, *via* the caudal vein once weekly.

RESULTS: The cell viability assay showed that GA inhibited proliferation of HT-29 cells in a dose- and time-dependent manner after treatment with GA (0.00, 0.31, 0.62, 1.25, 2.50, 5.00 or 10.00 μmol/L) for 24, 48 or 72 h. After 48 h, the percentage of apoptotic cells in cells treated with 0.00, 1.25, 2.50 and 5.00 μmol/L GA was 1.4% ± 0.3%, 9.8% ± 1.2%, 25.7% ± 3.3% and 49.3% ± 5.8%, respectively. Ultrastructural analysis of HT-29 cells treated for 48 h with 2.5μmol/L GA revealed apoptotic bodies and condensed and fragmented nuclei. Levels of caspase-8, -9 and -3 mRNAs were significantly increased after treatment with GA (1.25, 2.50 or 5.00 μmol/L) for 48 h (P < 0.05 for all). Protein levels of apoptosis-related factors Fas, FasL, FADD, cytochrome c, and Apaf-1 were increased in GA-treated cells, whereas levels of pro-caspase-8, -9 and -3 were significantly decreased (P < 0.05 for all). Furthermore, GA significantly and dose-dependently inhibited the

growth of HT-29 tumors in a mouse xenograft model ($P < 0.05$).

CONCLUSION: GA inhibits HT-29 proliferation *via* induction of apoptosis. The anti-cancer effects are likely mediated by death receptor (extrinsic) and mitochondrial (intrinsic) pathways.

Key words: Apoptosis; Death receptor pathway; Flow cytometry; Gambogic acid; Hoechst 33342; HT-29 cells; Mitochondrial pathway; MTT; Terminal deoxynucleotidyl transferase dUTP nick end labeling

Core tip: This study evaluated the effects of gambogic acid on colon cancer cells. Treatment of a human colon cancer cell line with gambogic acid inhibited proliferation *via* induction of apoptosis. Moreover, the growth of colon cancer cell xenograft tumors in mice was reduced by injections of gambogic acid. These anti-cancer effects were likely mediated through death receptor and mitochondrial pathways.

Huang GM, Sun Y, Ge X, Wan X, Li CB. Gambogic acid induces apoptosis and inhibits colorectal tumor growth *via* mitochondrial pathways. *World J Gastroenterol* 2015; 21(20): 6194-6205 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6194.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6194

INTRODUCTION

Colorectal cancer is the third leading cause of cancer and the fourth leading cause of cancer-related deaths worldwide[1,2]. Morbidity and mortality from colorectal cancer are increasing with continuing urbanization of the population. Apart from genetic causes, life and environmental factors determine the relative risk of the occurrence and development of colon cancer. Although the diagnostics for colon cancer have greatly improved, the molecular mechanisms of the disease are poorly understood[3,4]. Treatments for colon cancer include surgery, chemotherapy, and radiotherapy, or a combination of these treatments[5]. Chemotherapy is an effective treatment for colon cancer, but traditional chemotherapy has many serious side effects, including significant pain. At present, approximately half of the patients with a primary tumor can be cured by surgery, depending on the tumor location[6].

Gambogic acid (GA) is the major active ingredient in gamboge, which is extracted from various *Garcinia* species, including *Garcinia hanburyi* Hook f. (Tenghuang)[7]. GA has various biologic activities, such as anti-pyretic, analgesic, anti-inflammatory[7], autophagic[8] and anti-tumor activities[8-10]. Some research studies have shown that GA can inhibit the growth of many tumor cells both *in vitro* and *in vivo,* including cells in lung cancer[11,12], liver cancer[13,14], breast cancer[15-17], gastric cancer[18,19], pancreatic cancer[20], leukemia[21-23], melanoma[24], and glioblastoma[25]. GA is currently being investigated in clinical trials in China[25-27]. However, the effect of GA on the growth of human colon cancer cells remains unclear.

Apoptosis is the most important pathway for the anti-tumor effects of many compounds. GA has been shown to induce apoptosis by increasing nuclear condensation and DNA fragmentation[28,29], elevating levels of Bax and decreasing levels of Bcl-2[29,30], activating caspase-8, -9 and -3[31-33], suppressing NF-κB[33,34], and inhibiting matrix metalloproteinase-2 and -9[35] both *in vitro* and *in vivo*[22,36]. The aim of this study was to investigate the potential anti-cancer effects of GA on human colon cancer cells and identify the related molecular mechanisms.

MATERIALS AND METHODS

Chemicals and reagents

GA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, United States). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Hoechst 33342 staining kits were obtained from Beyotime Institute of Biotechnology (Haimen, China). Annexin V/propidium iodide (PI) was purchased from Biosea (Beijing, China). Real-time (RT)-PCR primers were purchased from Genscript Corp. (Piscataway, NJ, United States). M-MLV reverse transcriptase and reagents for RT-PCR were purchased from Promega Corp. (Madison, WI, United States). Antibodies against Fas, Fas ligand (FasL), Fas-associated with death domain protein (FADD), caspase-8, cytochrome c, apoptotic protease activating factor (Apaf)-1, caspase-9, caspase-3, GAPDH, and β-actin were obtained from Cell Signaling Technology Inc. (Danvers, MA, United States). Trizol and fluorescence-conjugated secondary antibodies were obtained from Invitrogen (of Thermo Fisher Scientific, Waltham, MA, United States). Other chemicals were purchased at the highest purity grade.

Cell culture

Human colon cancer cell line HT-29 was purchased from American Type Culture Collection (Manassas, VA, United States). The cells were cultured in complete RPMI-1640 medium (Hyclone of GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) supplemented with 10% heat-inactivated bovine serum (Gibco of Thermo Fisher Scientific, Waltham, MA, United States), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 ℃ with 5% CO_2 in a humidified atmosphere.

MTT assay of cell proliferation

HT-29 cells were seeded into a 96-well culture plate at 5000 cells/well for 16 h for attachment. The cells were then treated with GA (0.00, 0.31, 0.62, 1.25, 2.50 5.00, or 10.00 μmol/L) for 24, 48 or 72 h. MTT dye was added to each well at 37 ℃ and incubated for 4 h. The supernatant was then removed and the purple-colored formazan precipitates were dissolved in 150 μL of dimethyl sulfoxide and absorbance at 490 nm was measured on a multi-well plate reader. The background absorbance (medium without the cells) was subtracted. Percent viability was calculated using the formula: [(drug-treated group/control group) × 100]. Each assay was repeated three times, and the final results are expressed as mean ± SE.

Apoptotic cell detection by TUNEL and Hoechst 33342 staining

For TUNEL staining, HT-29 cells were incubated with GA (0.00, 1.25, 2.50 or 5.00 μmol/L) for 48 h in 96-well plates; the attached cells were then washed with PBS and fixed in freshly prepared 4% formaldehyde for 30 min. The cells were then washed twice with PBS and incubated with digoxigenin-conjugated dUTP in a terminal deoxynucleotidyl transferase-catalyzed reaction for 1 h at 37 ℃ in a humidified atmosphere. After the cells were immersed in stop/wash buffer for 10 min at room temperature and washed with PBS, they were incubated with anti-digoxigenin antibody-conjugated peroxidase for 30 min. Apoptotic cells with condensed and fragmented nuclei were stained brown after incubation with 3, 3′-diaminobenzidine for 5 min.

For Hoechst 33342 staining, HT-29 cells cultured on glass coverslips in 6-well plates were treated and fixed as described above. After rinsing twice with PBS, the cells were incubated in Hoechst 33342 staining solution for 5 min. Cells were washed twice with PBS and mounted using an antifade mounting medium. Apoptosis was detected by fluorescence microscopy.

Cellular ultrastructure analysis by transmission electron microscopy

HT-29 cells were incubated for 48 h with 0.0 or 2.5 μmol/L GA, harvested with trypsin, centrifuged, and washed with PBS. Cell samples were fixed in 2.5% (v/v) glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), post-fixed in 2% (w/v) buffered osmium tetroxide for 2 h, and dehydrated in ethanol. Specimens were embedded in Epon (Sigma-Aldrich), and thin sections were cut using an ultramicrotome and double stained with uranyl acetate and lead citrate.

Apoptosis quantification by flow cytometry

After incubation with GA (0.00, 1.25, 2.50 or 5.00 μmol/L) for 48 h, HT-29 cells were collected with trypsin, centrifuged, washed with PBS, stained with Annexin V-FITC and propidium iodide (PI) according to the manufacturer's protocol, and then analyzed using a FACScan flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, United States). Quantification was conducted from histogram plots, where early apoptotic cells stained with Annexin V-FITC are presented in the lower right quadrant and late apoptotic cells stained with both Annexin V-FITC and PI are presented in the upper right quadrant.

RNA isolation and quantitative RT-PCR analysis

Total RNA was extracted using Trizol and the concentration and purity were determined by measuring the optical density. RNA from each sample (1 μg) was used to generate cDNA using M-MLV reverse transcriptase according to manufacturer's specifications. After an initial denaturation step at 95 ℃ for 10 min using SYBR Green PCR Master Mix (Applied Biosystems of Thermo Fisher Scientific, Waltham, MA, United States), RT-PCR was cycled between 95 ℃ for 15 s and 60 ℃ for 1 min for 40 cycles. Amplification was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems) and products were routinely checked using dissociation curve software. Transcript quantities were compared by the relative Ct method, and the amounts of caspase-8, -9 and -3 were normalized to the endogenous control (*GAPDH*). The relative value to the control sample was given by $2^{-\Delta\Delta CT}$. RT-PCR primer sequences were as follows: caspase-8, (forward) 5′-GCCTCCCTCAAGTTCCT-3′, (reverse) 5′-CCTGGAGTCTCTGGAATAACA-3′; caspase-9, (forward) 5′-CGAACTAACAGGCAAGCAGC-3′, (reverse) 5′-ACCTCACCAAATCCTCCAGAAC-3′; caspase-3, (forward) 5′-TGGTTCATCCAGTCGCTTTG-3′, (reverse) 5′-CATTCTGTTGCCACCTTTCG-3′.

Western blot analysis

Following treatment with GA (0.00, 1.25, 2.50 or 5.00 μmol/L) for 48 h, HT-29 cells were washed twice with ice-cold PBS and collected in lysis buffer. The supernatant was collected by centrifuging at 13500 rpm for 20 min. Total protein concentration was quantified using a Bradford assay, and 120 μg of protein from each sample was separated on 10%, 12%, and 15% SDS-PAGE gels, and transferred onto nitrocellulose membranes. The nitrocellulose membranes were blocked with 5% non-fat milk powder (w/v) at room temperature for 2 h, then incubated with a primary antibody against Fas (1:500), FasL (1:500), FADD (1:500), caspase-8 (1:500), cytochrome c (1:500), Apaf-1 (1:500), caspase-9 (1:500), caspase-3 (1:500), GAPDH (1:1000), or β-actin (1:500), at 4 ℃ overnight. After washing, the membranes were incubated with fluorescence-conjugated secondary antibody (1:10000 anti-rabbit or anti-mouse; Invitrogen) at room temperature for 50 min. β-actin was used as an internal control to monitor protein loading and transfer of proteins from the gel to the membrane. Western blot bands were quantified

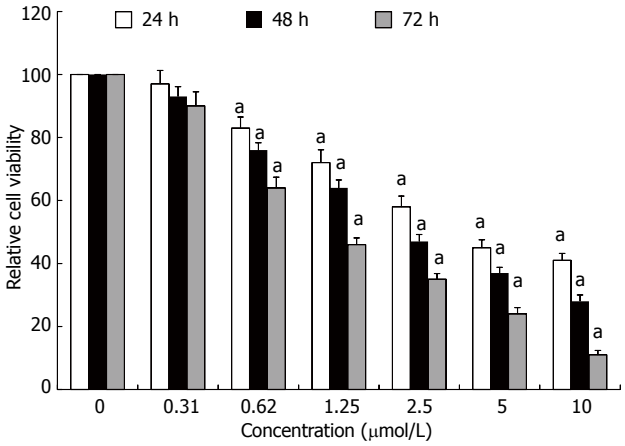


Figure 1 Gambogic acid-induced anti-proliferation of HT-29 cells. Relative cell viability (%) was evaluated by MTT assay after treatment for 24, 48 and 72 h. All data were normalized to 0 μmol/L, which was considered as 100%. $^{a}P < 0.05$ *vs* 0 μmol/L.

using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, United States). All results are representative of three independent experiments.

Xenograft assays in nude mice

Five-week-old BALB/c nude mice (n = 30) used for *in vivo* experiments were purchased from Vital River Laboratories (Beijing, China). The animal experimental protocol was approved by the ethics committee of Heilongjiang Province's Hospital (protocol number: 2008-010). The mice were housed in independent venting cases in a specific-pathogen free animal facility, with 6 mice in each case. The room temperature was keep at 20-25 ℃, humidity at 40%-70%, with a 12 h/12 h light/dark cycle. All animal procedures were in accordance with the Animal Research: Reporting of *In Vivo* Experiment guidelines. HT-29 cells (2×10^6 cells/mouse) were implanted by subcutaneous injection into the right armpit of the mice. When well-established HT-29 xenografts were palpable with a tumor size of 75 mm^3, mice were randomized into control and treatment groups, each containing 6 animals. All animals were checked twice a day. The animals in the GA group received caudal vein injections of GA (in saline; 5, 10 or 20 mg/kg) twice weekly for four weeks, whereas animals in negative and positive groups were given injections of the same volume of 0.9% saline and 10 mg/kg docetaxel, respectively, once weekly (0.1 mL/10 g).

All animals were weighed twice weekly, and mortality was monitored during the experimental period to assess toxicity of the treatments. Tumor volume was also measured twice weekly and calculated as: $0.5 \times ab^2$[24,37], where a and b refer to the longer and shorter dimensions, respectively. Relative tumor volume (RTV) was calculated according to the equation: Vt/V0, where V0 is the tumor volume and Vt is the tumor volume on day t. At the end of the experiment, all mice were euthanized and the subcutaneous tumors were weighed. The inhibition ratio of tumor weight (IR_{TW}) was calculated as: [(tumor weight of treatment group/tumor weight of saline group) × 100]. There were no animal deaths during the experiment and all tumor-implanted animals were humanely euthanized at the end of the experiment by overdose of pentobarbital (50 mg/kg; *ip*). The criteria for the humane endpoint were a tumor size > 20 mm in diameter with its weight more than 10% of the animal's body weight and/or the presence of ulceration, necrosis, or infection.

Statistical analysis

All statistical analyses were performed using SPSS software (Chicago, IL, United States). One-way analysis of variance (ANOVA) was used for comparison among groups, and two-way ANOVA was used for comparing two independent variables among groups followed by a Tukey's *post hoc* test. Data are shown as mean ± SE; $P < 0.05$ was considered to be significant.

RESULTS

GA-induced morphologic changes and anti-proliferation of HT-29 cells

HT-29 cellular morphology was observed and examined under a phase contrast microscope. Control cells showed a normal morphology with typical polygonal and cobblestone monolayer appearance, plump cell body, clear cell boundary, and transparent cytoplasm (data not shown). In the presence of GA, HT-29 cells appeared round with small wrinkles and broken debris, suggesting GA-induced toxicity.

Proliferation of HT-29 cells was assessed using the MTT assay (Figure 1). GA inhibited proliferation of HT-29 cells in a dose- and time-dependent manner, which was significant for concentrations of GA ⩾ 0.62 μmol/L at all times points ($P < 0.05$).

GA-induced apoptosis of HT-29 cells

Treatment of GT-29 cells with GA induced apoptosis as observed by TUNEL (Figure 2A) and Hoechst 33342 (Figure 2B) staining. Apoptotic HT-29 cells displayed round and shrunken cell bodies with condensed and fragmented nuclei. Transmission electron microscopy investigation revealed that HT-29 cells treated for 48 h with 2.5 μmol/L GA showed an abnormal subcellular morphology (Figure 3). The nuclear/cytoplasmic ratio was decreased, cells and nucleoli were shrunken, microvilli appeared on the cell membrane surface, apoptotic bodies appeared around the nuclear membrane, the nucleus was condensed and fragmented, the electron density deepened, and vacuolization in the cytoplasm became obvious.

Flow cytometric analysis was conducted to quantify GA-induced apoptosis; representative results are shown in Figure 4A. As shown in Figure 4B, only a small number of apoptotic cells (lower and upper right quadrants) was detected in the control group. Apoptosis rates at 48 h after treatment with 1.25,

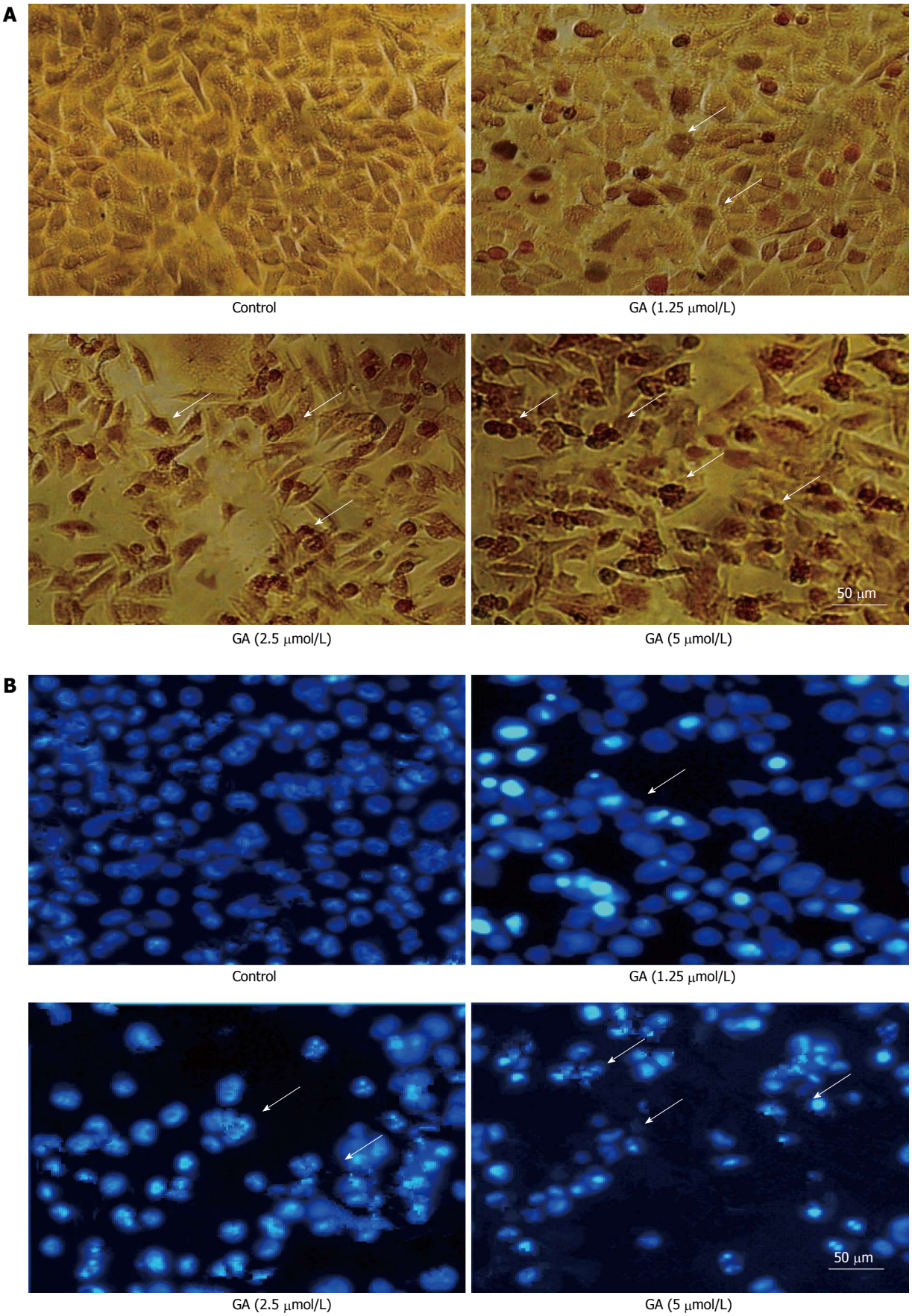


Figure 2 Gambogic acid-induced apoptosis. Apoptotic HT-29 cells (arrows) were observed by A: Terminal deoxynucleotidyl transferase dUTP nick end labeling; and B: Hoechst 33342 staining after treatment with gambogic acid (GA) for 48 h.

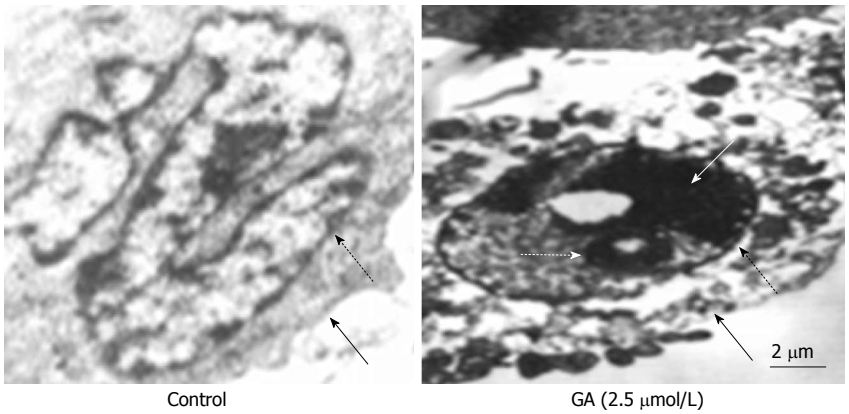


Figure 3 Ultrastructure of HT-29 cells. Transmission electron microscopy revealed ultrastructural changes in HT-29 cells after treatment with 2.5 μmol/L gambogic acid (GA) for 48 h; dashed black arrow shows the nuclear membrane; black arrow shows the cellular membrane; dashed white arrow shows the apoptotic body; and the white arrow shows the condensed nucleus.

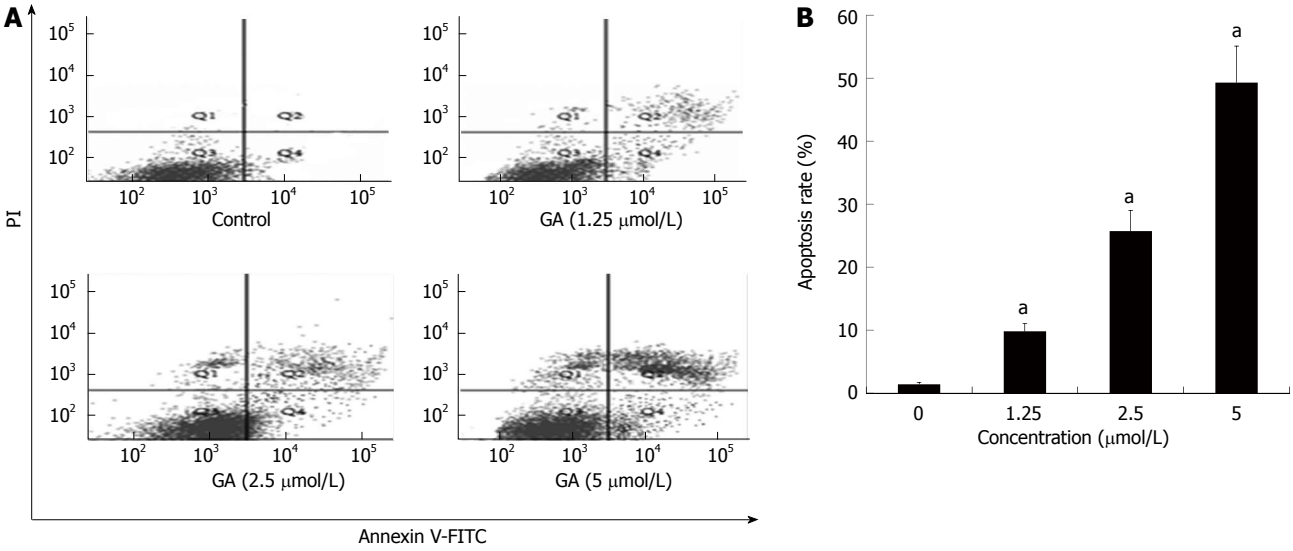


Figure 4 Quantification of gambogic acid-induced apoptosis by flow cytometry. A: HT-29 cells were treated for 48 h and sorted by flow cytometry to detect early (FITC-stained, lower right quadrant) and late [FITC- and propidium iodide (PI)-stained, upper right quadrant] apoptotic cells; B: The experiment was repeated three times and the average percentage of apoptotic cells (mean ± SE) is shown. $^{a}P < 0.05$ *vs* 0 μmol/L. GA: Gambogic acid.

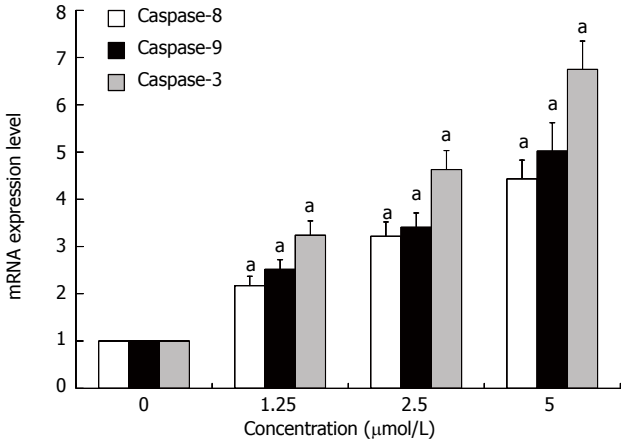


Figure 5 Gambogic acid increases the expression of caspase-9, -8 and -3 mRNAs in HT-29 cells. HT-29 cells were treated for 48 h and mRNA expression was analyzed by quantitative real-time PCR. Expression levels were normalized to *GAPDH* and are relative to 0 μmol/L. $^{a}P < 0.05$ *vs* 0 μmol/L.

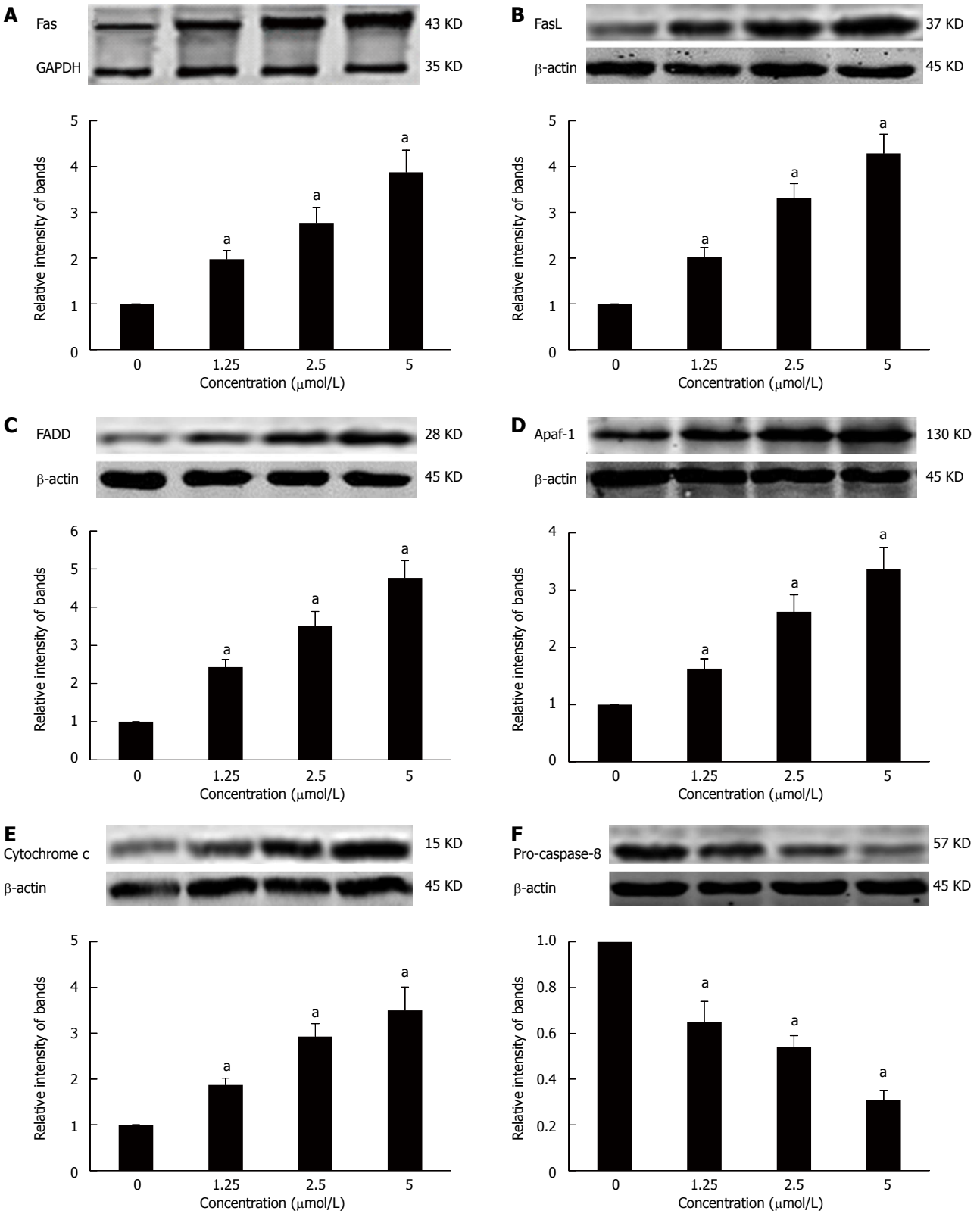
2.50 and 5.00 μmol/L GA were 9.8% ± 1.2%, 25.7% ± 3.3% and 49.3% ± 5.8%, respectively, which were significantly higher than that in the control condition (1.4% ± 0.3%; $P < 0.05$ for all).

GA increases mRNA expression of caspase-8, -9 and -3

The expression levels of caspase-8, -9 and -3 mRNAs in HT-29 cells were significantly increased after treatment with GA for 48 h as assessed by quantitative RT-PCR ($P < 0.05$ for all) (Figure 5).

Effects of GA on the death receptor and mitochondrial pathways in HT-29 cells

To further elucidate the molecular mechanism of GA-induced apoptosis in HT-29 cells, we examined the expression of proteins in the death receptor (extrinsic) and mitochondrial (intrinsic) apoptotic pathways. HT-29 cells treated for 48 h with GA expressed


A
Fas
GAPDH
43 KD
35 KD
Relative intensity of bands
Concentration (μmol/L)
B
FasL
β-actin
37 KD
45 KD
C
FADD
β-actin
28 KD
45 KD
D
Apaf-1
β-actin
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Cytochrome c
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F
Pro-caspase-8
β-actin
57 KD
45 KD

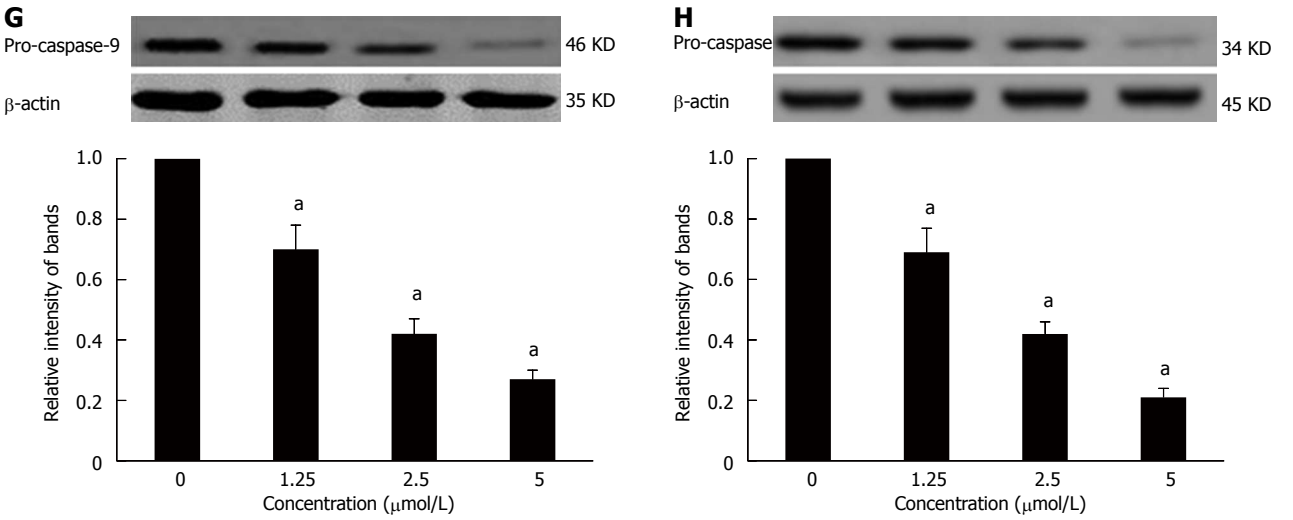

Figure 6 Effects of gambogic acid on the expression of apoptosis-related factors in HT-29 cells. HT-29 cells were treated with gambogic acid (GA) for 48 h and Western blot was performed to measure levels of Fas receptor (A); Fas ligand (FasL) (B); FADD (C); apaf-1 (D); cytochrome c (E); pro-caspase-8 (F); pro-caspase-9 (G); and pro-caspase-3 (H). Data are reported as the mean ± SE of at least three experiments. $^{a}P < 0.05$ *vs* 0 μmol/L.

significantly higher levels of Fas, FasL, FADD, Apaf-1, and cytochrome c ($P < 0.05$) (Figure 6A-E). At the same time, levels of pro-caspase-8, -9 -3 were significantly decreased ($P < 0.05$) (Figure 6F-H).

GA significantly inhibits growth of HT-29 tumors in a nude mouse xenograft model

The potential anti-tumor effect of GA *in vivo* was assessed using a human tumor xenograft mouse model. Compared with tumor growth in saline-treated mice, there was a dose-dependent decrease in tumor volume in mice treated with GA for the entire period of observation (Figure 7). Furthermore, there was a decrease in tumor weight on day 29 when the mice were euthanized, as evidenced by an increase in the IR_{TW}. GA was well tolerated at doses up to 20 mg/kg, with no signs of toxicity in this xenograft tumor model; loss of body weight after treatment was less than 10% in all treatment groups (data not shown).

DISCUSSION

This study demonstrates a dose- and time-dependent anti-proliferative effect of GA on human colon cancer cells *in vitro* and in an *in vivo* model. GA-induced apoptosis was previously reported in some other cell types, with relatively low toxicity and minimal side effects in normal cells[31,38,39]. As a physiologic mechanism, apoptosis kills cancer cells without imposing damage to normal cells or surrounding tissues[40]. Thus, inducing apoptosis in cancer cells has been a key mechanism for cancer treatment[41].

Apoptosis is a form of programmed cell death that typically leads to caspase activation *via* two major routes, the extrinsic death receptor and the intrinsic mitochondrial pathways[42]. Fas (CD95 or APO-1)[43] is a 36-kDa cell surface protein that belongs to the death receptor family, and has a pivotal role in apoptosis of breast[44], hepatocellular[45,46], colorectal[47,48] and nasopharyngeal[49] cancer cells *via* activation by its natural ligand, FasL. The death-inducing signaling complex (DISC) is rapidly formed after Fas stimulation, which consists of oligomerized Fas, FADD and pro-caspase-8. After binding to the DISC, pro-caspase-8 homodimers undergo a conformational change, and autocatalytic processing induces the generation of active caspase-8, leading to the activation of caspase-3. This caspase cascade leads to DNA damage and cell apoptosis[50-54]. In our study, we observed GA-induced increases in Fas, FasL, FADD, caspase-8 and caspase-3 expression, indicating that GA triggers apoptosis *via* the death receptor pathway.

Many factors, such as environmental stimuli and drugs, can induce mitochondrial dysfunction, leading to apoptosis *via* intrinsic pathways. Cytochrome c is released from dysfunctional mitochondria and accumulates in the cytoplasm where it binds to Apaf-1; pro-caspase-9 binds to Apaf-1 oligomers and leads to the formation of the apoptosome, followed by caspase-3 activation[55-57]. GA inhibits proliferation and induces apoptosis in many carcinoma cells *via* mitochondrial-dependent pathways[29-33]. Our data also show that GA induces upregulation of cytochrome c and Apaf-1, while downregulating pro-caspase-9 and -3. This result further supports an apoptotic effect of GA on HT-29 cells *via* the mitochondrial pathway.

GA has been long used for its anti-pyretic, analgesic and anti-inflammatory effects. However, the effect of GA on the growth of human colon cancer cells is still not very clear. From our tests, we can conclude that the death receptor and mitochondrial pathways are involved in the anti-tumor effect of GA in HT-29 cells. Our results, together with other studies, will provide a reference for clinical trials, though further studies are necessary.

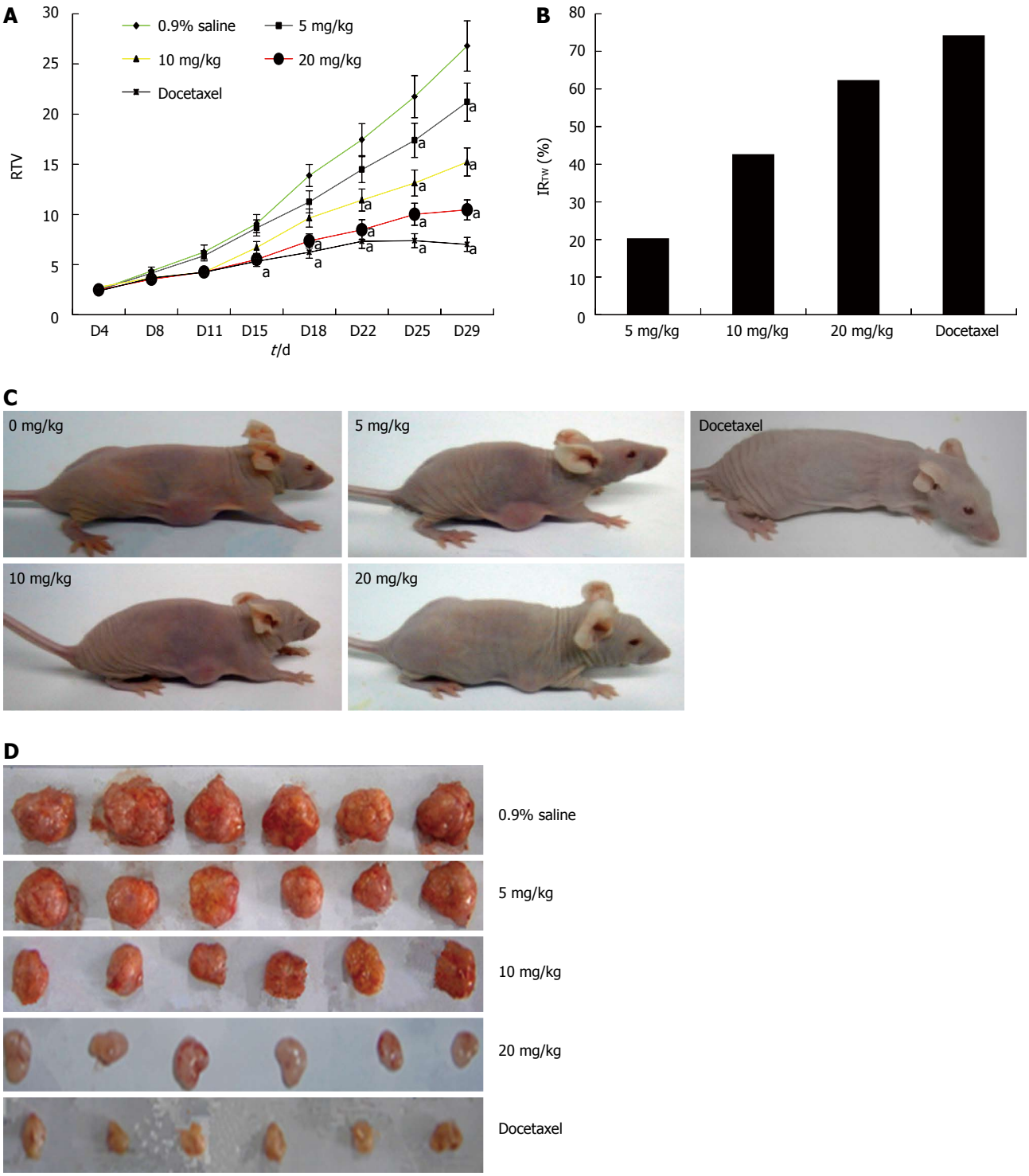

Figure 7 Gambogic acid inhibits the proliferation of HT-29 cells in BALB/c nude mice. A: Relative tumor volume (RTV) (mean ± SE) over the 29 d experiment; B: Inhibition ratio of tumor weight (IR_{TW}) from subcutaneously implanted HT-29 cells; Representative photographs of nude mice (C) and tumor samples (D) from the saline-, gambogic acid-, and docetaxel-treated groups. $^{a}P < 0.05$ *vs* saline.

COMMENTS

Background

Colorectal cancer is the third leading cause of cancer and the fourth leading cause of cancer-related deaths worldwide. Chemotherapy is an effective treatment for colon cancer, but traditional chemotherapy has many serious side effects, including significant pain.

Research frontiers

Gambogic acid (GA) is the major active ingredient in gamboge, extracted from various *Garcinia* species, including *Garcinia hanburyi* Hook f. (Tenghuang). GA has various biologic activities, including anti-pyretic, analgesic, anti-inflammatory, autophagic and anti-tumor activities. However, little is known regarding the effect of GA on the growth of human colon cancer cells.

Innovations and breakthroughs

This study is the first to report the effects of GA on the human colon cancer cell line HT-29. We observed a dose- and time-dependent anti-proliferative effect of GA on the cells. GA-induced apoptosis of HT-29 cells may be mediated by activation of the death receptor and mitochondrial pathways, as observed by

increased expression of apoptosis-related proteins. *In vivo*, GA significantly inhibited the growth of HT-29 tumors in a nude mouse xenograft model.

Applications

The results of this study suggest that GA inhibits HT-29 proliferation *via* induction of apoptosis and that the effects may be mediated by death receptor and mitochondrial pathways. The results will provide a reference for clinical trials, though further studies are necessary.

Terminology

Apoptosis is a programmed cell death process that cells undergo in response to certain signals.

Peer-review

This is a nice piece of work, where authors report that GA inhibits HT-29 proliferation *via* induction of apoptosis and these effects are possibly mediated by death receptor (extrinsic) and mitochondrial (intrinsic) pathways.

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ORIGINAL ARTICLE

Basic Study

Expression of COX-2 and HER-2 in colorectal cancer and their correlation

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Author contributions: Wu QB designed and performed research, analyzed data and wrote the paper; and Sun GP designed and contributed new reagents or analytic tools.
Ethics approval: The study was reviewed and approved by the First Affiliated Hospital of Anhui Medical University Institutional Review Board.

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Abstract

AIM: To detect the expression of COX-2 and HER-2 in colorectal cancer and to analyze their correlation and clinical significance.

METHODS: A total of 1026 colorectal cancer surgical specimens were collected from patients treated from December 2002 to December 2007 at the First Affiliated Hospital of Anhui Medical University. All specimens were made into 4-μm slices. The expression of COX-2 and HER-2 were detected by immunohistochemistry using the streptavidin-biotin-peroxidase method. The correlations between COX-2 and HER-2 expression and colorectal cancer clinical features were analyzed.

RESULTS: The positive rates of COX-2 and HER-2 expression in colorectal cancer were 77.97% (800/1026) and 46.20% (474/1026), respectively. There was a significant correlation between COX-2 and HER-2 expression in colorectal cancer ($P < 0.05$). In patients with tumor size $\geqslant$ 5 cm, the positive rates of COX-2 and HER-2 expression were 81.48% (308/378) and 57.94% (219/378), respectively. In patients with serosal invasion, the positive COX-2 and HER-2 expression rates were 80.53% (612/760) and 49.21% (374/760), respectively. In patients with lymph node metastasis, the positive expression rates were 85.04% (506/595) and 54.62% (325/595), respectively, and the positive expression rates differed significantly between patients with lymph node metastasis and those without ($P < 0.05$). In patients with Duke's C and D colorectal cancer, the positive COX-2 and HER-2 expression rates were 82.80% (443/535) and 57.94% (310/535), respectively. In patients with poorly differentiated colorectal cancer, the positive expression rates were 74.49% (210/282) and 52.84% (149/282), respectively ($P < 0.05$). In patients with distant metastasis, the positive expression rates were 82.27% (116/141) and 53.90% (76/141), respectively ($P < 0.05$). These findings suggest that COX-2 and HER-2 have synergistic effects in colorectal cancer. COX-2 and HER-2 expression had no significant correlation with sex, age, or tumor location.

CONCLUSION: COX-2 and HER-2 are important markers for invasion and metastasis of colorectal cancer, and they act together to regulate the invasion and metastasis of colorectal cancer.

Key words: Colorectal cancer; Correlation; COX-2; HER-2; Immunohistochemistry; Survival rate

Core tip: The relationship between and expression of COX-2 and HER-2 in colorectal cancer have not been fully elucidated. In this study, the expression of COX-2 and HER-2 was assessed in colorectal cancer tissues by immunohistochemistry, and the correlations between COX-2 and HER-2 expression and colorectal cancer clinical features were evaluated. Results demonstrated that COX-2 and HER-2 expression are significantly associated with serosal invasion, lymph node metastasis, Duke's stage, and poorly differentiated cancer. COX-2 and HER-2 have synergistic effects in colorectal cancer. Both COX-2 and HER-2 are important markers for invasion and metastasis of colorectal cancer.

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INTRODUCTION

Colorectal cancer is a very common malignant tumor of the digestive tract, with about 1.2 million new cases and 600000 deaths worldwide each year[1]. The incidence of colorectal cancer ranks second among various malignances in the Western developed countries. Although both the incidence and mortality of colorectal cancer rank between third and fifth in China, respectively, the incidence of colorectal cancer ranks second or third in large cities. Thus, colorectal cancer poses a serious threat to human health. As colorectal cancer has a complicated biologic behavior and is easy to relapse, metastasize, and develop resistance to chemotherapy drugs, the clarification of the mechanisms responsible for the development and progression of colorectal cancer and the development of early and effective diagnostic strategies and reasonable treatment strategies have always been focuses of research in the field of colorectal cancer[2].

Invasion and metastasis are the main biologic characteristics of malignant tumors. The poor therapeutic effects in colorectal cancer are often associated with tumor invasion and metastasis. Therefore, there is an emerging need to understand how to predict tumor invasion and metastasis and conduct early comprehensive therapy for tumors clinically[3]. In the present study, an immunohistochemical method was used to detect the expression of COX-2 and HER-2 in colorectal cancer, and the relationship between COX-2 and HER-2 expression and prognosis of colorectal cancer was analyzed, with an aim to provide a theoretical basis of pathologic diagnosis, prognosis evaluation, and treatment of this malignancy.

MATERIALS AND METHODS

Specimens

A total of 1026 colorectal cancer surgical specimens were collected from patients treated between December 2002 and December 2007 at the First Affiliated Hospital of Anhui Medical University. The patients ranged in age from 14 years to 88 years, with an average age of 57 years. There were 484 cases of colon cancer and 542 cases of rectal cancer. In terms of differentiation degree, 155 cases were well differentiated, 589 were moderately differentiated, and 282 were poorly differentiated. All specimens were fixed in 10% formalin, embedded in paraffin, and sectioned into 4-μm slices. Tissues 5 cm or above from the resection margin were used as controls. None of the patients received any radiotherapy, chemotherapy, or immunotherapy before surgery, and were confirmed pathologically after surgery. This study was approved by the Ethical Committee of Anhui Medical University.

Reagents

Rabbit anti-human COX-2 monoclonal antibody (SP21), rabbit anti-human HER-2 polyclonal antibody, and a color development kit were purchased from Zhongshan Golden Bridge Biotechnology (Beijing, China).

Immunostaining

Immunohistochemical staining was performed using the streptavidin-biotin-peroxidase method according to the manufacturer's instructions. For the negative control, the primary antibody was replaced with PBS. Tissues known to express the antigens of interest were used as positive controls.

Immunostaining evaluation

Immunostaining evaluation was performed using a semi-quantitative scoring system by estimating the percentage of cells stained and staining intensity. Immunohistochemically stained sections were evaluated independently by two experienced pathologists, with five high-power visual fields observed in each section. The percentage of cells stained was scored as follows: 0 = 0%-5%; 1 = 6%-25%; 2 = 26%-50%; 3 = 51%-75%; 4 = 76%-100%. Staining intensity was scored as follows: 1 = faintly yellow; 2 = brownish yellow; 3 = brown. A combined score was calculated as the sum of staining intensity and percentage of stained cells, and immunostaining was scored as negative (-; combined score = 0-1); positive (+; combined score = 2-3); moderately positive (++; combined score = 4-5);

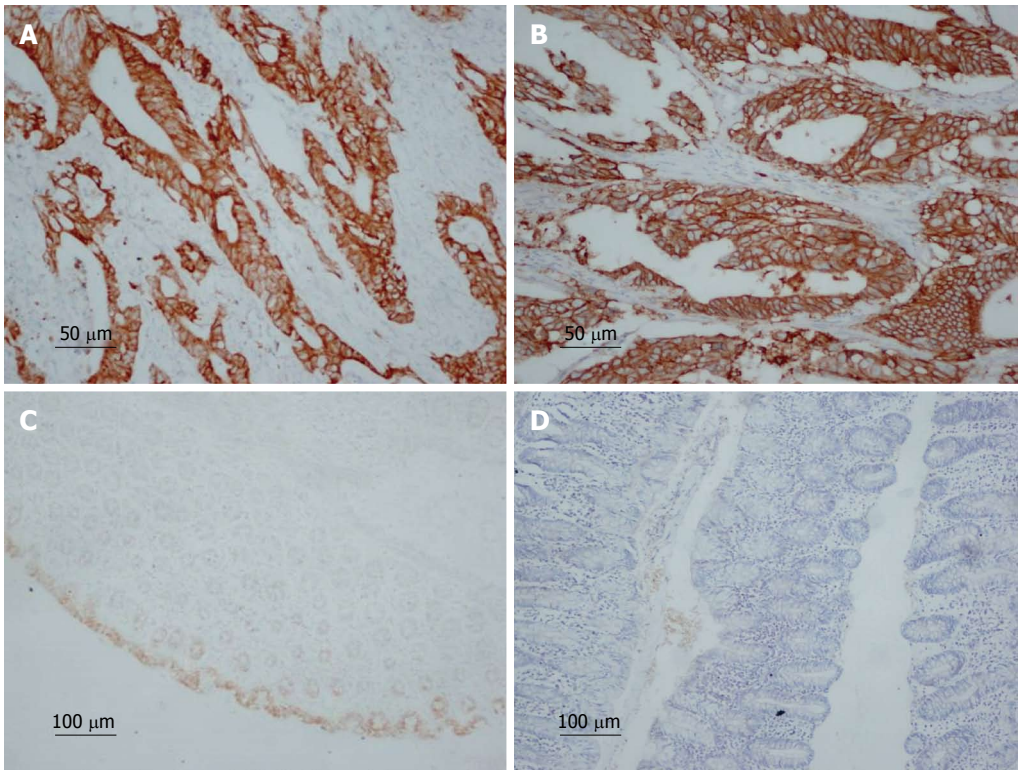

Figure 1 Immunohistochemical staining of COX-2 and HER-2 in colorectal cancer and normal colorectal tissue. A: COX-2-positive expression in colorectal cancer, magnification × 200; B: HER-2 positive expression in colorectal cancer, magnification × 200; C: COX-2-negative expression in normal colorectal tissue, magnification × 100; D: HER-2-negative expression in normal colorectal tissue, magnification × 100.

strongly positive (+++; combined score = 6-7).

Statistical analysis

Statistical analyses of the immunohistochemical staining were performed using SPSS16.0 software (SPSS Inc., Chicago, IL, United States). Differences were tested for statistical significance using the Mann-Whitney *U* test. Survival analysis was conducted using the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

RESULTS

COX-2 expression in colorectal cancer

COX-2-positive cells showed brownish yellow granules in the cytoplasm (Figure 1A). The positive rate of COX-2 expression was 77.97% (800/1026) in all the specimens. In patients with a tumor size ⩾ 5 cm, the positive rate of COX-2 expression was 81.48% (308/378). In patients with serosal invasion, the positive expression rate was 80.53% (612/760). In patients with Duke's C and D colorectal cancer, the positive expression rate was 82.80% (443/535). In patients with lymph node metastasis, the positive expression rate was 85.04% (506/595), and the positive expression rate differed significantly between patients with lymph node metastasis and those without ($\chi^2 = 41.213$; $P < 0.05$). High COX-2 protein expression was significantly correlated with tumor size, infiltration depth, Duke's stage, tumor differentiation, distant metastasis, and lymph node metastasis ($P < 0.05$), but not with sex, age, or tumor location (Table 1).

HER-2 expression in colorectal cancer

HER-2 was localized mainly on the membrane of cancer cells, and HER-2-positive cells showed brownish yellow granules on the membrane (Figure 1B). The positive rate of HER-2 expression in colorectal cancer was 46.20% (474/1026) in all the specimens. In patients with a tumor size ⩾ 5 cm, the positive rate of HER-2 expression rate was 57.94% (219/378). In patients with serosal invasion, the positive expression rate was 49.21% (374/760). In patients with Duke's C and D colorectal cancer, the positive expression rate was 57.94% (310/535). In colorectal adenocarcinoma patients with lymph node metastasis, the positive expression rate was 54.62% (325/595), and the positive expression rate differed significantly between patients with lymph node metastasis and those without ($\chi^2 = 40.430$; $P < 0.05$). High HER-2 protein expression was significantly correlated with tumor size, invasion depth, Duke's stage, tumor differentiation, distant metastasis, and lymph node metastasis ($P < 0.05$), but not with sex, age, or tumor location (Table 1).

Correlation between COX-2 and HER-2 expression in colorectal cancer

Of 800 COX-2 positive specimens, 350 were positive for HER-2 and 450 were negative. Of 226 COX-2

Table 1 Relationship between COX-2/HER-2 expression and clinicopathologic factors *n* (%)

Variable	*n*	COX-2				HER-2			
		Positive	Negative	χ^2	*P* value	Positive	Negative	χ^2	*P* value
Sex				2.300	0.129			2.769	0.096
Male	595	454 (76.3)	62 (20.0)			288 (48.4)	307 (51.6)		
Female	431	346 (80.2)	32 (15.1)			186 (43.2)	245 (56.8)		
Age (yr)				2.011	0.156			1.496	0.221
< 60	493	375 (76.1)	40 (16.3)			218 (44.2)	275 (55.8)		
⩾ 60	533	425 (79.7)	54 (19.6)			256 (48.0)	277 (52.0)		
Tumor site				2.007	0.157			2.499	0.114
Rectum	542	432 (79.7)	45 (16.3)			263 (48.5)	279 (51.5)		
Colon	484	368 (76.0)	49 (19.9)			211 (43.6)	273 (56.4)		
Tumor size				4.988	0.026			33.175	0.000
< 5 cm	648	489 (75.5)	159 (24.5)			255 (39.4)	393 (60.6)		
⩾ 5 cm	378	308 (81.5)	70 (18.5)			219 (57.9)	159 (42.1)		
Serosal invasion				11.130	0.001			10.697	0.001
No	266	188 (70.7)	78 (29.3)			100 (37.6)	166 (62.4)		
Yes	760	612 (80.5)	148 (19.5)			374 (49.2)	386 (50.8)		
Differentiation				6.553	0.038			6.911	0.032
Well	155	114 (73.5)	41 (26.5)			67 (43.2)	88 (56.8)		
Moderate	589	476 (80.8)	113 (19.2)			258 (43.8)	331 (56.2)		
Poor	282	210 (74.5)	72 (25.5)			149 (52.8)	133 (47.2)		
Duke's stage				15.191	0.000			62.045	0.000
A + B	491	357 (72.7)	134 (27.3)			164 (33.4)	327 (66.6)		
C + D	535	443 (82.8)	92 (17.2)			310 (57.9)	225 (42.1)		
Lymph node metastasis				14.865	0.000			40.430	0.000
Yes	595	506 (85.0)	89 (15.0)			325 (54.6)	270 (45.4)		
No	431	294 (68.2)	137 (31.8)			149 (34.6)	282 (65.4)		
Distal metastasis				3.928	0.047			3.901	0.048
Yes	141	119 (84.4)	22 (15.6)			76 (53.9)	65 (46.1)		
No	885	681 (76.9)	204 (23.1)			398 (45.0)	487 (55.0)		

Table 2 Relationship between COX-2 and HER-2 expression

COX-2 expression	HER-2 expression		Total	χ^2	*P* value
	Positive	Negative			
Positive	350	450	800	8.762	0.003
Negative	124	102	226		
Total	474	552	1026		

negative specimens, 124 were positive for HER-2 and 102 were negative. There was a significant positive correlation between COX-2 and HER-2 expression in colorectal cancer ($\chi^2 = 8.762$; $P < 0.05$) (Table 2).

COX-2 and HER-2 expression in normal colorectal tissues

A total of 50 tumor-adjacent colorectal tissues were used as normal colorectal tissues. Of these tissues, 6/50 (12.00%) showed positive COX-2 expression, and 1/50 (2.00%) showed positive HER-2 expression. Most of the normal tissues showed negative expression of COX-2 and HER-2 (Figure 1C and 1D).

Survival analysis

By February 28, 2011, 210/1026 (20.47%) patients were lost to follow-up, and 816/1026 (79.53%) had complete follow-up data. The follow-up duration ranged from 3 years to 5 years. The survival curves for COX-2 and HER-2 positive and negative patients are shown in Figure 2A and 2B, respectively. The survival curves for patients positive for both COX-2 and HER-2, those positive for either of them, and those negative for both are shown in Figure 3. These results showed that the survival time of COX-2 and HER-2 positive patients was significantly lower than that of COX-2 and HER-2 negative ones ($P < 0.05$).

DISCUSSION

According to the results of this study, the positive rate of COX-2 expression in colorectal cancer is 77.97%, significantly higher than in normal colorectal tissues. COX-2 expression was significantly associated with lymph node metastasis[4]. This may be because COX-2 can: (1) increase the production of prostaglandins and inhibit the body's immune response; (2) inhibit tumor cell apoptosis and promote cell proliferation; (3) regulate cell cycle progression; (4) promote tumor angiogenesis; (5) increase the expression of matrix metalloproteinases in tumor cells; and (6) induce activation of precursors of carcinogenic substances. As high COX-2 expression exists in precancerous lesions and carcinoma *in situ* and is significantly higher than in the normal tissue, it is generally believed that high COX-2 expression is an early event in tumorigenesis.

COX-2 is not or is lowly expressed in normal tissues; however, COX-2 expression is increased in inflammatory and tumor tissues and plays an

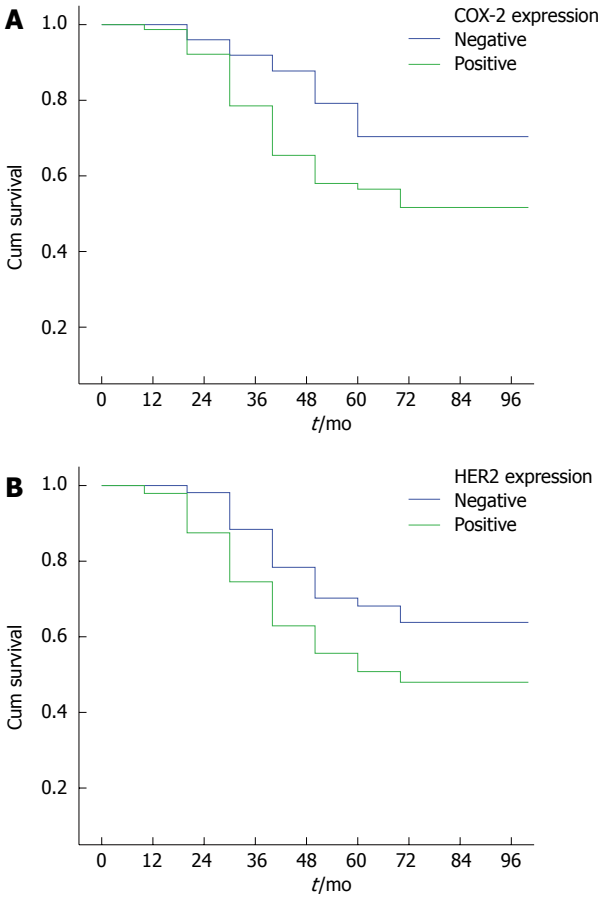

Figure 2 Survival curves for patients with colorectal cancer. A: Patients with positive and negative COX-2 expression; B: Patients with positive and negative HER-2 expression.

important role in inflammation, cell proliferation, and differentiation, suggesting that COX-2 is involved in the initiation and progression of cancer. Brown *et al*[4] believed that most colorectal cancers have COX-2 overexpression, which can induce tumor angiogenesis, damage the immune system, and promote tumor invasion. Tuynman *et al*[5] incubated lymphocytes with COX-2-positive or -negative colorectal cancer cells and found that lymphocyte proliferation index was significantly reduced in COX-2-positive colorectal cancer cells. They also found that this effect could be inhibited by the COX-2 inhibitor NS-398, suggesting that COX-2 expression in colorectal cancer can inhibit the proliferation of lymphocytes to make the tumor evade the host immune response.

Elzagheid *et al*[6] performed an immunohistochemical analysis of 145 stage Ⅰ-Ⅳ colorectal cancer specimens collected from 1981 to 1990 at Finland Turku University Hospital and found that patients with higher TNM stage ($P < 0.06$) and those with higher Duke's stage ($P < 0.045$) had higher levels of COX-2 expression, though COX2 expression was not significantly associated with age, sex, tumor histologic grade, or lymph node metastasis. Research shows that regulation of COX-2 expression is a key step in colorectal carcinogenesis. As COX-2 expression

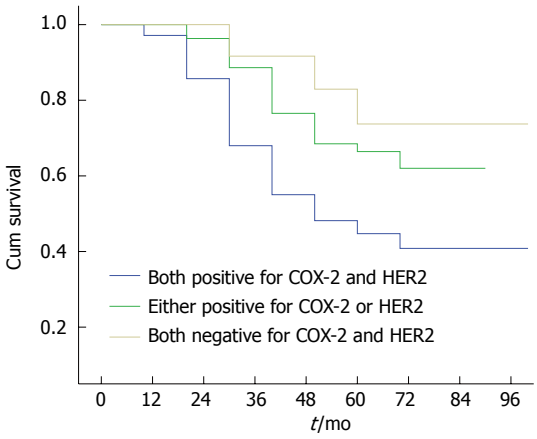

Figure 3 Survival curves of colorectal cancer patients positive for both COX-2 and HER-2, positive for either of them, and negative for both. Compared with patients positive for both markers, patients negative for both had better survival.

is significantly associated with tumor stage, it is considered a prognostic factor for colorectal cancer. The increase in the activity of COX-2 promotes the progression of colorectal cancer.

Peng *et al*[7] conducted a meta-analysis of 23 studies involving 4567 colorectal cancer patients that evaluated the relationship between COX-2 expression detected by immunohistochemistry and patient survival, and they found that high expression of COX-2 was associated with slightly poorer survival. The present study draws a similar conclusion. Of 1026 colorectal cancer tissues, the positive rate of COX-2 expression is 77.97%. In colorectal adenocarcinoma patients with lymph node metastasis, the positive expression rate is 85.04%, significantly higher than in patients without lymph node metastasis. In addition, COX-2 protein expression is significantly associated with tumor size, infiltration depth, tissue differentiation, Duke's stage, and distant metastasis, confirming that high COX-2 protein expression in colorectal cancer tissue may enhance the ability of tumor growth. This result is similar to many previous studies reporting that COX-2 overexpression in colorectal cancer tissue is closely related to tumor invasion, lymph node metastasis, and poor prognosis.

HER-2 is a protein with tyrosine kinase activity encoded by the cellular oncogene *ERBB2*, which is located on chromosome 17q21[8]. Under normal circumstances, the oncogene is inactive and is the normal component of the cellular genome, participating in the regulation of cell growth, differentiation, and division. The main forms of *ERBB2* activation are abnormal gene amplification, abnormal transcription regulation, and mRNA overexpression, leading to excessive expression of its protein product and producing tumor transformation activity[9]. Numerous studies have shown that its overexpression is associated with pathologic morphology and biologic behavior in a wide variety of tumors, especially breast,

gastric, ovarian, and colorectal cancers. Studies confirm that high expression of this protein in breast cancer is closely related to high S-phase fraction, high mitosis index, high thymine labeling index, and DNA heteroploidy.

Ma *et al*[10] found that the peptide fragments of HER-2 after enzymatic digestion were almost completely consistent with those of the epidermal growth factor receptor (EGFR). Even in the absence of ligands, HER-2 can still lead to a sustainable activation of EGFR protein kinase, make the cells grow out of control, and result in tumor occurrence. At present, HER-2-targeted therapy has been widely used for the treatment of breast cancer; however, its role in colorectal cancer is rarely reported. The present study shows that the positive rate of HER-2 expression in colorectal cancer is 46.20%, suggesting that HER-2 has a very important role in the growth, invasion, and metastasis of colorectal cancer. Theoretically, HER-2-targeted drugs can also restrain invasion and metastasis of colorectal cancer in which HER-2 is overexpressed. A better understanding of the biologic characteristics of colorectal cancer may open a new avenue for targeted therapy of colorectal cancer[11].

This study was performed in two stages. In the first stage, 522 specimens were collected from December 2005 to December 2007, and the experiments were performed between March 2010 and September 2010. In the second stage, 504 specimens were collected from December 2002 to November 2002, and the experiments were performed between September 2010 and March 2010. When comparing the results between the two stages, the positive rates of COX-2 and HER-2 expression were slightly lower in the second stage. This might be due to prolonged specimen storage and the increase in the number of specimens. According to the pathologic reports, the 1026 tumors were roughly classified into the following histologic types: papillary adenocarcinoma, tubular adenocarcinoma, mucous adenocarcinoma, signet ring cell carcinoma, undifferentiated carcinoma, adenosquamous carcinoma, squamous cell carcinoma, carcinoid, and small cell carcinoma. In addition, there is currently controversy over whether cancer nodules should be classified as regional lymph node metastasis or distant metastasis and whether liver metastasis should be regarded as direct invasion or distant metastasis. Upon consideration, we finally decided to classify papillary adenocarcinoma and carcinoid as well-differentiated tumors, and classify mucous adenocarcinoma, signet ring cell carcinoma, undifferentiated carcinoma, and neuroendocrine carcinoma as poorly differentiated tumors. Liver metastases of hepatic flexure colon cancer were regarded as direct invasion of adjacent organs, while other liver metastases of colon cancer were regarded as distant metastasis. For Duke's staging, TxN0M0 was regarded as A + B, and N1-3 or M1 as C + D. In terms of tumor differentiation, moderately or poorly differentiated adenocarcinoma was classified as moderately differentiated adenocarcinoma in the first stage, but was later classified as poorly differentiated adenocarcinoma in the second stage after consultation with several experienced pathologists. In terms of distant metastasis, cancer nodules were initially staged as N1, but later adjusted to M1 after literature search. These made the *P* values in preliminary experimental results in both groups > 0.05, which showed a slight difference from the results obtained using large samples. After adjustment, the *P* values became < 0.05 in both groups. This is consistent with multiple previous reports.

Many genes are activated in tumor development and progression, and these genes interact with each other to promote the growth and malignant transformation of tumors. There is a large body of evidence that upregulation of inducible COX-2 may promote the occurrence of colorectal cancer, but the mechanism responsible for regulating its expression is not clear. Previous studies show that there are two possible growth factor signaling pathways associated with COX-2 expression: HER-2/neu and transforming growth factor-β/Smad. Kiguchi *et al*[12] found that overexpression of HER-2/neu in the bile duct epithelium of transgenic mice induced upregulation of COX-2 expression. Vadlamudi *et al*[13] found that in human colorectal cancer cell lines, HER-2/neu activated factors promoting COX-2, causing COX-2 mRNA and protein expression and the accumulation of prostaglandin E2. Lucarelli *et al*[14] discovered that the positive rates of COX-2 in invasive breast cancer, ductal carcinoma *in situ*, and normal breast epithelial cells were 87%, 85%, and 75%, respectively, and those of HER-2 in invasive breast cancer and ductal carcinoma *in situ* detection were both 34%, suggesting that HER-2 and COX-2 may regulate each other.

Hirokazu *et al*[15] showed that the positive rate of COX-2 expression in gastric cancer was 54.8% and that of HER-2 was 86.4%. The present study examined the correlation between COX-2 and HER-2 expression in colorectal cancer. The results show that both COX-2 and HER-2 are highly expressed in colorectal cancer, and there is a significant correlation between the expression of COX-2 and HER-2. In addition, the positive rate of COX-2 expression in HER-2 positive patients is significantly higher than in HER-2 negative ones. Taken together, these findings suggest that HER-2 may upregulate the expression of COX-2. Studies have shown that the HER-2 inhibitor trastuzumab significantly prolongs survival of patients with colorectal cancer. Thus, whether inhibition of COX-2, a downstream protein of HER-2, could improve the therapeutic effect of HER-2 inhibitor deserves further research.

Studies have shown that high expression of both COX-2 and HER-2 increase the expression of vascular

endothelial growth factor C (VEGF-C) in tumor cells. Su *et al*[16] discovered that transfection with COX-2 gene or exposure to prostaglandin E2 in lung adenocarcinoma cells significantly upregulated the expression of VEGF-C protein, whereas COX-2-specific inhibitors reduced the expression of endogenous VEGF-C. The authors suggested that COX-2 upregulated VEGF-C through prostaglandin receptor EP1 and HER-2. The present study found that COX-2 and HER-2 expression have a significant correlation, and both are significantly associated with lymph node metastasis, tumor infiltration depth, and Duke's stage. We speculate that upregulation of COX-2 expression may promote the secretion of VEGF-C by tumor cells, thus promoting lymphangiogenesis via the VEGF-C/VEGFR-3 signaling pathway, and lead to lymphatic spread of tumor cells.

COX-2 expression in tumor tissue is correlated with prognosis. Smakman *et al*[17] found that in colon cancer cells with high COX-2 expression, the adhesion of the cells to matrix and the potential of cancer cells to metastasize increase, both of which are conducive to the development, progression, and metastasis of cancer. Zhang *et al*[18] detected the expression of COX-2 in 64 normal mucosal specimens, 116 primary colon cancer specimens, and 16 colon cancer metastases, and they found that the positive rate of COX-2 expression was 12% in normal mucosal tissues, 72% in primary tumors, and 100% in colon cancer metastases. The present study shows that the two- and four-year survival rates are significantly lower in the COX-2-positive group than in the COX-2-negative group, indicating that COX-2 overexpression is positively correlated with the recurrence and metastasis of colorectal cancer, and negatively with the prognosis of colorectal cancer.

Al-Maghrabi *et al*[19] found that 56% of patients with colorectal cancer showed positive cytoplasmic expression of COX-2, and COX-2 expression was positively associated with lymph node involvement and distant metastasis. In addition, high COX-2 expression was associated with a higher rate of tumor recurrence, suggesting that COX-2 expression provides useful prognostic information in colorectal cancer and may help screen patients with a high risk of recurrence. The present study indicates that the survival time of COX-2-negative patients is significantly higher than of COX-2-positive ones, further confirming that COX-2 expression is associated with a poor prognosis.

Dixon *et al*[20] showed that high COX-2 expression is an important factor contributing to colorectal carcinogenesis. COX-2 inhibitors (*e.g.*, celecoxib) can reduce the risk of relapse of colorectal adenomas. Nonsteroidal anti-inflammatory drugs are known to reduce the risk and mortality of colorectal cancer by inhibiting cyclooxygenases. Kasper *et al*[21] performed an immunohistochemical analysis of COX-2 expression in colorectal cancer and liver metastases in 57 patients and found that COX-2 was consistently involved in the occurrence of metastatic colorectal cancer. COX-2 inhibitors exert their antitumor effects possibly by altering the signaling pathways related to cell sensitivity and apoptosis.

Rahman *et al*[22] analyzed 130 cases of colorectal cancer and found that high COX-2 expression was associated with resistance to chemotherapy drugs and faster tumor cell growth. If 5-fluorouracil is given together with celecoxib, the latter may inhibit multidrug resistance and improve the chemotherapy sensitivity of drug-resistant cells. Thus, COX-2 inhibitors combined with currently used 5-fluorouracil-based regimens may have potentially positive benefits. Roelofs *et al*[23] found high COX-2 mRNA expression in almost 80% of colorectal cancer cases, suggesting that COX-2 is a potential biomarker of cancer risk, and COX-2 inhibitors may prevent colon cancer. Kraus *et al*[24] conducted many animal experiments showing that COX-2 inhibitors prevent the formation of adenomas or delay their development, and reduce the morbidity and mortality of colorectal cancer. However, cancer prevention is still being ignored in the cancer research field.

HER-2 has malignant transforming activity, and overexpression of HER-2 often suggests high degree of malignancy and poor prognosis. Researchers from both China and other countries have reported that colorectal cancer patients with high HER-2 expression had earlier lymph node metastasis, poorer prognosis, and shorter survival[25,26]. The five-year survival rate of patients with HER-2 overexpression was significantly lower than those with negative HER-2 expression[27], and HER-2 overexpression can be used as a reliable indicator of colorectal cancer prognosis. However, Li *et al*[28] performed a meta-analysis and found that HER-2 overexpression may have little impact on survival of patients with colorectal cancer. Lu *et al*[29] believed that EGFR and HER2 may be used as potential biomarkers for lymph node metastasis and prognosis of colorectal cancer. EGFR or HER2 is a potential predictor of poor clinical prognosis of colorectal cancer. The present study also found that the survival of HER-2-positive patients is significantly lower than of HER-2-negative ones.

In conclusion, the findings presented here suggest that there is a positive correlation between COX-2 and HER-2 expression, and simultaneous expression of COX-2 and HER-2 can enhance the metastasis and invasion ability of colorectal cancer, and suggests poor prognosis in patients with colorectal cancer. Joint detection of COX-2 and HER-2 expression in colorectal cancer tissue can be used an effective index for evaluating prognosis and screening patients with a high risk of metastasis.

COX-2- and HER-2-targeted drugs provide another effective, comprehensive, individualized treatment option for patients with colorectal cancer. However, the following problems need to be solved before they

can be used clinically: (1) the relationship between targeted drug therapy and expression of COX-2 and HER-2 in colorectal cancer needs to be further defined. It remains to be investigated how to formulate standardized indications for use of targeted drug therapy in colorectal cancer based on the expression of COX-2 and HER-2. This requires a multi-disciplinary approach; (2) the timing and dose of targeted drug therapy for colorectal cancer should be determined; and (3) the combined use of targeted drugs with surgery, chemotherapy, and radiotherapy, as well as the joint use of multiple molecular-targeted drugs should be explored. The better understanding of the biologic characteristics of colorectal cancer and the further research of targeted drug therapy will provide a new avenue for the treatment of colorectal cancer.

As COX-2 and HER-2 expression was detected using the immunohistochemical method in the present study, the results may be influenced by the quality of antibody reagents and the skill of operators. Also, quantitative detection could not be performed, though the method is simple. In addition, due to the large number of specimens and the long time span of the study, laboratory conditions such as temperature and humidity were different. As a result, the positive rates of COX-2 and HER-2 expression were slightly different from those reported in the literature. More stable detection methods, such as fluorescence *in situ* hybridization and quantitative PCR, and standardized reagents (*e.g.*, fixed manufacturers) should be used in the future to overcome the above-listed problems.

COMMENTS

Background

Colorectal cancer is a very common malignancy and the second leading cause of cancer deaths worldwide. Although significant advances have been made in the understanding and therapy of colorectal cancer in last decade, no effective targeted therapy drugs have been discovered. More attention should be focused on finding early diagnosis and prognosis markers of colorectal cancer. COX-2 and HER-2 are known to be involved in the progression of many cancers. However, the associations of COX-2 and HER-2 expression with the progression and prognosis of colorectal cancer have not been fully elucidated.

Research frontiers

In recent years, several interesting and promising molecular markers of colorectal cancer have been discovered, including the epidermal growth factor receptor and the vascular endothelial growth factor receptor. These molecules will help to identify early cancers, screening programs, and target therapies.

Innovations and breakthroughs

In this study, the authors assessed the expression of COX-2 and HER-2 in colorectal cancer tissues by immunohistochemistry, and subsequently analyzed the correlation of COX-2 and HER-2 expression with clinical features of colorectal cancer. They also found that COX-2 and HER-2 had synergistic effects in colorectal cancer.

Applications

Based on the finding that expression of COX-2 and HER-2 are significantly associated with clinical features of colorectal cancer, this study may trigger the interest of COX-2 and HER-2 as important prognostic markers and potential molecular targets in colorectal cancer therapy.

Peer-review

The authors conducted a descriptive study and revealed that the expression levels of the two molecules are associated with clinical features of colorectal cancer. The findings are expected to contribute to the development of molecularly targeted therapy for colorectal cancer.

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ORIGINAL ARTICLE

Basic Study

CD97 promotes gastric cancer cell proliferation and invasion through exosome-mediated MAPK signaling pathway

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Conflict-of-interest: The authors have declared that no competing interests exist.
Data sharing: The technical appendix, statistical code, and dataset are available from the corresponding author at wuyulian@medmail.com.cn or chenli_hz@yahoo.com.

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Abstract

AIM: To investigate the mechanism underlying the promoting role of CD97 in gastric cancer cell proliferation and invasion.

METHODS: Two types of exosomes released by gastric cancer cells with high (SGC/wt) or low (SGC/kd) CD97 expression were isolated by ultracentrifugation and identified by electron microscopy and western blot analysis. The influences of the two exosomes on gastric cancer cell proliferation and invasion were investigated by proliferation and Matrigel invasion assays. Exosomal miRNAs were subsequently isolated from the two samples and their miRNA profiles were compared *via* microarray assay analysis. Reverse transcription-quantitative real-time polymerase chain reaction was used to validate the microarray assay. Target genes of the differently expressed microRNAs were predicted based on five independent algorithms and were then subjected to gene oncology enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis. After identifying the pathway that was the most likely altered, tumor cells were treated with the two exosomes at different concentrations, and the pathway activation was identified through western blot analysis.

RESULTS: Exosomes isolated from SGC/wt cells significantly promoted tumor cell proliferation in a dose-dependent manner *in vitro*. SGC/wt exosomes

also significantly elevated the invasiveness of both SGC/wt (129.67 ± 8.327 *vs* 76.00 ± 5.292, $P < 0.001$) and SGC/kd (114.52 ± 9.814 *vs* 45.73 ± 4.835, $P < 0.001$) cells as compared to the exosomes released by SGC/kd cells. Microarray assay of the two exosomes revealed that 62 miRNAs were differently regulated with a signal intensity of > 500 and a false discovery rate < 0.05. The following KEGG analysis defined the MAPK signaling pathway as the most likely candidate pathway that regulated tumor cell proliferation and invasion. Through western blot analysis, significant up-regulations of phosphorylated MAPKs, including extracellular signal-regulated kinase, Jun NH2-terminal kinase, and p38 mitogen-activated protein kinase, were detected in a dose-dependent manner in the SGC/wt exosomes treated groups, confirming activation of the MAPK signaling pathway stimulated by SGC/wt exosomes.

CONCLUSION: CD97 promotes gastric cancer cell proliferation and invasion *in vitro* through exosome-mediated MAPK signaling pathway, and exosomal miRNAs are probably involved in activation of the CD97-associated pathway.

Key words: CD97; Exosome; Proliferation; Invasion; miRNA; Gastric cancer

Core tip: CD97, belongs to the epidermal growth factor-seven-transmembrane subfamily, and has been found to promote proliferation and invasion of gastric cancer cells. However, the underlying mechanism is poorly understood. In this study, we found that exosomes isolated from gastric cancer cells with high CD97 expression promoted tumor cell proliferation and invasion. Furthermore, through microarray and western blot analyses, MAPK signaling pathway activation was observed when cells were treated with those exosomes. These results indicated that CD97 promotes gastric cancer cell proliferation and invasion *in vitro*, at least in part, through the exosome-mediated MAPK signaling pathway, and exosomal miRNAs are probably involved in activation of the CD97-associated pathway.

Li C, Liu DR, Li GG, Wang HH, Li XW, Zhang W, Wu YL, Chen L. CD97 promotes gastric cancer cell proliferation and invasion through exosome-mediated MAPK signaling pathway. *World J Gastroenterol* 2015; 21(20): 6215-6228 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6215.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6215

INTRODUCTION

CD97 is a member of the epidermal growth factor-seven-transmembrane subfamily, which belongs to the class B G-protein-coupled receptors[1,2]. Originally, CD97 was found to be expressed by hematopoietic cells[3,4], then abundantly detected in numerous carcinomas including gastric, colorectal, thyroid, esophageal, pancreatic, and oral squamous cell carcinomas[5-10]. In gastric cancer, previous studies have demonstrated that enhanced CD97 expression is associated with the dedifferentiation and aggressiveness of tumor cells and directly correlates with the clinicopathological parameters such as TNM stage[11,12]. Recently, we revealed that CD97 small isoform was associated with increased invasiveness *in vitro* as well as elevated local growth and metastatic spread of gastric cancer *in vivo*[13]. However, the mechanisms underlying these promoting roles of CD97 are still poorly understood.

Exosomes are spherical and bilayer vesicles with a diameter of 30-100 nm, which are released extracellularly upon fusion of multivesicular bodies with the plasma membrane[14,15]. Many types of cells including tumor cells, lymphocytes, epithelial cells, and stem cells produce exosomes[15-18]. During the past few years, an increasing number of studies have demonstrated that tumor-derived exosomes play important roles in tumor formation and progression. In breast and gastric cancer, it was observed that tumor exosomes could enhance tumor cell proliferation[19,20]. Moreover, exosomes from breast and pancreatic cancer were found to contribute to the formation of a niche which promoted tumor metastasis[21,22]. Based on these observations, we speculate that tumor exosomes may be involved in the CD97-dependent promotion of biological behaviors of gastric cancer cells.

In addition, exosomes are enriched in proteins, lipids and nucleic acids including miRNA, mRNA and other non-coding RNAs[23,24]. When internalized by recipient cells, exosomes deliver these biological molecules, which still maintain their bioactivity, to other cells, thus mediating intercellular communication[18,25]. One topic of considerable interest is that these transferred exosomal miRNAs may participate in the regulation of biological behaviors of target cells. Yang *et al*[26] found that macrophages regulated the invasiveness of breast cancer cells through exosome-mediated miRNA delivery. Similarly, Rana *et al*[27] reported that transferred exosomal miRNAs predominantly modulated pre-metastatic organ cells, thus facilitating metastasis of pancreatic cancer. In gastric cancer, tumor cells also produce a large number of exosomes that contain abundant miRNAs[20,28]. Thus, it will be of great significance to further investigate the possible role of exosomal miRNAs in the CD97-dependent regulation of tumor cell behavior.

In the present study, by employing ultracentrifugation, two types of exosomes from gastric cancer cells with high CD97 expression and cells with stable CD97 knockdown were isolated, and their effects on cell proliferation and invasion were investigated. Moreover, miRNA profiles of the two exosomes were

Table 1 Four miRNA candidates and negative control sequences of CD97

Oligo	Sequence (5′-3′)
1F	TGCTGATGACATTCTGGATGGTGACCGTTTTGGCCACTGACTGACGGTCACCACAGAATGTCAT
1R	CCTGATGACATTCTGTGGTGACCGTCAGTCAGTGGCCAAAACGGTCACCATCCAGAATGTCATC
2F	TGCTGTATCTTCAAGGTTTGAGAGCAGTTTTGGCCACTGACTGACTGCTCTCACCTTGAAGATA
2R	CCTGTATCTTCAAGGTGAGAGCAGTCAGTCAGTGGCCAAAACTGCTCTCAAACCTTGAAGATAC
3F	TGCTGAAGAAAGTAGAGCTCCAGGCCGTTTTGGCCACTGACTGACGGCCTGGATCTACTTTCTT
3R	CCTGAAGAAAGTAGATCCAGGCCGTCAGTCAGTGGCCAAAACGGCCTGGAGCTCTACTTTCTTC
4F	TGCTGAAGATGAACAGGCCAAAGACCGTTTTGGCCACTGACTGACGGTCTTTGCTGTTCATCTT
4R	CCTGAAGATGAACAGCAAAGACCGTCAGTCAGTGGCCAAAACGGTCTTTGGCCTGTTCATCTTC
Negative control	
Negative-F	TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT
Negative-R	CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTC

compared by microarray assay and significantly regulated signaling pathways related to exosomes were predicted and verified.

MATERIALS AND METHODS

Cell culture

The stomach adenocarcinoma cell line SGC-7901 was purchased from ATCC (www.atcc.org). SGC-7901 cells were cultured in RPMI-1640 medium (Genom Biologic, Hangzhou, China) supplemented with 10% fetal bovine serum (FBS, Sijiqing Biologic, Hangzhou, China) and 1% penicillin/streptomycin (Genom Biologic). Cells were incubated in a standard humidified incubator in 5% CO_2 at 37 ℃ and passaged every 3-5 d using trypsin-EDTA (Genom Biologic).

Transfection and clone selection

Four miRNA candidate target sequences of the human CD97 gene and the non-silencing sequence (Table 1) were designed and cloned into the pcDNA6.2-GW/EmGFP-miR vector (Invitrogen, Shanghai, China). The SGC-7901 cells were transfected and selected using 4 μg/mL of Blasticidin S HCl (Life Technologies) as described previously[13]. Down-regulation of CD97 was confirmed by reverse transcription polymerase chain reaction (RT-PCR) and western blot analysis.

Total RNA extraction and reverse transcription-polymerase chain reaction

Total RNAs from gastric cancer SGC-7901 wild-type cells (SGC/wt), non-silencing vector bearing cells (SGC/ns) and stable CD97 knockdown cells (SGC/kd) cells were extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. cDNA synthesis and PCR assay were performed as previously described using primers suitable for amplification of all three CD97 isoforms[13]. PCR products were visualized on a 1% agarose gel with 0.05% ethidium bromide.

miRNA extraction and reverse transcription-quantitative real-time polymerase chain reaction

Total RNAs including miRNA were extracted from exosomes of SGC/wt and SGC/kd using the miRNeasy Mini kit (Qiagen) following the manufacturer's instructions. cDNA was synthesized using the miScript Ⅱ RT Kit (Qiagen). The reverse transcription reaction system included 4 μL of 5 × miScript HiSpec buffer, 2 μL of 10 × miScript nucleics mix, 2 μL of miScript reverse transcriptase mix, 5 μL of template RNA and 7 μL of RNase-free water. The mixture was incubated for 60 min at 37 ℃ and followed by 95 ℃ for 5 min to inactivate the reaction. qPCR was performed using the miScript SYBR Green PCR Kit (Qiagen) in an ABI PRISM Stepone Plus Sequence Detection System (Applied Biosystems, Foster City, CA, United States) in accordance with the manufacturer's protocol. Briefly, 12.5 μL of 2 × QuantiTect SYBR Green PCR master mix, 2.5 μL of 10 × miScript universal primer, 2.5 μL of 10 × miScript primer assay, 2 ng of diluted template cDNA and RNase-free water added to 20 μL were mixed. The mixture were then incubated in a 96-well plate at 95 ℃ for 15 min, followed by 40 cycles of 94 ℃ for 15 s, 55 ℃ for 30 s and 70 ℃ for 30 s. The differences in expression of miRNAs between SGC/wt and SGC/kd exosomes were analyzed using the ΔΔCT method and were normalized by RNU6B expression[29].

Exosome isolation

Cells were cultured until 80% confluent, the growth medium was replaced by serum-free medium, and the medium was collected after 2 d of incubation. Exosomes were then isolated by differential centrifugation as previously described[30]. Briefly, the conditioned medium was centrifuged at 300 *g* for 10 min at 4 ℃ to pellet the cells and then 16500 *g* for 20 min to further remove cells and cell debris. The supernatants were then filtered through 0.20 μm filters to remove particles larger than 200 nm. The exosomes were pelleted by ultracentrifugation (Beckman Coulter, Fullerton, CA, United States) at 120000 *g* for 70 min at 4 ℃, washed in PBS and pelleted by ultracentrifugation at 120000 *g* for 70 min at 4 ℃. The final exosome pellets were resuspended in PBS and stored at -80 ℃ until use. The total exosomal protein concentration was measured using the Enhanced BCA Protein Assay

Kit (Beyotime, China).

Exosome identification by electron microscopy

Exosomes obtained *via* differential centrifugation were resuspended in 1% glutaraldehyde in PBS (pH 7.4). A 20 μL drop of suspension was pipetted onto an electron-microscopy grid coated with formvar carbon and allowed to stand for 1 min at room temperature. Excess fluid was removed with a piece of Whatman filter paper. The sample was then stained with 2% phosphotungstic acid for 1 min, allowed to dry under an electric incandescent lamp for 10 min and viewed using Philips Tecnai 10 transmission electron microscopy (Philips, Netherlands). Exosome size was measured by scale bar.

Cell proliferation assay

Tumor cell proliferation was determined by 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays. Cells were plated in 96-well plates at 5×10^3 cells/well and cocultured with exosomes at different concentration for 24 h. At the appropriate time-point, the medium was replaced by 100 μL serum-free medium, and 20 μL MTS solution (Promega, Madison, WI, United States) was added to each well. After incubated in a humidified incubator for another 2 h, the plates were measured at 490 nm using a Microplate Reader (Bio-rad, Hercules, CA, United States). Cell proliferation was expressed using the optical densities obtained at each concentration.

Cell invasion assays

Invasion assays were performed in 24-well Transwell™ chambers (Costar), which were separated by polycarbonate filters with 8 μm pore size between the upper and lower culture compartments. For tumor cell invasion, the upper chamber was coated with Matrigel matrix (0.8 mg/mL, BD Biosciences) before seeding the cells. 1.0×10^5 exosome-treated cells in RPMI-1640 medium were added to the upper chamber and the lower chamber was filled with medium containing 10% FBS. After 36 h incubation in a 5% CO_2 atmosphere at 37 ℃, the non-invading cells were removed using cotton swabs and the invading cells were fixed and stained with 0.2% crystal violet (Sigma). Invaded cells were counted by light microscopy (Leica) in four separate high-power fields per filter.

Western blot analysis

Proteins were harvested and resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a PVDF membrane (Millipore, Billerica, MA, United States). After blocking with 5% non-fat milk for 2 h, the membranes were incubated overnight at 4 ℃ with antibodies specific against CD97 (Abnova), β-actin, CD9, hsp70, total-p38, -ERK, -JNK or phosphorylated -p38, -ERK, and -JNK (all from Santa Cruz), respectively. Horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit IgG was applied as secondary antibody for 1 h at room temperature. The immunoreactive protein bands were identified by luminescent visualization using an ECL kit (Millipore). Signal intensity was measured using a Bio-Rad XRS chemiluminescence detection system (Bio-Rad).

MicroRNA microarray assay

Total RNA from exosomes was isolated using the miRNeasy Mini Kit (Qiagen, Germany). MicroRNA microarray analysis following the miRbase v20.0 was performed by LC Sciences (Hangzhou, China; http://www.lc-bio.com/). Briefly, total RNA samples were 3'-extended with a poly(A) tail and hybridized overnight at 34 ℃ on a μParaflo microfluidic chip using 100 L 6 × SSPE buffer (0.90 mol/L NaCl, 60 mmol/L Na_2HPO_4, 6 mmol/L EDTA, pH 6.8) containing 25% formamide. After hybridization, tag-conjugating Cy3 dye was circulated through the microfluidic chip for dye staining. Fluorescence images were then collected and digitized using Array-Pro image analysis software (Media Cybernetics, Bethesda, MD, United States). Data analysis was started by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). The normalized microarray data were validated by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR).

Target genes prediction of differently expressed microRNAs

Five computational prediction algorithms (TargetScan, miRanda, PITA, RNAhybrid and microTar) were used to predict targets of the significant changed miRNAs identified in the microarray analysis. Following a comparison of all datasets, a subset of genes that were targeted by more than four algorithms was generated.

GO enrichment and Kegg pathway analysis of target genes

To comprehensively describe the properties of the targets, the putative genes were subjected to gene oncology (GO) enrichment and Kyoto encyclopedia of genes and genomes (KEGG) pathway analysis based on DAVID 6.7 software (http://david.abcc.ncifcrf.gov/home.jsp). The Fisher's exact test and χ^2 test were used to select the significant GO categories and signaling pathways. The threshold of significance was defined by the P value, with $P < 0.001$ or $P < 0.05$ regarding as significance for GO and KEGG analysis, respectively.

Statistical analysis

Statistical analysis was performed with the SPSS 19.0 software. Differences were examined using Student's

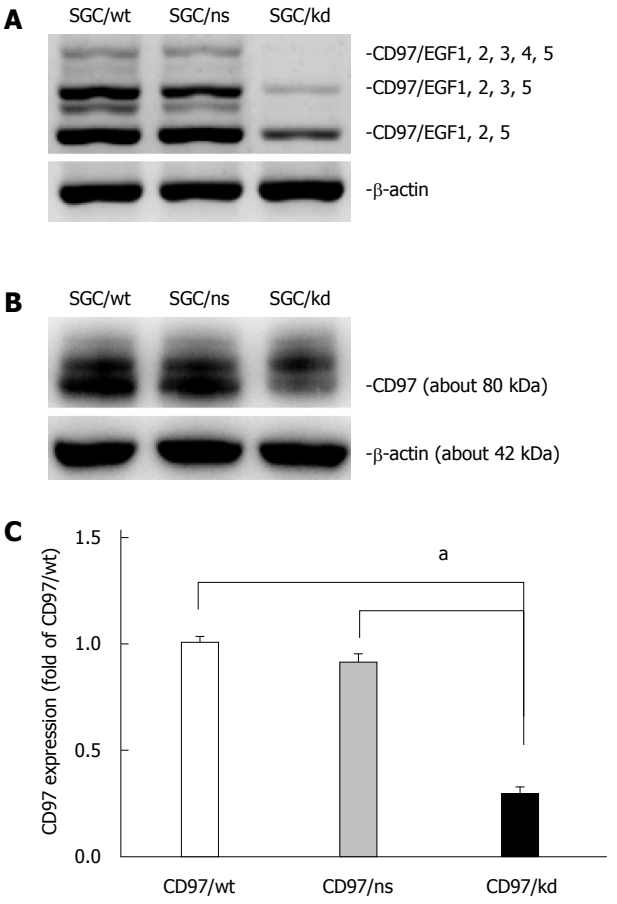


Figure 1 Identification of transfectants with stable CD97 knockdown. A: RT-PCR analysis was used to assess CD97 mRNA levels of SGC/wt, SGC/ns and SGC/kd. This result showed a decreased CD97 level in SGC/kd compared to SGC/wt and SGC/ns; B: The -80 kDa CD97 protein was detected by western blot analysis in total cellular extracts of SGC/wt, SGC/ns and SGC/kd; C: Western blot analysis showed a significant decrease in CD97 protein in SGC/kd. Data are expressed as mean ± SD. $^{a}P < 0.05$ *vs* SGC/wt or SGC/ns. SGC/wt: Wild-type gastric cancer cells SGC-7901; SGC/ns: Non-silencing vector bearing cells; SGC/kd: Stable CD97 knockdown cells generated from SGC-7901.

t-test or one-way analysis of variance. All experiments were performed at least in triplicate and the data are presented as mean ± SD with a *P* value of 0.05 or less considered statistically significant.

RESULTS

Generation of transfectants with stable CD97 knockdown

For this purpose, the human gastric carcinoma cell line SGC-7901, which shows relatively high CD97 expression[13,31], was selected for stable transfection with miR-vectors targeting the site of CD97. After transfection, stable clones were selected and CD97 levels were evaluated by RT-PCR and western blot analysis. As shown in Figure 1, the CD97 knockdown cells (SGC/kd), which were generated from candidate 4, displayed a significant loss of CD97 in both mRNA and protein level compared to the wild-type cells (SGC/wt) or the non-silencing vector bearing cells (SGC/ns). Therefore, these cells were selected for subsequent experiments.

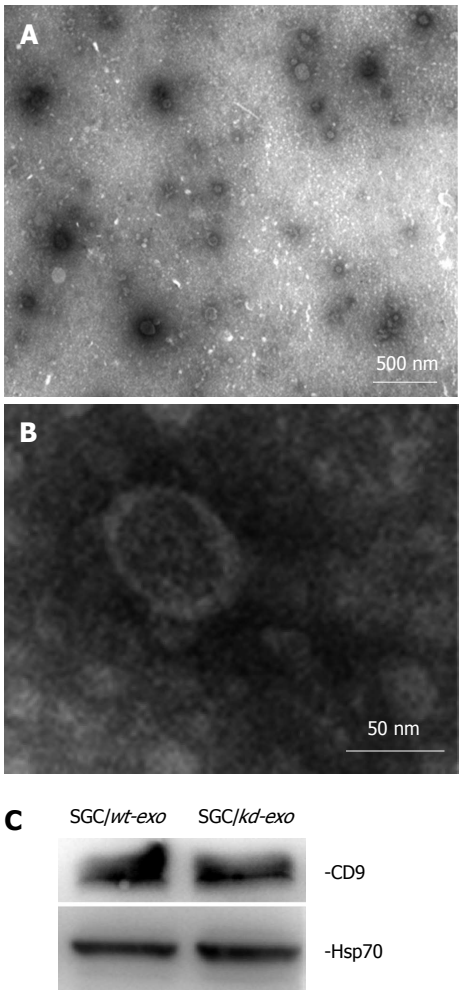


Figure 2 Characterization of tumor-derived exosomes by electron microscopy and western blot analysis. A and B: Exosomes released by SGC/wt or SGC/kd cells were isolated and observed with an electron microscope. The exosomes are small round vesicles limited by a lipid bilayer, and their diameters are between 30 and 100 nm. Scale bars are as indicated; C: Western blot analysis of exosomal lysate proteins. The published exosomal markers, Hsp70 and CD9, were detected in exosomes derived from gastric cancer cells, indicating successful exosome isolation. SGC/*wt-exo*: Exosomes isolated from SGC/wt cells; SGC/*kd-exo*: Exosomes isolated from SGC/kd cells.

Isolation and identification of tumor-derived exosomes

Based on their unique size and density, exosomes released by SGC/wt and SGC/kd cells were isolated by ultracentrifugation and observed by electron microscopy. The isolated exosomes were small closed vesicles limited by a lipid bilayer, and their diameters ranged from 30 to 100 nm under electron microscopy (Figure 2A and B), which was consistent with the reported size of exosomes[15]. In addition, Hsp70 and CD9, the published exosomal markers[32,33], were detected in these membrane vesicles (Figure 2C), further confirming the successful isolation of tumor exosomes.

SGC/wt-derived exosomes promote tumor cell proliferation in vitro

To investigate the effects of the two exosomes on proliferation of gastric cancer cells, we measured the viability of cells treated with different concentrations of exosomes (0, 50, 100, 200 and 400 μg/mL) for 24 h *in vitro*. The MTS assay showed that SGC/wt-derived exosomes (*wt-exo*) increased proliferation of both SGC/wt and SGC/kd cells in a dose-dependent manner. However, in the SGC/kd-derived exosomes (*kd-exo*)-treated groups, no significant difference was observed. It is noteworthy that the promoting effect of *wt-exo* on SGC/wt cells was not as significant as on SGC/kd clones, indicating that SGC/kd cells were more sensitive to *wt-exo* stimulation than SGC/wt cells (Figure 3A).

SGC/wt-derived exosomes promote tumor cell invasion in vitro

To evaluate the effects of the two types of exosomes on gastric cancer cell invasion, SGC/wt and SGC/kd cells were incubated for 4 h with serum-free medium containing 200 μg/mL of the indicated exosomes or an equal amount of bull serum albumin (BSA) as a control, then seeded on chambers coated with Matrigel matrix. After 36 h incubation in the upper chamber, the cells that had invaded through the Matrigel to the undersurface of Transwell filters were stained and counted. When compared with *kd-exo* or BSA control, *wt-exo* significantly enhanced the invasiveness of both SGC/wt and SGC/kd cells, especially SGC/kd cells where invasiveness was 2.5-fold greater than that in SGC/wt cells. However, in the *kd-exo* stimulated groups, cell invasiveness in SGC/wt cells and SGC/kd cells was not significantly altered (Figure 3B and C).

MicroRNA microarray analysis and validation of differently expressed miRNAs by RT-qPCR

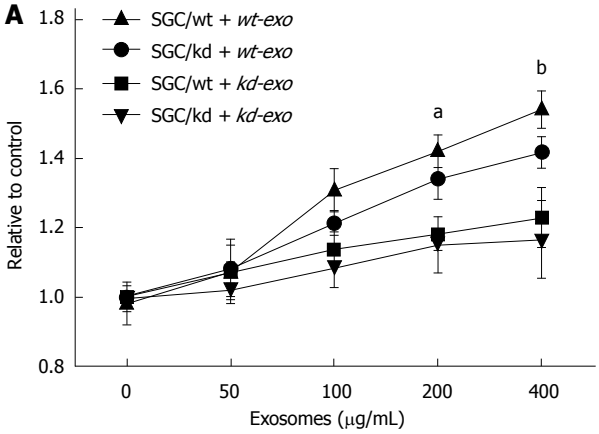
Exosomes are abundant in miRNAs and can be transferred from one cell to another *via* exosome secretion and internalization. These transferred miRNAs maintain their biological functions in the recipient cells. To determine whether exosomal miRNAs were relevant in cell biological behavior, we compared their miRNA profiles across SGC/wt and SGC/kd exosomes *via* microarray assay. Analysis of microarray data indicated that a total of 265 detectable miRNA transcripts were present in SGC/wt and SGC/kd exosomes, of which 83 miRNAs had a signal intensity of > 500. In order to reduce the false negative rate, we considered differently regulated miRNAs as those with a fold change of more than 1.5 and a false discovery rate (FDR) of less than 0.05 according to the microarray data. As shown in Figure 4A, 62 miRNAs were differently regulated, of which 36 were up-regulated and 26 were down-regulated in SGC/wt exosomes compared to SGC/kd exosomes. To verify the microarray assay data, four miRNAs displaying either increased (miR-2861, miR-4734) or decreased (miR-4728-5p, miR-6165) expression were selected to perform RT-qPCR analysis. The outcomes confirmed the microarray data and indicated a positive correlation between the quantity of transcripts measured by microarray and the RT-qPCR assay (Figure 4B).

Target gene prediction and GO and KEGG pathway enrichments of the predicted genes

To identify potential target genes of the 62 miRNAs differently expressed between SGC/wt and SGC/kd exosomes, we performed target gene prediction using five established algorithms. A total of 5746 putative targets were identified (data not shown). To better understand the functions of the predicted genes, those targets were subjected to GO functions from three ontologies: cellular component, molecular function and biological process. As shown in Figure 5, the high-enriched GO categories were nucleotide binding, plasma membrane, regulation of RNA metabolic process, regulation of transcription, and intracellular signaling cascade. These categories were mainly involved in cell metabolism, proliferation, signal transduction, apoptosis, and homeostatic processes. Furthermore, these targets were classified according to KEGG functional annotations to identify pathways that might be actively regulated. The results suggested 38 relevant KEGG pathways, and the top three pathways enriched were pathways in cancer, cytokine-cytokine receptor interaction and the MAPK signaling pathway (Table 2). It is worth noting that, among the list of highly-enriched pathways, pathways in cancer were prominent, especially the MAPK signaling pathway. Thus, it is necessary to further investigate whether CD97 promotes tumor cell proliferation and invasion through an exosome-mediated MAPK signaling pathway.

Verification of activation of the MAPK signaling pathway by western blot analysis

To verify MAPK signaling pathway activation, the expression of three best-characterized MAPKs, including extracellular signal-regulated kinase ERK), Jun NH2-terminal kinase (JNK), and p38 mitogen-activated protein kinase (p38), were determined by western blot analysis. The results revealed that SGC/wt exosomes caused significant up-regulation of phosphorylated ERK, JNK and p38 expression in a dose-dependent manner. On the other hand, although SGC/kd exosomes enhanced the expression of these three phosphorylated proteins, their expression was much weaker compared to the SGC/wt exosomes stimulated groups (Figure 6). These observations demonstrated that exosomes derived from SGC/wt cells, which show high CD97 expression, could activate the MAPK signaling pathway, and activation of this pathway may lead to elevated proliferation and invasion of tumor cells.


A
SGC/wt + wt-exo
SGC/kd + wt-exo
SGC/wt + kd-exo
SGC/kd + kd-exo
Relative to control
1.8
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1.4
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a
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0
50
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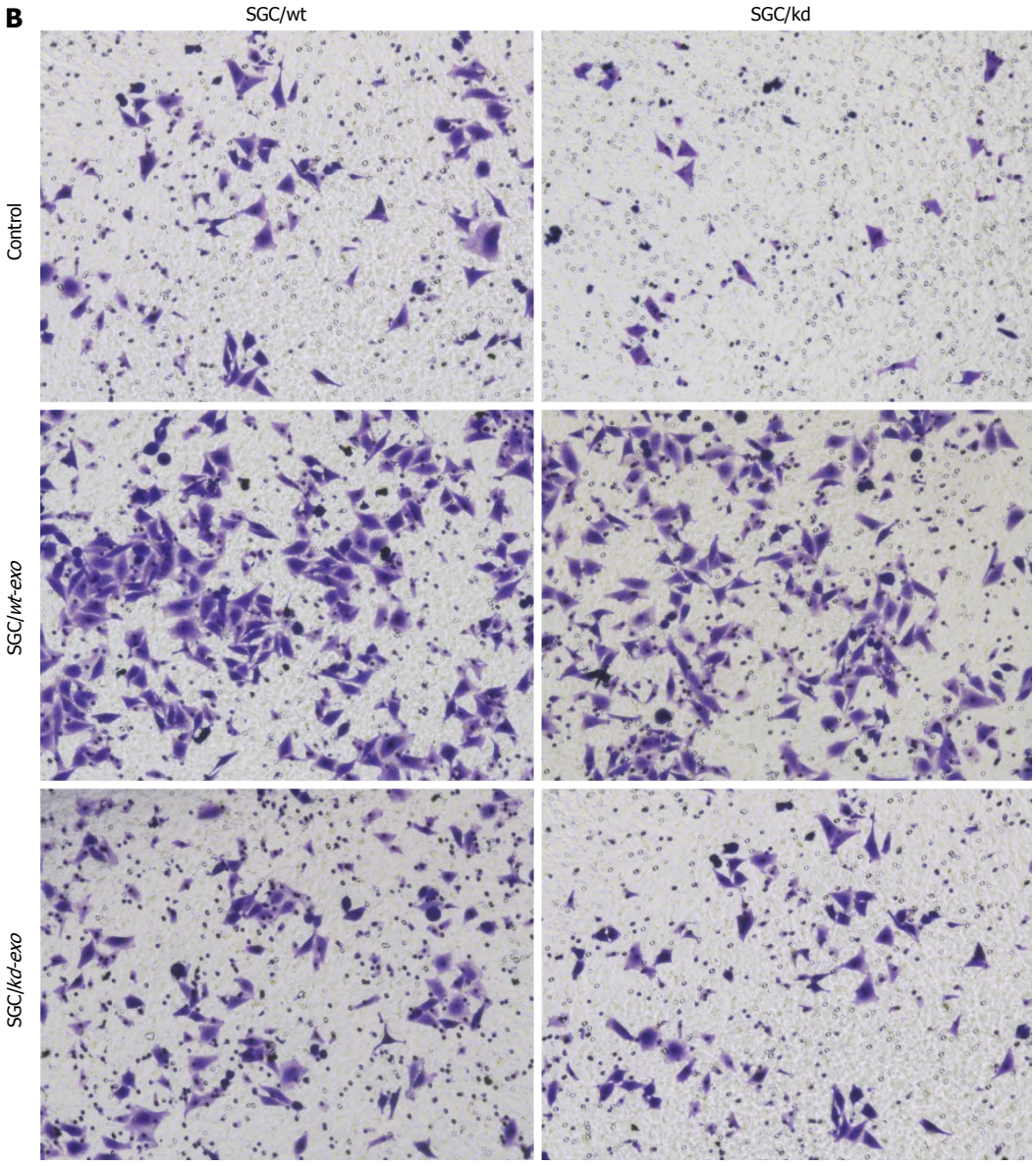
Exosomes (μg/mL)


B
SGC/wt
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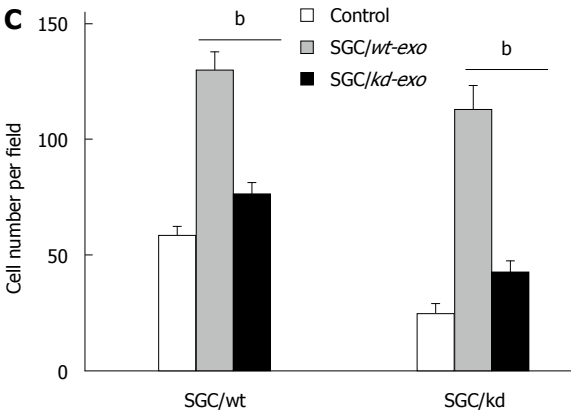

Figure 3 SGC/wt cells-derived exosomes promoted tumor cell proliferation and invasion *in vitro*. A: A total of 5 × 10^3/well SGC/wt and SGC/kd cells were seeded in 96-well plates in RPMI 1640 medium without fetal bovine serum (FBS). After incubating overnight, the cells were treated with *wt-exo* or *kd-exo* at the concentrations of 0, 50, 100, 200 and 400 μg/mL for 24 h, and cell proliferation was measured by MTS assay. The results showed that *wt-exo* significantly promoted proliferation of both SGC/wt and SGC/kd cells in a dose-dependent manner. $^aP < 0.05$, $^bP < 0.01$ *vs kd-exo* group; B: SGC/wt and SGC/kd cells were treated with 200 μg/mL of indicated exosomes for 4 h, and seeded on Matrigel matrix coated chambers for the invasion assay; C: Invaded cell counting revealed that the number of penetrated cells in the wt-exo group was significantly higher than that in the *kd-exo* group. $^bP < 0.01$ *vs kd-exo* group. Representative crystal violet staining after 36 h invasion showed invaded cells in each group. Data are expressed as mean ± SD. *wt-exo*: SGC/wt-derived exosomes; *kd-exo*: SGC/kd-derived exosomes.

DISCUSSION

Although it is well studied that overexpressed CD97 plays important roles in gastric cancer formation and progression, the mechanisms underlying these functions are poorly investigated. In order to identify the possible mechanism involved, we isolated exosomes from wild-type gastric cancer SGC-7901 cells with high CD97 expression and from SGC-7901 cells with stable CD97 knockdown. We demonstrated that exosomes from wild-type cells, but not from CD97 knockdown cells, significantly promoted tumor cell proliferation and invasion. Furthermore, through microarray and western blot analyses, we found that enhanced proliferation and invasion ability may be induced by exosome-mediated activation of the MAPK signaling pathway.

During the past few years, many studies have demonstrated that tumor-derived exosomes are involved in a wide range of tumor pathophysiological processes in numerous ways, such as promoting tumor growth and metastasis, activating certain signaling pathways, and exerting detrimental effects on the anti-tumor immune system[19-21,34,35]. The present study further suggested that the effects of exosomes on tumor biological behavior depend on their parent cells. It was shown that exosomes from SGC/wt cells with high CD97 expression, significantly increased gastric cancer cell proliferation and invasion; whereas, exosomes from SGC/kd cells with low CD97 expression did not show similar effects. From these observations, it is arguable that CD97 increases the proliferation and invasion of tumor cells, at least in part, by releasing CD97-related exosomes. We speculate that those two exosomes contain different biological molecules, thus biomaterials in SGC/wt exosomes have higher malignant-promoting capacities as compared to that of SGC/kd exosomes; and upon internalization, these biomaterials are transferred to the recipient cells, resulting in elevated malignant behavior.

miRNAs are small, non-coding RNA molecules that regulate the activity of complementary mRNAs and play important roles in a wide range of physiologic and pathologic processes[36]. Microarray analysis of the two exosomes showed significant differences in their exosomal miRNA profiles. The GO enrichments showed that the predicted targets were mainly located in the cell membrane and organelles and significantly involved in cell metabolism, proliferation and signal transduction. The results of KEGG analysis suggested that the MAPK signaling pathway was prominent. Subsequent western blotting verified that MAPK signaling pathway activation was induced by SGC/wt exosomes. Thus, it is reasonable that the promoting role of CD97-related exosomes depends on activation of the MAPK signaling pathway and this activation is probably indirectly induced by the exosomal miRNAs.

In addition to nucleic acids, exosomes contain abundant proteins. In a study conducted by Liang *et al*[37], thousands of proteins were identified in exosomes released by two ovarian cancer cell lines, OVCAR-3 and IGROV1, and some proteins were tissue specific or associated with tumorigenesis or metastasis. Furthermore, exosomes from human breast and colorectal cancer cells were reported to contain full-length, signaling-competent epidermal growth factor receptor (EGFR) ligands[38]. In that study, the researchers found that exosomes expressing individual EGFR ligands displayed different bioactivities. Specifically, exosomes with higher amphiregulin levels exhibited greater invasive potential than other EGFR ligand-containing exosomes, indicating that exosomes participate in diverse tumor biological activities *via* certain exosomal proteins. In the present study, although microarray analysis showed that the

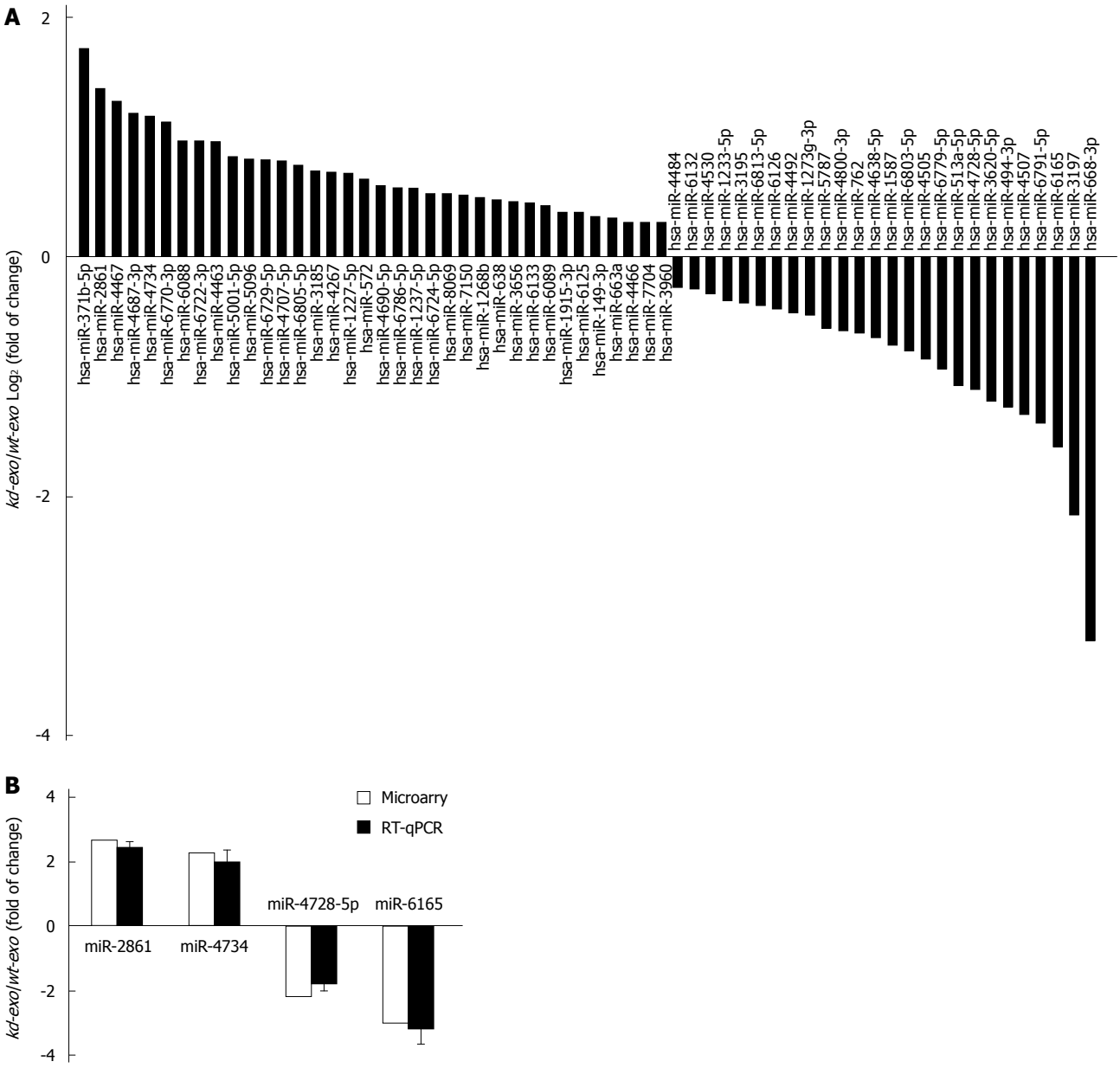

Figure 4 Comparison of miRNA profiles of the two exosomes by microarray analysis and validation of differently expressed miRNAs by RT-qPCR analysis. A: Bar graph showed differently expressed miRNAs in *kd-exo* by microarray as compared to *wt-exo*; B: Four miRNAs that displayed either an increased or decreased expression were selected for RT-qPCR validation of microarray assay. The same expression trends were observed between microarray and RT-qPCR for all the miRNAs. *wt-exo*: SGC/wt-derived exosomes; *kd-exo*: SGC/kd-derived exosomes.

exosomal miRNAs were involved in MAPK signaling pathway activation, the possibility that exosomal proteins directly regulated the signaling pathway and/or tumor biological behavior could not be excluded. Thus, further studies focused on the relationships between exosomal proteins and the diverse biological behaviors of gastric cancer related to CD97 are required.

Apart from the effects exerted on proliferation and invasion, tumor-derived exosomes were also proved to contribute to the establishment of a pre-metastatic niche, which is a suitable microenvironment in distant metastatic organs generated by primary tumor prior to arrival of metastatic cells[22,39]. Jung *et al*[22] reported that depending on CD44v6, tumor exosomes prepared the pre-metastatic niche in distant organs, which allowed embedding and growth of highly metastatic pancreatic ASML cells. Recently, Rana *et al*[27] suggested that exosomal miRNAs were recovered in pre-metastatic organs, where they significantly influenced mRNA translation and predominantly modulated pre-metastatic organs to prepare stromal cells for tumor cell hosting. Thus, tumor exosomes play important roles in the cross-talk between primary tumor and metastatic organs. Our previous study demonstrated that CD97 small isoform facilitated gastric cancer metastasis in an orthotopically implanted mouse model[13]. Therefore, the contribution

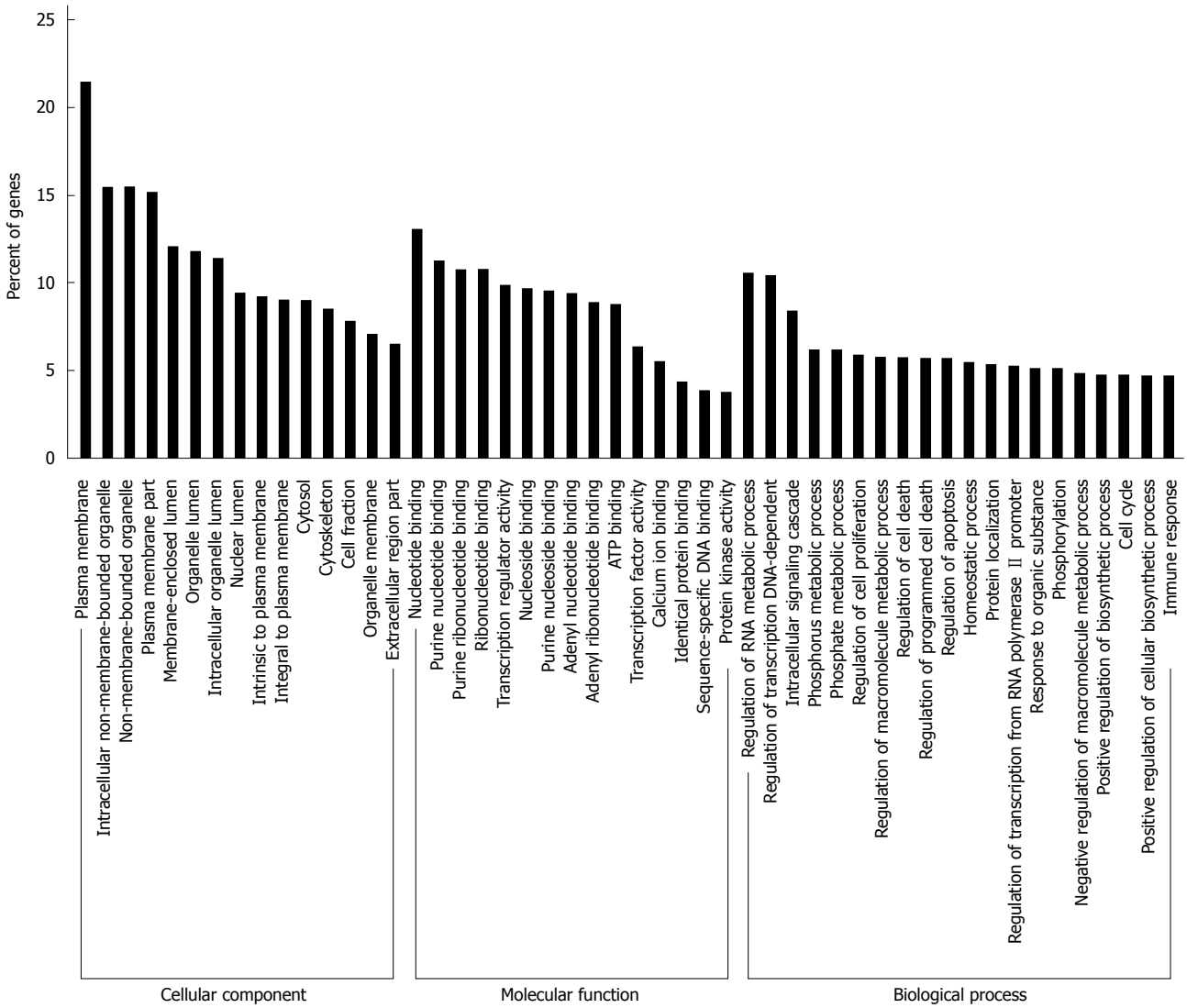

Figure 5 Gene ontology analysis of the significant predicted genes of differently expressed miRNAs. The results were classified into three main categories: cellular component, molecular function, and biological process. The vertical axis indicated the percent of genes in each category. The horizontal axis is the enrichment of Gene ontology (GO).

Table 2 Significant Kyoto encyclopedia of genes and genomes pathways enriched for target genes of differently expressed miRNAs

No.	Pathway ID	Pathway description	Target genes with pathway annotation, *n* (%)	*P* value
1	hsa05200	Pathways in cancer	179 (2.37)	5.50×10^{-3}
2	hsa04060	Cytokine-cytokine receptor interaction	152 (2.01)	4.00×10^{-4}
3	hsa04010:	MAPK signaling pathway	146 (1.93)	1.13×10^{-2}
4	hsa04080	Neuroactive ligand-receptor interaction	139 (1.84)	1.81×10^{-2}
5	hsa04510	Focal adhesion	121 (1.60)	2.00×10^{-4}
6	hsa04810	Regulation of actin cytoskeleton	116 (1.54)	3.79×10^{-2}
7	hsa04062	Chemokine signaling pathway	107 (1.42)	5.60×10^{-3}
8	hsa04020	Calcium signaling pathway	95 (1.26)	5.88×10^{-2}
9	hsa04142	Lysosome	72 (0.95)	2.3×10^{-3}
10	hsa04670	Leukocyte transendothelial migration	68 (0.90)	2.36×10^{-3}

of CD97 to metastasizing tumor cell settlement is probably facilitated by the formation of the pre-metastatic niche, which may depend on CD97-related exosomes or exosomal miRNAs. However, to the best of our knowledge, there are few studies describing the relationship between CD97-related exosomes and tumor metastasis, which also merits investigation in future *in vivo* studies.

In conclusion, using exosomes isolated from cells with high and low CD97 expression, we demonstrate that CD97 promotes gastric cancer cell proliferation and invasion *in vitro*, at least in part, through

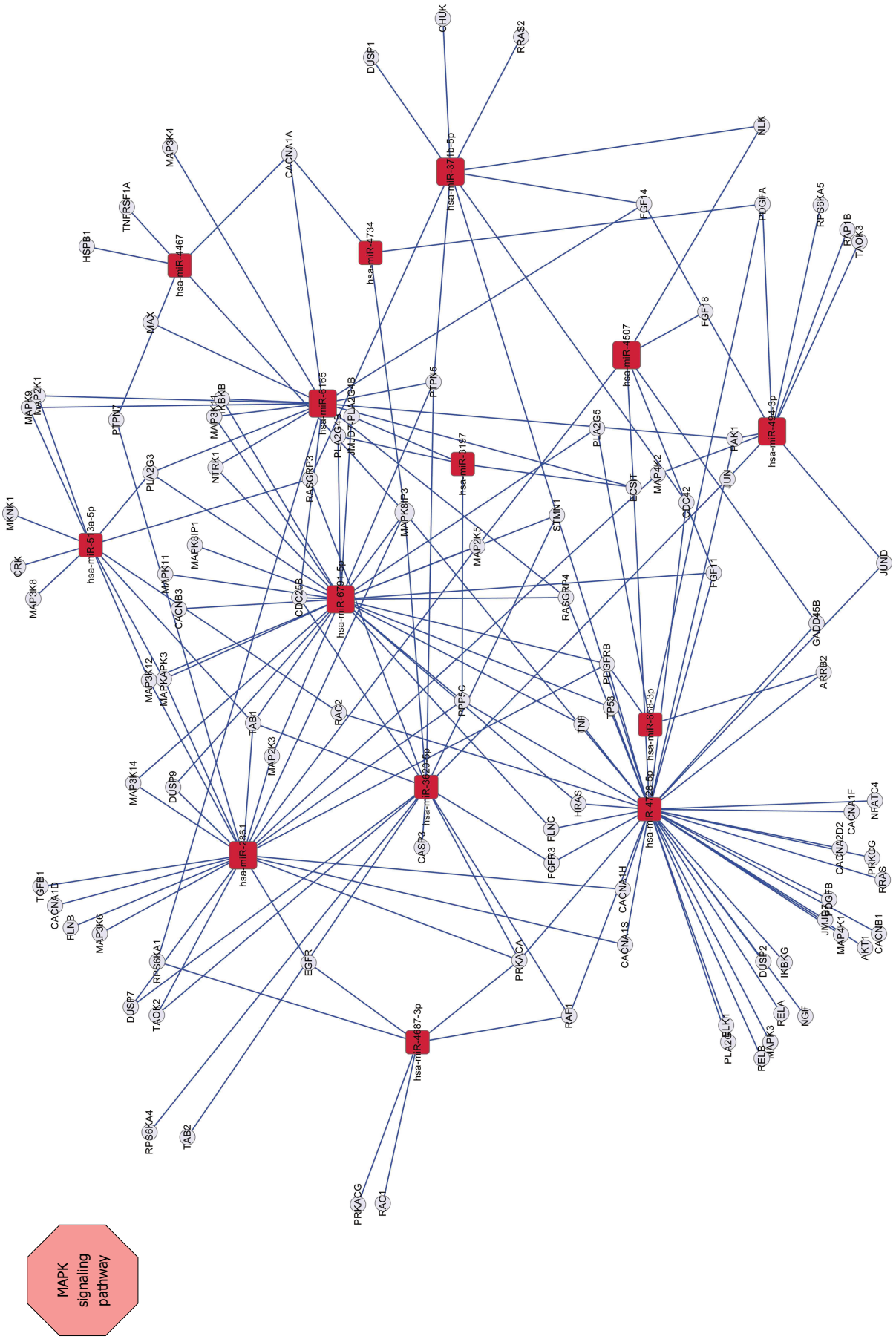

Figure 6 Network between MAPK signaling pathway-related genes and miRNAs. Squares represent miRNAs; circles represent target genes; straight lines represent miRNA-MAPK signaling pathway-related gene relationship. The size of the square represents the degree of miRNAs, a larger degree has a larger number of target genes.

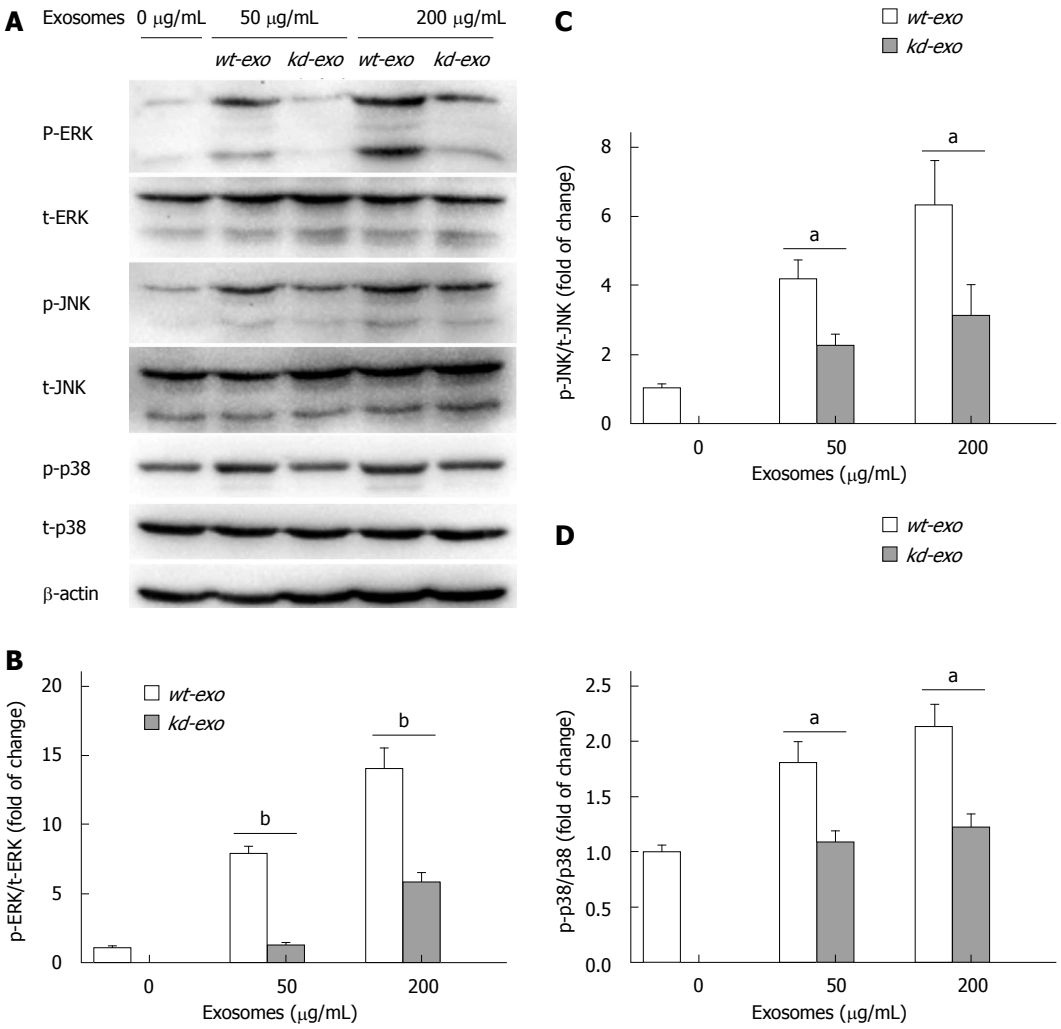

Figure 7 Modulation of JNK, ERK, and p38 MAPK expression by tumor exosomes. SGC/kd cells were stimulated with *wt-exo* or *kd-exo* at certain concentrations for 6 h in serum-free medium. Cells were then lysed, and an equal amount of protein from each group was examined by immunoblotting using specific antibodies against phosphorylated (p-) ERK, JNK, p38 and total (t-) ERK, JNK and p38. A: Representative immunoblot of JNK, ERK, and p38 MAPK protein levels; B-D: Bar graph shows the expression ratio of p-ERK over t-ERK, p-JNK over t-JNK, and p-p38 over t-p38 by densitometric quantification, respectively. Data are expressed as mean ± SD. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs kd-exo* group. *wt-exo*: SGC/wt-derived exosomes; *kd-exo*: SGC/kd-derived exosomes.

exosome-mediated MAPK signaling pathway, and that exosomal miRNAs are probably involved in activation of the CD97-associated pathway. Although shown *in vitro*, these findings should be confirmed in other gastric cancer cell types as well as in animal models to have translational relevance.

COMMENTS

Background

Gastric cancer is one of the most common and lethal malignancies worldwide. Understanding gastric cancer formation and progression is of significant importance in the treatment of this carcinoma. As a member of the epidermal growth factor-seven-transmembrane subfamily, CD97 has been found to be overexpressed in the majority of gastric carcinoma tissues and has been demonstrated to promote gastric cancer cell proliferation and invasion. However, the underlying mechanism remains unclear.

Research frontiers

During the past few years, an increasing number of studies have demonstrated that tumor-derived exosomes play important roles in tumorigenesis and tumor progression. In gastric cancer, it was observed that tumor exosomes could enhance tumor cell proliferation.

Innovations and breakthroughs

Previous studies have demonstrated that CD97 is involved in gastric cancer progression, but the underlying mechanism is unknown. In the present study, the authors combined CD97 and exosomes, and found that CD97 promotes gastric cancer cell proliferation and invasion *in vitro* through exosome-mediated MAPK signaling pathway. In addition, they suggested that exosomal miRNAs are involved in activation of the CD97-associated pathway. These findings will be helpful in understanding the role of CD97 in gastric cancer formation and progression.

Applications

In understanding the mechanism of CD97-dependent promotion of proliferation and invasion in gastric cancer, the results of this study may provide a future strategy for CD97 as a diagnostic biomarker and/or a way to improve clinical treatment of gastric cancer.

Terminology

Exosomes are spherical and bilayer vesicles with a diameter of 30-100 nm, which are released extracellularly upon fusion of multivesicular bodies with the plasma membrane. Many types of cells including tumor cells, lymphocytes, epithelial cells, and stem cells can produce exosomes.

Peer-review

It is a well-written and a well-designed study, which showed, in an elegant manner, the potential role of CD97 and MAPK signaling in gastric cancer cell proliferation and invasion. This is thought to be interesting and excellent

novel study. These findings must be demonstrated *in vivo* to have translational relevance.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Cholangiographic characteristics of common bile duct dilatation in children

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Abstract

AIM: To investigate whether children with congenital common bile duct dilatation (CBDD) differ from children with obstructive CBDD in cholangiographic characteristics.

METHODS: In this retrospective cohort study, the baseline data and the results of imaging analyses were reviewed among children who had endoscopic retrograde cholangiopancreatography (ERCP) due to CBDD. ERCP was performed on all pediatric patients by experienced pediatric endoscopists. The maximal transverse diameter of the common bile duct (CBD) was measured on ERCP. To assess whether age-adjusted CBDD could be used for differential diagnosis, a CBDD severity index (SI) was calculated by dividing the measured CBD diameter by the age-corrected maximal diameter of a normal CBD.

RESULTS: A retrospective medical chart review revealed that 85 consecutive children under 16 years of age with hepatobiliary disease and CBDD were referred to Seoul Asan Medical Center. Fifty-five (64.7%) children had congenital CBDD and 30 (35.3%) had obstructive CBDD. The two groups did not differ significantly in terms of clinical characteristics except for sex. The congenital and obstructive CBDD groups did not differ significantly in terms of mean CBD diameter (19.3 ± 9.6 mm *vs* 12.2 ± 4.1 mm, $P > 0.05$). However, congenital CBDD cases had a significantly higher mean SI than obstructive CBDD cases (3.62 ± 1.64 *vs* 1.98 ± 0.71, $P = 0.01$). In multivariate analysis, an SI value $\geqslant$ 2.32 and comorbidity with anomalous union of pancreaticobiliary duct (APBDU) in ERCP independently predicted congenital CBDD.

CONCLUSION: Measuring the CBD may aid the differential diagnosis of both CBDD and APBDU in

children.

Key words: Endoscopic retrograde cholangiopancreatography; Common bile duct; Choledochal cyst; Choledolithiasis; Children

Core tip: A severity index calculated by measuring the diameter of the common bile duct (CBD) adjusted for age was a better method to discriminate between congenital common bile duct dilatation (CBDD) and secondarily obstructive CBDD in children compared with simply measuring the diameter of the CBD.

Oh SH, Chang SH, Kim HJ, Cho JM, Hwang JH, Namgoong JM, Kim DY, Cho YA, Yoon CH, Kim KM. Cholangiographic characteristics of common bile duct dilatation in children. *World J Gastroenterol* 2015; 21(20): 6229-6235 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6229.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6229

INTRODUCTION

The causes of common bile duct dilatation (CBDD) may differ according to age and geography. In adults, CBDD is generally caused by intrinsic luminal obstruction and thus investigative methods focus mainly on the causes of obstruction, such as biliary tract stones and pancreaticobiliary malignancies[1,2]. In children, congenital CBDD (*i.e.*, choledochal cyst) must be considered when investigating the causes of CBDD[1,2]. However, in Western countries, pediatric choledochal cyst is rare, accounting for only 1.2%-8.6% of pediatric patients who undergo endoscopic retrograde cholangiopancreatography (ERCP) for pancreatic and biliary disease; by contrast, pancreatitis and choledolithiasis are diagnosed much more commonly in this pediatric population[3-5]. However, in East Asia, the most frequent diagnosis in children who are investigated by ERCP for pancreatic and biliary disease is choledochal cyst[6-8]. Moreover, the frequency of choledochal cyst differs depending on the patient's country of origin: one-third of the patients who are reported to have this condition in the world are from Japan[6-9].

The clinical manifestations of choledochal cyst also differ depending on patient age[9]. In Japan, choledolithiasis has been reported in 18%-70% of adults with choledochal cyst. By contrast, only 9% of pediatric patients with choledochal cyst were reported to have choledolithiasis[9,10], although recent findings suggest that this prevalence may be higher than was previously believed (17%-29%)[9-12]. Such relatively high choledolithiasis co-morbidity may initially complicate the differential diagnosis of CBDD from choledolithiasis alone. Choledolithiasis can lead to CBDD that can be initially misdiagnosed as choledochal cyst in both children and adults[13-15]. Thus, choledochal cyst should be diagnosed on the basis of both the clinical features and the results of various diagnostic modalities[6,9,16].

In adults with CBDD, the diameter of the common bile duct (CBD) is considered to be of no clinical significance[17-19]. This is because the studies in adults aimed to differentially diagnose stones from mitotic lesions in the biliary tree. To our knowledge, studies of pediatric CBDD that examine whether bile duct size can help to differentiate between obstructive and congenital causes of CBDD have not yet been performed. Therefore, the aim of this study was to determine whether children with congenital CBDD differ from children with other CBDD causes in terms of cholangiographic characteristics.

MATERIALS AND METHODS

A retrospective medical chart review revealed that 85 consecutive children under 16 years of age with hepatobiliary disease and CBDD were referred to Seoul Asan Medical Center, a tertiary referral center in Seoul, South Korea between January, 2000 and January, 2012[6,16]. The baseline data and the results of imaging analyses were documented. All children were screened by trans-abdominal ultrasonography (TUS) and more than one imaging modality, such as computed tomography and magnetic resonance cholangiopancreatography (MRCP). They underwent a total of 123 ERCP procedures. The study protocol was approved by the institutional review board of Asan Medical Center, Seoul.

ERCP was performed on all pediatric patients by experienced pediatric endoscopists[6]. The maximal transverse diameter of the CBD between the insertion of the cystic duct and the head of the pancreas was measured along its longitudinal axis *via* a cholangiogram[20,21]. Measurements were not taken within 5 mm of the origin of the CBD. The reference cut-off value for the normal maximum diameter of the CBD relative to age was obtained from the intravenous cholangiographic data of Witcombe *et al*[20]. To assess whether age-adjusted CBDD could be used for differential diagnosis, a CBDD severity index (SI) was calculated by dividing the measured CBD diameter by the age-corrected maximal diameter of a normal CBD. To avoid variation due to other causes, patients with a previous history of cholecystectomy, obstructive cholestasis, and premedication such as with opioids were excluded. Moreover, measurements were made on unmagnified cholangiograms by using electronic calipers. Neonatal cases were also excluded due to technical difficulties in ERCP. Although contrast dye

Table 1 Etiological classification of common bile duct dilatation in children

Causes	*n* = 85	Descriptions
Congenital CBDD	*n* = 55 (64.7%)	
Choledochal cyst	*n* = 55	Type Ⅰ (*n* = 26)
		Type Ⅳa (*n* = 29)
Obstructive CBDD	*n* = 30 (35.3%)	
Choledolithiasis	*n* = 23	Idiopathic (*n* = 10)
		Chronic pancreatitis (*n* = 6)
		Leukemia (*n* = 2)
		G6PD deficiency (*n* = 2)
		Spherocytosis (*n* = 2)
		Trauma (*n* = 1)
Miscellaneous	*n* = 7	Chronic pancreatitis (*n* = 3)
		Lymphoma/pancreatic cancer (*n* = 3)
		Trauma (*n* = 1)

CBDD: Common bile duct dilatation.

was gently flushed into the CBD, the dilatation caused by direct dye injection into the duct was ignored. All ERCP findings were reviewed by radiologists and the diagnosis of choledochal cyst was confirmed by surgical excision and intraoperative cholangiography. To reduce intraobserver and interobserver variability, measurement of the diameter was performed three times and average values of the diameter were used. These were validated by gastroenterologists and radiologists at the same institution. This process led to patients being classified into those with congenital CBDD and those with obstructive CBDD due to secondary causes. The morphological descriptions of the CBD were based on Todani's classification system[22,23].

Statistical analysis

For univariate analysis, continuous variables were assessed by using independent sample *t*-tests and categorical variables were assessed by using χ^2 tests. For multivariate analysis, a logistic regression model was used to generate odds ratios (ORs), the corresponding 95% confidence intervals (95%CIs), and the *P* values. The optimal cut-off of CBD that allowed congenital CBDD to be differentiated from obstructive CBDD was determined by using a receiver operating characteristic (ROC) curve. All statistical calculations were performed by using SPSS software (SPSS for Windows, version 14.0; SPSS Inc., Chicago, IL). A *P* value less than 0.05 was considered to indicate statistical significance.

RESULTS

In total, 33 boys (38.8%) and 52 girls (61.2%) were diagnosed with CBDD according to our study criteria. The mean patient age was 6.3 ± 3.6 years. The indications for ERCP are summarized in Table 1. Fifty-five (64.7%) children had congenital CBDD and 30 (35.3%) had CBDD due to other secondary causes. The clinical and cholangiographic characteristics of the 85 patients are summarized in Table 2. The most common presenting clinical manifestations in both groups at the time of diagnosis were abdominal pain and jaundice. Some patients in both groups also presented with pancreatitis. This was regarded as a complication in the patients with choledochal cyst but as the underlying disease in the patients with obstructive CBDD. The two groups did not differ significantly in terms of clinical characteristics except for sex: the patients with congenital CBDD were significantly more likely to be female than the patients with obstructive CBDD (80% *vs* 26.7%, *P* = 0.032).

The congenital and obstructive CBDD groups did not differ significantly in terms of mean CBD diameter (19.3 ± 9.6 mm *vs* 12.2 ± 4.1 mm). However, the congenital group had a significantly higher mean CBDD SI (3.62 ± 1.64) than the obstructive CBDD group (1.98 ± 0.71). In addition, as the SI increased, so did the prevalence of choledochal cyst among children with CBDD (Figure 1). All patients with an SI of ⩾ 3 had a choledochal cyst, unlike patients with an SI of 1-2 or 2-3 (both *P* < 0.05). ROC analysis showed that an SI of 2.32 could serve as a cut-off value with a sensitivity of 68%, a specificity of 96.7%, and an area under the curve of 0.87. Of the 55 children with congenital CBDD, 34 (61.8%) had a SI of ⩾ 2.32. By contrast, only one of 30 children (3.3%) with obstructive CBDD had such a high SI.

Despite the high specificity of the CBDD SI ⩾ 2.32, its low sensitivity means that additional efforts are needed to distinguish between the two types of CBDD in children with SI < 2.32. We observed that anomalous union of pancreaticobiliary duct (APBDU) was very common in congenital CBDD: of the 21 children with congenital CBDD and SI < 2.32, 15 (71.4%) had APBDU. Multivariate analysis revealed that SI ⩾ 2.32 (OR = 2.4, 95%CI: 1.2-5.52) and APBDU comorbidity (OR= 5.7, 95%CI: 1.92-24.81) were independent factors that predicted congenital CBDD (Table 2).

The two groups did not differ significantly in terms of any of the other cholangiographic findings. The patients with obstructive CBDD tended to have CBDs with cylindrical-fusiform features more frequently than the patients with congenital CBDD (93.3% *vs* 78.2%) but this difference did not achieve statistical significance. Moreover, patients with congenital CBDD tended to have cystic features more frequently than the patients with obstructive CBD (21.8% *vs* 6.7%) but this too did not achieve statistical significance. The two cases of obstructive CBDD with cystic features had had severe choledolithiasis, which had normalized after endoscopic removal of the stone; recurrence was not observed during the follow-up period. Notably, although choledolithiasis occurred in three-quarters of the children with obstructive CBDD (76.6%), more than half of the congenital CBDD cases (61.8%) also

Table 2 Clinicocholangiographic characteristics of children with common bile duct dilatation *n* (%)

Characteristics total (*n* = 85)	Congenital CBDD (*n* = 55)	Obstructive CBDD (*n* = 30)	Univariate *P* value	Multivariate *P* value
Clinical				
Age, mean ± SD (mo)	63.8 ± 36.4	82.4 ± 46.0	NS	
Sex, M:F	11:44	22:8	0.042	
Abdominal pain	50 (91.0)	24 (80.0)	NS	
Jaundice	23 (41.8)	13 (43.3)	NS	
Abdominal mass	2 (3.6)	0 (0)	NS	
Vomiting	11 (20.0)	4 (13.3)	NS	
Cholangitis	5 (9.0)	1 (3.3)	NS	
Pancreatitis	23 (43.6)	9 (30.0)	NS	
Cholangiographic				
CBD diameter	19.3 ± 9.6	12.2 ± 4.1	NS	
Severity index ⩾ 2.32	34 (61.8)	1 (3.3)	0.012	0.024
Cystic features	14 (21.8)	2 (6.7)	NS	
Cylindrical-fusiform feature	43 (78.2)	28 (93.3)	NS	
APBDU	39 (70.9)	0 (0)	0.005	0.001
Choledolithiasis	34 (61.8)	23 (76.6)	NS	
Cholelithiasis	3 (5.5)	7 (23.3)	NS	
Pancreatic duct dilatation	5 (9)	2 (6.7)	NS	

APBDU: Anomalous union of pancreaticobiliary duct; CBD: Common bile duct; CBDD: Common bile duct dilatation; NS: Not significant.

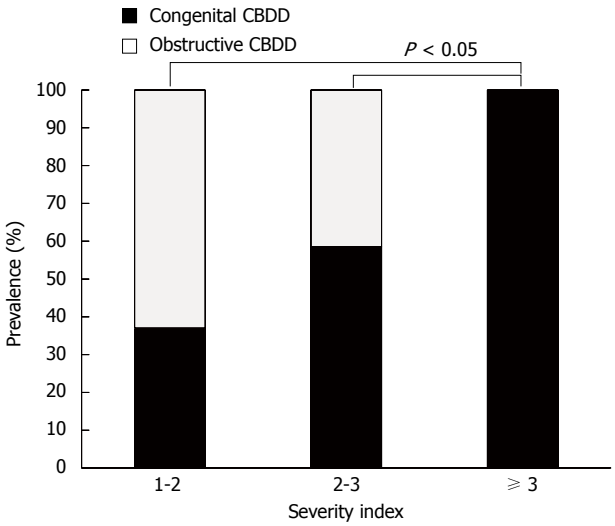

Figure 1 Prevalence of congenital common bile duct dilatation relative to common bile duct severity index. CBDD: Common bile duct dilatation.

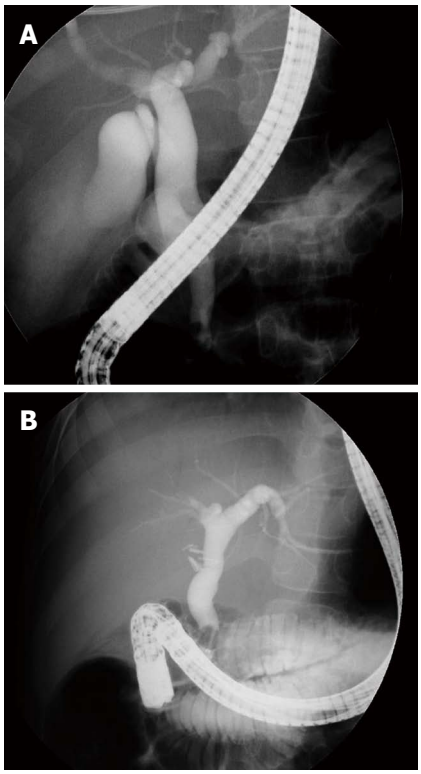

Figure 2 Two children with different etiologies show similarities in terms of endoscopic retrograde cholangiopancreatography findings. A: Congenital common bile duct dilatation (CBDD) with choledolithiasis; B: Obstructive CBDD due to hereditary spherocytosis.

had choledolithiasis. Thus, choledolithiasis was not useful for differential diagnosis in CBDD. It was also difficult to differentiate between the obstructive and congenital CBDD patients with SI < 2.32 on the basis of their CBDD features: there were 46 patients with SI < 2.32, of whom 14 had congenital CBDD and 28 had obstructive CBDD. Ten of the 14 congenital CBDD patients (71.4%) and 21 of the 28 obstructive CBDD patients (75%) had cylindrical-fusiform CBDD features. Indeed, in our experience, it was sometimes difficult to differentiate between congenital and obstructive CBDD by only measuring their CBDD diameters (Figure 2).

DISCUSSION

The present study indicates that several cholangiographic features may be helpful for assessing CBDD, especially for the differential diagnosis of CBDD in children. Firstly, the SI of CBDD was helpful for discriminating congenital CBDD from obstructive CBDD. Secondly, APBDU comorbidity was also an important factor for this differential diagnosis. This

close relationship between congenital CBDD and APBDU is already well-known in the literature. Thirdly, the presence of choledolithiasis was not useful for the differential diagnosis of the two CBDD types.

This is the first time that CBDD has been expressed as an SI that incorporated an age-corrected reference. In the present study, the SI of CBDD showed high specificity in terms of differentiating congenital CBDD from obstructive CBDD. This indicated the importance of using age-related CBD reference diameters to assess CBDD in children. Several other studies have also noted this. In a pediatric study of APBDU, most cases of the non-dilated CBD type of APBDU were found to actually have CBDD when the CBD diameter was corrected by an age-related reference[24]. Two TUS studies also provided cut-off CBD diameter references that would allow the identification of CBDD: 2 mm in neonates and 3 mm in children under 13 years of age[25,26]. However, when we employed these TUS references in the present study, the congenital CBDD group could not be differentiated from the obstructive CBDD group.

It is generally known that APBDU is often accompanied by congenital CBDD[12,27]. Although Todani's classification system divides congenital CBDD into five types[22,23], most patients with choledochal cysts have types Ia, Ic, and IVa CBDD, and all of these types are accompanied by APBDU in almost all adults with this condition[12]: 50%-80% have type I congenital CBDD while 15%-35% have type IV congenital CBDD[28]. Moreover, 76% of children with types I and IV CBDD had APBDU[29]. In the present study, APBDU played a critical role in the differential diagnosis of children with cylindrical-fusiform CBDD. Of the 21 children with congenital CBDD with SI < 2, 20 (71.4%) had APBDU, which facilitated the differential diagnosis of our patients with cylindrical-fusiform CBDD.

No consensus has yet been reached regarding the best approach for identifying APBDU and CBDD in pediatric patients. In addition, APBDU has not been defined in children in relation to the age-corrected size of the common channel[30]. In adults, however, MRCP and endoscopic ultrasonography have been shown to be useful for diagnosing APBDU[31,32]. One study showed that MRCP diagnosed APBDU in adults with a sensitivity of 83% and a specificity of 90%[16]. Since much less is known about APBDU in children, it is unclear whether ERCP can be replaced by MRCP in these patients[33,34]. Indeed, in a study of children with known or suspected APBDU, only 70% were identified by MRCP[33]. The use of endoscopic ultrasonography in children has not been widely established[35]. As a result, ERCP remains the standard diagnostic modality for biliary disease in children, even though its usefulness is limited in neonates and by ERCP-associated complications.

While choledolithiasis with congenital CBDD was initially thought to be rare in Japan, it was then found to be more common than originally believed when it was assessed on the basis of local referral patterns[6,9,10,33]. The present study also showed a high prevalence of choledolithiasis among congenital CBDD children (61.8%). The rate of this comorbidity may depend on the age at diagnosis and the degree of pathological progression, as evidenced by the fact that adult patients with congenital CBDD have CBD stones more frequently than pediatric patients (50% *vs* 28.6%)[9].

In conclusion, the SI of CBDD, as measured by ERCP, together with APBDU comorbidity, may aid the differential diagnosis of congenital CBDD and obstructive CBDD. However, the study has several limitations. Firstly, it was not adequately powered because of the small cohort size, its retrospective study design, the discrepancy between the numbers of patients in each group, and the etiological heterogeneity of the obstructive CBDD group. In addition, this study may have been limited by selection bias, namely, patients with more complications tend to be referred to a tertiary hospital. Therefore, it is not yet possible to state conclusively that ERCP-measured CBD diameter is useful for differentially diagnosing congenital CBDD in children. However, further study is warranted given that the accurate diagnosis of children with choledochal cyst on the basis of bile duct measurements would facilitate their early and appropriate surgical management and thus result in low morbidity rates and a good prognosis. Given the current scarcity of related studies, a collaborative study that investigates the usefulness of ERCP for diagnosing children with CBDD is needed.

COMMENTS

Background

While the investigation of common bile duct dilatation (CBDD) in adults focuses mainly on causes of secondary obstruction of common bile duct (CBD), congenital CBDD must be prioritized in the diagnosis of CBDD in children. ERCP remains the standard diagnostic modality for CBDD in children. However, no consensus has been reached regarding the best approach for identifying CBDD and the diagnosis of CBDD is based on the morphology of CBD.

Research frontiers

No study to measure the diameter of CBD and to adjust its degree of CBDD according to age has been done in children.

Innovations and breakthroughs

This study shows that rather than simple measurement of dilatation of the CBD, a calculated index adjusted for age would be more specific for differentiating obstructive form congenital pathology.

Applications

Not only evaluating the morphology of the CBD, but also measuring the diameter of CBD may be helpful in the differential diagnosis of CBDD in children.

Terminology

CBDD is mainly caused by a congenital anomaly in children, while in adults it is caused by obstruction secondary to cancer and choledolithiasis.

Peer-review

This is an interesting concept regarding discriminating between congenital

pathology and secondarily obstructive causes by utilizing a calculated index of the CBD diameter adjusted for age. However, this concept remains in need of further study to validate the applicability in children with bile duct dilatation.

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ORIGINAL ARTICLE

Retrospective Study

Utility of the low-accelerating-dose regimen in 182 liver recipients with recurrent hepatitis C virus

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Author contributions: Lim KBL and Schiano TD participated in research design, writing of the manuscript, performance of the research, data analysis; Sima HR, Fiel MI and Khaitova V participated in performance of the research, data analysis; Doucette JT participated in research design and data analysis manuscript revision; Chernyiak M, Ahmad J, Bach N, Chang C, Grewal P, Kim-Schluger L, Liu L, Odin J, Perumalswami P and Florman SS participated in research design/manuscript revision.

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Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest: No potential conflicts of interest relevant to this article were reported.

Data sharing: No additional data are available.

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Abstract

AIM: To describe our experience using a low-accelerating-dose regimen (LADR) with pegylated interferon alpha-2a and ribavirin in treatment of hepatitis C virus (HCV) recurrence.

METHODS: From 2003, a protocolized LADR strategy was employed to treat liver transplant (LT) recipients with recurrent HCV at our institution. Medical records of 182 adult patients with recurrent HCV treated with LADR between 1/2003 and 1/2011 were reviewed. Histopathology from all post-LT liver biopsies were reviewed in a blinded fashion. Paired recipient and

donor IL28B status were assessed. A novel technique was employed to ascertain recipient and donor IL28B (rs12979860) Gt data using DNA extracted from archival FFPE tissue from explanted native livers and donor gallbladders respectively. The primary endpoint was SVR; secondary endpoints examined include (1) patient and graft survival; (2) effect of anti-viral therapy on liver histology (fibrosis and inflammation); (3) incidence of on-treatment development of ACR, CDR, or PCH; (4) association of recipient and donor IL28B genotype with SVR; and (5) incidence of anti-viral therapy-associated adverse events (anemia, leukopenia, thrombocytopenia, depression) and hepatic decompensation.

RESULTS: The overall SVR rate was 38% (29% Gt1, 67% Gt2, 86% Gt3 and 58% Gt4). HCV Gt ($P < 0.0001$), donor age ($P = 0.003$), cytomegalovirus mismatch ($P = 0.001$), baseline serum bilirubin ($P = 0.002$), and baseline viral load ($P = 0.04$) were independent predictors for SVR. SVR rates were significantly higher in the recipient-CC/donor-non CC pairs ($P = 0.007$). Neither baseline fibrosis nor change in fibrosis stage after anti-viral therapy were associated with SVR. Fibrosis progressed in 72% of patients despite SVR. Median graft survival was 91 mo. Five-year patient survival was superior in patients who achieved SVR (97% *vs* 82%, $P = 0.001$). Pre-treatment ALP $\geqslant$ 150 U/L ($P = 0.01$), total bilirubin $\geqslant$ 1.5 mg/dL ($P = 0.001$) and creatinine $\geqslant$ 2 mg/dL ($P = 0.001$) were independently associated with patient survival. Only 13% of patients achieving SVR died during the follow-up period. Treatment discontinuation and treatment-related mortality occurred in 35% and 2.2% of patients, respectively. EPO, G-CSF and blood transfusion were needed in 89%, 40% and 23% of patients, respectively. Overall hospitalization rate for treatment-related serious adverse events was 21%. Forty-six (25%) of the patients were deceased; among those who died, 25 (54%) were due to liver-related complications, and 4 deaths (9%) occurred while receiving therapy (2 patients experienced hepatic decompensation and 2 sepsis).

CONCLUSION: LADR strategy remains relevant in managing post-LT recurrent HCV where access to DAAs is limited. SVR is associated with improved survival, but fibrosis progression still occurs.

Key words: Hepatitis C recurrence; Liver transplant; Low accelerating dose regimen; Peginterferon α-2a; IL28B

Core tip: This study represents the largest single center experience in treating recurrent hepatitis C virus (HCV) in LT recipients utilizing a low-accelerating-dose regimen (LADR) protocol of PEG alpha-2a and RBV; achieving 38% SVR and superior five-year patient survival in patients with SVR (97% *vs* 82%, $P = 0.001$). A novel technique was used to ascertain recipient and donor IL28B (rs12979860) Gt data using archival FFPE tissue from explanted native livers and donor gallbladders. A comprehensive blinded review of available liver histology was performed. LADR strategy remains relevant in managing recurrent HCV where access to DAAs is limited. SVR is associated with improved survival, but fibrosis progression still occurs.

Lim KBL, Sima HR, Fiel MI, Khaitova V, Doucette JT, Chernyiak M, Ahmad J, Bach N, Chang C, Grewal P, Kim-Schluger L, Liu L, Odin J, Perumalswami P, Florman SS, Schiano TD. Utility of the low-accelerating-dose regimen in 182 liver recipients with recurrent hepatitis C virus. *World J Gastroenterol* 2015; 21(20): 6236-6245 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6236.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6236

INTRODUCTION

Post-liver transplant (LT) treatment of recurrent hepatitis C virus (HCV) with pegylated (PEG)-based regimens is challenging, labor intensive, and carries sub-optimal SVR rates, and poor patient tolerability[1,2]. The optimal time to commence anti-viral therapy is controversial and adds extra complexity to this cohort of difficult-to-treat patients. Data on donor and recipient IL28B genotypes have shed some light on their impact on HCV recurrence and treatment response, with increasing awareness and relevance of donor IL28B status[3-6]. Although the advent of DAA has increased the success rate of post-LT antiviral therapy, improved SVR rates must be balanced against drug-drug interactions with immunosuppressive agents and increased adverse events resulting in treatment discontinuation[7,8]. Emerging data using interferon-free regimens have shown initial promise with improved response rates and tolerability[9,10]. However, interferon-containing regimens may still be needed in post-LT patients who do not respond to newer regimens, or when access to DAAs are limited due to financial and resource constraints[11,12].

To date, published studies on post-LT HCV therapy have typically been single center series with heterogeneous treatment strategies[13-16]. LADR is well described in cirrhotic HCV patients in the pre-LT setting[17,18], but experience of low-accelerating-dose regimen (LADR) in the post-transplant setting is limited. We herein report our experience in treating 182 LT recipients with HCV recurrence over a 9-year period using a standardized LADR protocol of PEG α-2a and RBV. To our knowledge, this represents the largest single-center treatment study of post-LT HCV using a uniform strategy. The data presented will be of utility in guiding therapy in interferon-containing regimens when the newer oral agents are ineffective,

not available or are restricted for financial reasons.

MATERIALS AND METHODS

Starting in 2003, a protocolized LADR strategy was employed to treat all LT recipients with recurrent HCV at our institution. The decision to commence anti-viral therapy was based on clinical need as determined by the primary hepatologist. Patients were started on 90 mcg PEG α-2a weekly and RBV 7 mg/kg daily in 2 divided doses (50% of optimal dose). Doses were increased at week 4 to PEG α-2a 135 mcg weekly and RBV 10 mg/kg daily thereafter doses were PEG alpha-2a 180 mcg weekly and RBV 14 mg/kg daily from week 8 onwards if tolerated, for a total of 48 wk regardless of HCV Gt. EPO 40000 IU once weekly was started when the hemoglobin fell below 10 g/dL. RBV dose reduction occurred if EPO was unsuccessful at maintaining hemoglobin above 10 g/dL. Patients received blood transfusions if they experienced symptomatic anemia despite EPO supplementation. G-CSF 300 mcg once weekly (up to maximum dose of 300 mcg three times weekly) was commenced when the WBC was < 1500/mm and/or ANC was < 750/mm. Dose reduction of PEG α-2a by 50% occurred when the platelet count < 50000/mm; PEG α-2a was discontinued when platelet count was less than 25000/mm.

Study design

The medical records of LT recipients age > 18 years with recurrent HCV, treated with LADR between 1/2003 and 1/2011 were reviewed. The following were excluded from the analysis: LT recipients treated with non-pegylated interferon, HCV patients co-infected with HBV or HIV, and patients enrolled in other HCV study protocols. Patients with uncontrolled psychiatric illness, poorly controlled diabetes, symptomatic cardiopulmonary disease, concomitant autoimmune disease, moderate to advanced chronic kidney disease (> CKD stage 2) and SLKT recipients were typically not treated with antiviral therapy. We defined recurrent HCV in LT recipients as the presence of typical histological features of HCV and contemporaneously detectable HCV RNA in the serum, after excluding concurrent other etiologies. LT recipients with fibrosing cholestatic hepatitis (FCH) were included in our analysis. The diagnosis of FCH was determined histologically by the presence of cholestasis, ductular reaction, mild portal inflammation, portal fibrosis and delicate fibroconnective tissue accompanying and surrounding proliferating bile ductules, as well as periportal fibrosis and exclusion of large duct biliary obstruction[19].

Clinical data including recipient and donor demographics, review of hospitalizations occurring during therapy, HCV Gt, serious adverse events, use of growth factors (EPO and G-CSF) and anti-depressants were recorded. Quantitative HCV RNA by polymerase chain reaction (Cobas TaqMan, Roche Laboratories), liver chemistries and complete blood count, at specific time points during HCV therapy, were collected. All transplanted patients received intra-operative methylprednisolone 500 mg. Patients with serum creatinine > 2 mg/dL or requiring dialysis prior to LT received basiliximab induction therapy on the day of LT and post-operative day-4. LT recipients were maintained on tacrolimus-based immunosuppression and MMF in the setting of renal dysfunction. Oral steroid therapy was tapered and discontinued during the 6 mo after LT. Severe ACR was treated with pulse methylprednisolone 500 mg daily over 3 d; mild and moderate ACR were treated by optimizing tacrolimus levels and MMF dosing.

The primary endpoint was SVR, defined as absence of HCV RNA at 6 mo after completion of anti-viral therapy. Secondary endpoints were (1) patient and graft survival; (2) effect of anti-viral therapy on liver histology (fibrosis and inflammation); (3) incidence of on-treatment development of ACR, CDR, or PCH; (4) association of recipient and donor IL28B genotype with SVR; and (5) incidence of anti-viral therapy-associated adverse events (anemia, leukopenia, thrombocytopenia, depression) and hepatic decompensation. On-treatment responses were analyzed at weeks 4 (RVR), 8, 12 (EVR), 24 and 48 (ETR). This study was reviewed and approved by the Icahn School of Medicine at Mount Sinai, NY Institutional Review Board.

Histological assessment

Archival (FFPE) liver tissue from all post-LT biopsies was obtained and independently reviewed. Liver biopsy specimens were stained with hematoxylin and eosin and Masson trichrome. The latest liver biopsy before starting anti-viral therapy, any liver biopsy performed while receiving therapy, and the first biopsy after anti-viral therapy was concluded, were considered as the pre-, on- and post-treatment biopsies, respectively. All biopsies were reviewed by a single hepatopathologist (MIF) blinded to patient identity and timing of the liver biopsy. Inflammation and fibrosis were graded and staged using the Scheuer classification[20]. Biopsies having ACR, CR, PCH, FCH and steatosis were identified and graded utilizing standard histological classification systems[21-24].

IL28B genotyping

Surviving liver recipients were tested for IL28B (rs12979860) genotype using peripheral venous blood samples. If recipient serum was not available, DNA extracted from FFPE tissue from explanted native livers was used to determine the IL28B genotype. In addition, because donor sera were not available for testing, archival FFPE tissue from the donor gallbladder was used in order to obtain adequate tissue for DNA extraction. Approximately 3 to 5 five-micron thick sections were cut from the FFPE blocks.

After standardization of DNA isolation, amplification using qPCR was performed. When testing the DNA using TaqMan assay, approximately 100-200 ng DNA per reaction was required. Allelic discrimination for the "C" and "T" was analyzed using the DNA sample extracted[25-27]. The results obtained from this novel method were cross-referenced with IL28B results obtained from recipient serum in a subset of cases and showed 100% concordance, providing validation of this technique.

Statistical analysis

Statistical methods and analysis were performed by an experienced biostatistician (John T Doucette) from Icahn School of Medicine at Mount Sinai. Descriptive statistics were produced for all study variables to examine their univariate distributions. Bivariate associations with SVR were assessed using Pearson's χ^2 (or Fisher's exact test, when appropriate) for categorical factors, and *t*-tests for continuous variables. All significance tests were two-sided with a level of $\alpha = 0.05$. Logistic regression models were fitted to identify independent predictors of SVR. To examine predictors of survival, log-rank tests were used for bivariate associations and Cox proportional hazards models were fitted to identify independent predictors. For both the logistic regression and Cox models, a modified stepwise procedure was employed with a significance level of $\alpha = 0.05$ for both entry and removal. Candidates for the stepwise procedure were all baseline characteristics that had a bivariate association with each outcome at the $\alpha = 0.20$ level. Because some candidate variables had missing data, the models selected by the stepwise procedure were then re-fitted with each unselected candidate added one at a time to reassess significance while allowing inclusion of the maximum number of observations. Although patients who achieved SVR were compared to those who did not with respect to survival in bivariate analysis, SVR was not a candidate predictor for the survival models because it is not a baseline characteristic.

RESULTS

Twelve hundred forty-one patients underwent LT for HCV during the study period, and 158 LT recipients were treated with non-LADR protocols, either as part of study protocols, or using non-pegylated interferon with or without RBV prior to 2003. One hundred eight-two patients with recurrent HCV were treated using the LADR protocol. Patients were predominantly male (80%), Caucasian (50%), with a median age of 52 years. One hundred forty one (77%) were infected with HCV Gt1. HCV Gt sub-types were available in 131 patients with Gt1; Gt1a was more common than Gt1b in our cohort (57% *vs* 36%). Seventy-four (75%) had pre-treatment baseline HCV RNA > 1000000 IU/mL. Other baseline characteristics of these 182 patients are summarized in Table 1. The median time from LT to commencing anti-viral therapy was 20.5 mo; median age when therapy started was 56 years; 119 (65%) patients completed 48 wk of anti-viral therapy. One hundred thirty-five (74%) patients tolerated peak PEG α-2a dose of 180 mcg/wk, and 34 (19%) achieved RBV doses > 1000 mg/d. Median peak weekly PEG α-2a dose was 180 mcg; median daily RBV dose was 800 mg.

Response rates

The overall SVR rate was 38% (70/182 patients). SVR stratified by HCV Gt 1, 2, 3, 4 was 29%, 67%, 86%, and 58%, respectively. Gt4 patients showed superior SVR (58%) compared to Gt1 patients (29%) (P = 0.05). No difference in SVR was observed between Gt1a (24%) and Gt1b (28%) (P = 0.6), nor between Gt2 (67%) and Gt 3 (86%) (P = 0.5).

Despite receiving lower doses from the outset, 25 (14%) patients still achieved RVR; 53 (29%) patients were HCV RNA negative at 8 wk of anti-viral therapy; 75 (41%) patients achieved EVR. Thirty-one percent who achieved RVR went on to attain SVR, while 69% of those without RVR still went on to achieve SVR (P = 0.0003). Seventy-two percent who achieved EVR had SVR; 11% of those who did not achieve EVR went on to SVR (P < 0.0001). Among patients who were HCV negative at week 8, 72% went on to achieve SVR, while only 20% who were HCV positive at week 8 achieved SVR (P < 0.0001) (Table 2). Forty-eight percent of patients failed to achieve a 2-log decline in serum HCV RNA between baseline and week 12 of anti-viral treatment (classified as non-responders); relapse and non-response rates were 20% and 41%, respectively. SVR rates were 40%, 29%, 42% for recipient IL28B genotype CC, CT and TT respectively, and 36%, 37%, 33% for donor IL28B genotype CC, CT and TT, respectively. Significantly different SVR rates were observed between the various pairs of donor and recipient IL28B. SVR rates were significantly higher in the recipient-CC/donor-non CC (72%) *vs* other recipient and donor combinations (41%, 43%, 24% for recipient-CC/donor-CC, recipient-non CC/donor-CC and recipient-non CC/donor-non CC respectively) (P = 0.007).

Factors associated with SVR and survival on univariate analysis are summarized in Table 3. Multivariate analysis found HCV Gt (P < 0.0001), donor age (P = 0.003), cytomegalovirus (CMV) mismatch (P = 0.001), baseline serum bilirubin (P = 0.002), and baseline viral load (P = 0.04) to be independent predictors for SVR. Pre-treatment ALP $\geqslant$150 U/L (P = 0.01), total bilirubin $\geqslant$ 1.5 (P = 0.001) and creatinine $\geqslant$ 2 mg (P = 0.001) were independently associated with patient survival (Table 4).

One hundred thirty-six (75%) patients were alive at time of data censure; 24 had progressed to cirrhosis or

Table 1 Baseline characteristics of the study patients *n* (%)

Recipient characteristics	*n*	
Median age (yr)	182	52 ± 8.2
Male gender	182	145 (80)
Ethnicity	182	
Caucasian		91 (50)
Black		18 (10)
Hispanic		52 (29)
Asian		14 (8)
Arabic		6 (3)
Others		1 (1)
IL28B	122	
CC		40 (33)
CT		51 (42)
TT		31 (25)
HCC (pre-LT)	182	75 (41)
Diabetes mellitus	182	71 (39)
CMV positivity	181	126 (70)
Duration from LT to anti-viral therapy (mo)	182	20.5 ± 43.5
Median peak PEG dose (mcg/wk)	181	180 ± 31.6
Median peak RBV daily dose (mg)	181	800 ± 294
Median treatment duration (wk)	182	48 ± 21.5
HCV genotype	182	
1		141 (77)
1a		80/141 (57)
1b		51/141 (36)
Subtype not available		10/141 (7)
2		15 (8)
3		14 (8)
4		12 (7)
Median baseline lab values		
ALT (IU)	181	84 ± 128.3
AST (IU)	181	80 ± 112.5
ALP (IU)	180	131 ± 144.3
Total bilirubin (mg)	181	0.9 ± 3.1
HCV RNA (IU/mL)	181	3870000 ± 23602918
Hemoglobin (g/dL)	180	13 ± 1.7
White cell count	180	4.3 ± 1.9
Platelets	181	123 ± 73
Creatinine	181	1.2 ± 1.3
Immunosuppression at start of LADR[1]	182	
Tacrolimus		146 (80)[1]
Cyclosporine		25 (14)
Sirolimus		4 (2)
Mycophenolate mofetil		66 (36)
Prednisone		9 (5)
Donor characteristics		
Male gender	175	99 (57)
Median age	177	48 ± 18
CMV positivity	178	117 (66)
Ethnicity	182	
White		118 (65)
Black		26 (14)
Hispanic		12 (7)
Asian		6 (3)
American indian/alaskan native		3 (2)
Other/unknown		17 (9)
Donor IL28B	122	
CC		66 (54)
CT		35 (29)
TT		21 (17)

[1]Percentages add to > 1 due to combination therapy. HCC: Hepatocellular carcinoma; LT: Liver transplant; CMV: Cytomegalovirus; HCV: Hepatitis C virus; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; LADR: Low-accelerating-dose regimen.

Table 2 Viral kinetics and SVR

	Percentage	Percentage who went on to achieve SVR	*P* value
RVR			0.0003
Yes	14	31	
No	86	69	
Week 8 negativity			< 0.0001
Yes	29	72	
No	71	20	
EVR			< 0.0001
Yes	41	72	
No	59	11	

Table 3 Univariate analysis

Factors associated with SVR	SVR (%)	*P* value
HCV genotype 1 *vs* non-1	29 *vs* 71	< 0.0001
HCV viral load < 1 million *vs* ⩾ 1 million IU/mL	54 *vs* 33	0.009
Recipient IL28B-CC *vs* non-CC	55 *vs* 34	0.03
Paired IL28b recipient CC and donor non-CC *vs* other recipient and donor combinations	72 *vs* 36	0.007
Pre-treatment total bilirubin < 1.5 mg *vs* ⩾ 1.5 mg	44 *vs* 25	0.02
Pre-treatment ALP < 150 *vs* ⩾ 150	44 *vs* 29	0.04
Treatment duration ⩾ 48 wk *vs* < 48 wk	45 *vs* 27	0.02
Peak RBV dose ⩾ 800 mg *vs* < 800 mg	44 *vs* 25	0.02
Administration of MMF *vs* No MMF	30 *vs* 43	0.09
RVR *vs* no RVR	34 *vs* 66	0.0006
EVR *vs* no EVR	85 *vs* 15	< 0.0001
Week 8 HCV RNA undetectable *vs* detectable	72 *vs* 20	< 0.0001
Donor age ⩽ 40 *vs* > 40	58 *vs* 29	0.0002
Matched recipient and donor ethnicity *vs* unmatched	46 *vs* 33	0.09
Mismatched CMV status (D+/R-) *vs* other combinations	54 *vs* 35	0.04
Pre-treatment fibrosis stage 0-2 *vs* 3-4	43 *vs* 26	0.08
Factors associated with patient survival	10-year survival (%)	
Week 8 HCV RNA undetectable *vs* detectable	71 *vs* 44	0.003
Week 12 HCV RNA undetectable *vs* detectable	73 *vs* 44	< 0.001
Pre-treatment fibrosis stage 0-2 *vs* 3-4	70 *vs* 31	0.004
Pre-treatment ALP < 150 *vs* ⩾ 150	67 *vs* 42	< 0.001
Pre-treatment total bilirubin < 1.5 mg *vs* ⩾ 1.5 mg	62 *vs* 46	< 0.001
Pre-treatment creatinine < 2 *vs* ⩾ 2	61 *vs* 0	< 0.001

HCV: Hepatitis C virus; ALP: Alkaline phosphatase; RBV: Ribavirin.

graft failure requiring re-LT. Among these 24 patients, 58% were non-responders, 29% relapsed and 13% achieved SVR. Median graft survival was 91 mo. Five-year patient survival was superior in patients who achieved SVR (97% *vs* 82%, $P = 0.001$) (Figure 1). Forty-six (25%) of the patients were deceased. Among those who died, 25 (54%) were due to liver-related complications, and 4 deaths (9%) occurred while receiving therapy (2 patients experienced hepatic decompensation and 2 sepsis).

Sixty (35%) patients discontinued therapy before 48 wk. On-treatment anemia, defined as a fall in Hb > 2 g/

Table 4 Multivariate analysis

Factors associated with SVR	Comparison	Adjusted OR for SVR	95%CI		*P* value
			Lower	Upper	
HCV genotype	2 *vs* 1	11.40	2.8	47.3	< 0.0001
	3 *vs* 1	43.10	6.5	286.3	
	4 *vs* 1	10.70	2.4	48.8	
Pre-treatment total bilirubin	≥ 1.5 *vs* < 1.5	0.21	0.08	0.57	0.002
Donor age	Each 10 yr	0.69	0.54	0.88	0.003
CMV mismatch	Donor +/recipient - *vs* all others	4.80	1.8	12.6	0.001
HCV viral load (baseline)	Each 1 million	0.97	0.94	0.999	0.040
		Adjusted HR			
Pre-treatment ALP	≥ 150 *vs* < 150	2.01	1.18	3.43	0.010
Pre-treatment total bilirubin	≥ 1.5 *vs* < 1.5	2.49	1.47	4.21	0.001
Pre-treatment creatinine	≥ 2 *vs* < 2	5.88	2.68	12.92	< 0.001

CMV: Cytomegalovirus; HCV: Hepatitis C virus; ALP: Alkaline phosphatase.

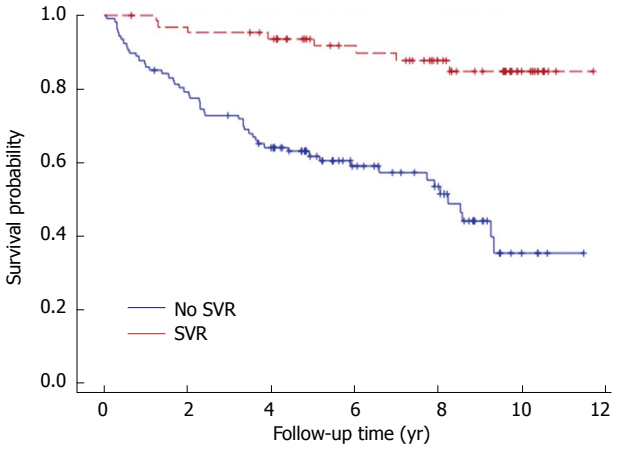

Figure 1 Association of patient survival and achieving SVR. Five-year patient survival was superior in patients who achieved SVR (97% *vs* 82%, *P* = 0.001). Blue curve: Patients who did not achieve SVR; Red curve: Patients who achieved SVR.

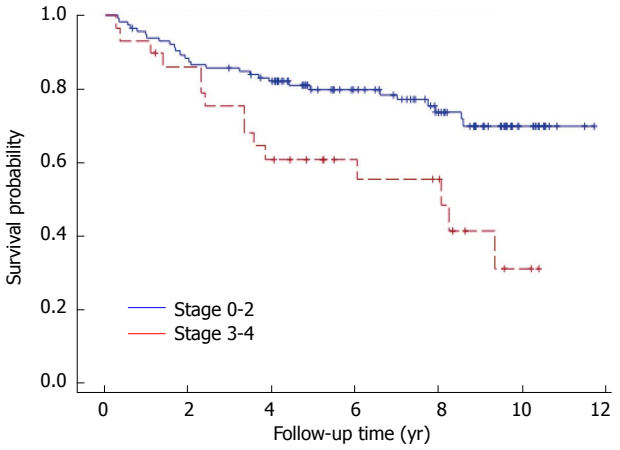

Figure 2 Association of patient survival and baseline fibrosis. Five-year patient survival was superior in patients with low baseline fibrosis stage (*P* = 0.004). Blue curve: Patients with low baseline fibrosis stage; Red curve: Patients with moderate to advanced baseline fibrosis stage.

dL from baseline or Hb < 10 g/dL, occurred in 123 (69%). Leukopenia, defined as WBC < 1500, occurred in 18 (10%). EPO, G-CSF and blood transfusion were needed in 89%, 40% and 23% of patients, respectively. Development of anemia and leukopenia were not significantly associated with SVR or patient survival. Thirty-one percent were given anti-depressants due to clinically significant PEG α-2a-induced depression. The overall hospitalization rate for treatment-related serious adverse events was 21%. Fifteen percent of patients required two or more hospitalizations. The three most common reasons for hospitalization were anemia, pulmonary complications and infection.

Histology

One hundred fifty-three (84%) patients had a liver biopsy prior to HCV therapy. Among the 182 patients included in this study, 153 pre-treatment, 90 on-treatment and 89 post-treatment liver specimens were available for review. Seventy-six paired pre- and post-treatment biopsies were available for analysis. Univariate analysis showed baseline fibrosis stage was significantly associated with survival (*P* = 0.004) (Figure 2); a trend to significance was observed between baseline fibrosis and SVR (*P* = 0.08). Baseline fibrosis was not significantly associated with SVR and survival on multivariate analysis. Fibrosis was observed to progress despite anti-viral therapy, while no change was seen in necroinflammatory grade, steatosis grade and stage (Table 5). Change in fibrosis stage after anti-viral therapy was not associated with SVR (*P* = 0.08) (Table 6). Fifty-nine patients had histology proven rejection in pre-, on-, and post-treatment biopsies (Table 7). PCH occurred in 30 patients. No association was found between paired donor and recipient IL28B status on development of on-treatment ACR, inflammation grade (HAI score), and fibrosis stage in the pre-treatment liver biopsies. Seven patients had FCH prior to anti-viral therapy. Among these difficult-to-treat FCH patients, three achieved SVR, one relapsed and three were non-responders. Three FCH patients died but four were still alive at time of data censure. All three FCH patients who

Table 5 Histological characteristics (76 patients) *n* (%)

	Pre-anti viral treatment	Post-anti viral treatment	*P* value
Fibrosis stage (n = 76) (based on the Scheuer scheme)			
Stage 0-2	65 (86)	41 (54)	< 0.001
Stage 3-4	11 (14)	35 (46)	
Grade (n = 76)			
Grade 0-2	51 (67)	46 (61)	0.33
Grade 3-4	25 (33)	30 (39)	
Degree of steatosis (brunt classification) (n = 78)			
Score 0-1	69 (88)	73 (94)	0.29
Score 2-3	9 (12)	5 (6)	
Steatohepatitis grade (n = 75)			
Grade 0	69 (92)	71 (95)	0.73
Grade 1-2	6 (8)	4 (5)	
Steatohepatitis stage (n = 75)			
Stage 0-1	72 (96)	73 (97)	1.00
Stage 2-4	3 (4)	2 (3)	

Table 6 Change in fibrosis stage after anti-viral treatment (76 cases) *n* (%)

Fibrosis		SVR (n = 32) P = 0.08	Survived (n = 60) P = 0.03
Progressed			
one stage	32 (42)	17 (53)	27 (45)
two stages	10 (13)	5 (16)	9 (15)
⩾ three stages	5 (7)	1 (3)	2 (4)
Unchanged	23 (30)	5 (16)	17 (28)
Improved	6 (8)	4 (12)	5 (8)

Table 7 Frequency of rejection, plasma cell hepatitis and fibrosing cholestatic hepatitis *n* (%)

	Pre-anti viral treatment n = 153	On-anti viral treatment n = 90	Post-anti viral treatment n = 89
Rejection	39 (25)	12 (13)[1]	8 (9)
ACR	39 (25)	10 (11)	7 (8)
CDR	0	5 (6)	1 (1)
Plasma cell hepatitis	7 (5)	11 (12)	12 (13)
Fibrosing cholestatic hepatitis	7 (5)	5 (6)	1 (1)

[1]Three cases developed acute cellular rejection (ACR) and CDR in different on-anti viral treatment biopsies.

achieved SVR were still alive.

DISCUSSION

Treatment of recurrent HCV in LT recipients remains challenging. SVR rates from published studies using interferon and RBV based therapies range from 8% to 48%[15-18]. These studies are limited by small sample size, and significant heterogeneity in study design, time from LT to start of antiviral treatment and degree of fibrosis at baseline. Our study represents the largest single center experience to date in the treatment of recurrent HCV in LT recipients utilizing a uniform LADR protocol of PEG α-2a and RBV. The LADR approach achieved 38% SVR. We included patients with FCH in our analysis, which may account for the lower SVR compared to other studies which excluded these difficult-to-treat patients[28]. Five-year patient survival was superior in patients who achieved SVR (97% *vs* 82%, P = 0.001. We found HCV RNA negativity at week 8 more predictive than RVR for achieving SVR. One explanation for this observation is the lower starting doses of PEG α-2a and RBV utilized in the LADR approach which resulted in a longer time to achieve HCV RNA negativity. RVR and EVR data from the current study may help guide therapy for other PEG α-2a and RBV-containing regimens used in the post-LT setting. Despite the LADR approach, treatment discontinuation occurred in 35%, and anemia remained a significant problem (69%) with up to 89% of patients requiring EPO and 23% requiring blood transfusion. Our data suggest that initiating antiviral therapy in patients with less fibrosis, as well as in the setting of a creatinine < 2.0 appears warranted. Thus, even with the new all oral antiviral regimens, starting HCV treatment earlier before more advanced fibrosis or renal dysfunction develops may be beneficial. It also appears reasonable to initiate HCV therapy earlier in patients receiving an older donor liver, as both their survival and chance of SVR were appreciably lower in the current study.

The advent of potent DAAs has added further complexity to the management of this post-LT population. Recently published multi-center studies[7,8,29,30] involving treatment of recurrent HCV with telaprevir or boceprevir achieved an overall SVR of 50%-63%. The improved SVR however was counterbalanced against reduced patient tolerability, increased rates of treatment discontinuation and potential adverse events including risk of death, significant drug-drug interactions[31-33], cost, and access to therapy. Coilly *et al*[8] observed a 43% treatment discontinuation rate, anemia in 92% of patients with 35% requiring red blood cell transfusions, and treatment-related mortality of 8%. Burton *et al*[29] found that 57% of patients required blood transfusions during the first 16 wk of therapy, 27% required hospitalization and there was 9% mortality. These observations may have implications on future therapies, underscoring the fact that adopting a LADR approach in combination with DAA therapy in LT recipients may be a useful strategy to mitigate anti-viral therapy-associated adverse events and reduce early treatment discontinuation. Whether a 12%-25% increase in SVR with DAA triple therapy compared to conventional therapy with PEG and RBV in post-LT patients justifies the cost, adverse events and potential life threatening consequences is debatable. The lower treatment-related mortality rate associated with LADR may influence some transplant physicians to pursue this treatment strategy until more

effective, safer and shorter duration therapies emerge.

Although early data from interferon-free regimens in the post-LT setting appear promising, longer-term relapse rates and the incidence of viral resistance remain unknown. With the current and projected financial burden of approved and evolving DAA, these interferon-free regimens may remain out of reach to individuals in some geographic areas. The administration of first generation DAA in conjunction with PEG and RBV may be the best available therapy in some patients, and the use of PEG α-2a and RBV with newer DAA may still be warranted in patients failing all-oral therapy. Anemia remains an issue in the treated patients, mostly ascribable to RBV-its dosing could not be optimized in the majority of our patients. Use of a LADR protocol may be more appropriate when using the first generation DAA because of the anemia, rather than starting with standard doses of PEG α-2a and RBV. Based on the current study's data, a LADR protocol could also be considered in conjunction with newer DAAs, possibly for harder to treat Gt3 patients.

To date, few studies on recurrent HCV have distinguished Gt 1a and 1b frequency and outcomes. We observed a greater proportion of subtype 1a compared to 1b (61% *vs* 39%), with similar SVR rates (24% *vs* 28%). Our analysis demonstrated 71% SVR in liver recipients with HCV Gt 2-4, and 86% SVR Gt 3. The better-than-expected SVR in LT recipients with Gt3 warrants further study to confirm if longer courses of therapy are indeed needed and if PEG alpha-2a will remain necessary in the treatment regimen for Gt3 recurrent HCV with the availability of all-oral regimens. There is a paucity of data on antiviral therapy in Gt4 liver recipients; the current study shows appreciable therapeutic efficacy in this group.

A unique strength of this study was the employment of a novel technique to ascertain both donor and recipient IL28B (rs12979860) Gt data using archival FFPE tissue from donor gallbladders and explanted native livers. Adequate amounts of DNA were obtained in all patients. This enabled us to obtain 122-paired donor and recipient IL28B Gt data for analysis. Previously published studies show inconsistent associations between recipient, donor and paired donor/recipient IL28B genotype with HCV recurrence and SVR in transplant recipients[34-37]. We observed the highest SVR rates (72%) in the recipient CC/donor-non CC pairs; while donor CC was associated with 41%-43% SVR and recipient-non CC/donor-non CC pairs were associated with the poorest SVR rates of 24%. The significance of donor and recipient IL28B needs to be re-evaluated with the advent of DAA and interferon-free regimens.

This study undertook a comprehensive review of liver biopsies performed prospectively in a blinded fashion by a single hepatopathologist in pre-, on- and post-treatment liver biopsies. Our analyses of paired pre- and post-treatment liver histology showed no significant change in necroinflammatory scores but did show progression of fibrosis. Liver fibrosis progressed in 72% of patients despite achieving SVR. We found that baseline fibrosis was not significantly associated with SVR but was with overall survival. This is in contrast to a previous study of two non-contemporaneous cohorts which showed that baseline fibrosis was significantly associated with SVR[28]. All patients in our study who were cirrhotic at baseline did not achieve SVR. This is an important reminder to LT physicians in order to still aggressively treat other potential causes of liver fibrosis-namely NASH. The search for potent anti-fibrotic agents is still warranted and represents a clinical need in the post-LT setting. The number of patients with plasma cell hepatitis (PCH) doubled during and after therapy; longer term follow up of these patients is necessary in order to ascertain if their ultimate survival was negatively impacted and whether they had fibrosis progression[38]. Only 13% of patients achieving SVR died during the follow up period, underscoring the benefits of successful antiviral therapy in this population.

Limitations

The retrospective nature of this study resulted in some unavailable data at selected time points. We were not able to accurately ascertain the number of patients who required dose reductions of PEG α-2a and RBV. Some pre- and post-treatment liver biopsies were unavailable to assess post-treatment necroinflammatory and fibrosis scores. The decision to treat recurrent HCV was based on the hepatologist's discretion and not fibrosis stage; however this treatment algorithm is employed in large transplant programs in the US and thus is reflective of current practice.

In conclusion, this single-center study is the largest to date that examines the utility of a LADR protocol in the treatment of recurrent HCV in liver transplant recipients. We have shown that utilizing a LADR strategy achieved an overall SVR of 38%. Patients with GT 2, 3, and 4 had excellent SVR rates, suggesting that this regimen may still be an option for such patients failing all-oral antiviral therapy, especially when using the newer DAA as part of a LADR strategy. Major strengths of this study were the extensive review of pre-treatment and post-treatment liver histology and the utility of a novel method of obtaining IL28B genotype data from donor gallbladder. Our analysis suggests that baseline fibrosis was not associated with improved SVR, although patients having more advanced fibrosis had decreased overall survival. This makes the case for treatment of recurrent HCV at all stages of fibrosis, but especially earlier after LT before fibrosis has progressed and appreciable renal dysfunction (often with accompanying anemia) have developed. Healthcare reimbursement systems in some countries may limit the availability of DAAs. Until interferon-free HCV regimens are more extensively studied in liver recipients and become more affordable

and widely available, PEG α-2a and RBV will continue to have a role in the armamentarium used to treat recurrent HCV, especially in resource-limited regions around the world.

COMMENTS

Background

Post-LT treatment of recurrent hepatitis C virus (HCV) with PEG-based regimens is challenging, labor intensive, and carries sub-optimal SVR rates, and poor patient tolerability. Emerging data using interferon-free DAA regimens have shown improved response rates and tolerability. However, interferon-containing regimens may still be needed in post-LT patients when access to DAAs is limited due to financial and resource constraints.

Research frontiers

The low-accelerating-dose regimen has proven to be an effective therapeutic strategy in treatment of HCV in cirrhotic patients. The utility of low-accelerating-dose regimen in the largest post-LT cohort in a single center is described herein.

Innovations and breakthroughs

Comprehensive review of pre-, on- and post-treatment liver histology was undertaken and provides insights in treatment-related changes in liver histology. Novel techniques were employed to ascertain recipient and donor IL28B genotype using archival FFPE tissue from explanted native livers and donor gallbladders.

Applications

The study results suggest that the low-accelerating-dose regimen can be successfully employed as a treatment strategy for liver transplant recipients with recurrent HCV.

Terminology

The low-accelerating-dose regimen involves commencing patients on 90 mcg PEG α-2a weekly and RBV 7 mg/kg daily in 2 divided doses (50% of optimal dose). Doses were increased at week 4 to PEG α-2a 135 mcg weekly and RBV 10 mg/kg daily thereafter doses were PEG α-2a 180 mcg weekly and RBV 14 mg/kg daily from week 8 onwards if tolerated, for a total of 48 wk regardless of HCV genotype.

Peer-review

This retrospective study highlights the utility of the low-accelerating-dose regimen of pegylated alpha-interferon α-2-a and RBV in patients with recurrent HCV infection following liver transplantation. This is the largest reported single-center experience to date. This is an important paper highlighting that interferon-based regimens remain relevant in the era of DAA regimens where access to these costly drugs is limited.

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ORIGINAL ARTICLE

Retrospective Study

Laboratory test variables useful for distinguishing upper from lower gastrointestinal bleeding

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Abstract

AIM: To distinguish upper from lower gastrointestinal (GI) bleeding.

METHODS: Patient records between April 2011 and March 2014 were analyzed retrospectively (3296 upper endoscopy, and 1520 colonoscopy). Seventy-six patients had upper GI bleeding (Upper group) and 65 had lower GI bleeding (Lower group). Variables were compared between the groups using one-way analysis of variance. Logistic regression was performed to identify variables significantly associated with the diagnosis of upper *vs* lower GI bleeding. Receiver-operator characteristic (ROC) analysis was performed to determine the threshold value that could distinguish upper from lower GI bleeding.

RESULTS: Hemoglobin (P = 0.023), total protein (P = 0.0002), and lactate dehydrogenase (P = 0.009) were significantly lower in the Upper group than in the Lower group. Blood urea nitrogen (BUN) was higher in the Upper group than in the Lower group (P = 0.0065). Logistic regression analysis revealed that BUN was most strongly associated with the diagnosis of upper *vs*

lower GI bleeding. ROC analysis revealed a threshold BUN value of 21.0 mg/dL, with a specificity of 93.0%.

CONCLUSION: The threshold BUN value for distinguishing upper from lower GI bleeding was 21.0 mg/dL.

Key words: Logistic regression analysis; Likelihood analysis; Receiver-operator characteristic analysis; Blood urine nitrogen; Hemoglobin

Core tip: Differentiation of upper *vs* lower gastrointestinal (GI) bleeding is crucial. Laboratory test variables were investigated for their ability to distinguish upper from lower GI bleeding from retrospective analysis. Total protein, hemoglobin, and lactate dehydrogenase were lower and blood urea nitrogen (BUN) was higher in patients with upper GI bleeding. The threshold BUN value for distinguishing upper from lower GI bleeding was 21.0 mg/dL, with a specificity of 93.0%.

Tomizawa M, Shinozaki F, Hasegawa R, Shirai Y, Motoyoshi Y, Sugiyama T, Yamamoto S, Ishige N. Laboratory test variables useful for distinguishing upper from lower gastrointestinal bleeding. *World J Gastroenterol* 2015; 21(20): 6246-6251 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6246.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6246

INTRODUCTION

Upper gastrointestinal (GI) bleeding is defined as bleeding that occurs proximal to the Treitz ligament, and lower GI bleeding occurs distal to the Treitz ligament. Causes of upper GI bleeding include gastric ulcers, duodenal ulcers, and gastric cancer[1]. Causes of lower GI bleeding include diverticula, angiodysplasia, and colorectal cancer[2]. The mortality rate of upper GI bleeding ranges from 3.5% to 7.4%[3,4] and that of lower GI bleeding is 1.9%[5,6]. Lower GI bleeding is less severe than upper GI bleeding[7]. Upper GI bleeding is diagnosed and treated with endoscopy, using methods such as clipping and bipolar electrocoagulation[8]. When patients do not respond to these therapies, arteriography with embolization is performed[9,10]. Lower GI bleeding ceases spontaneously[7], although 8%-37% of patients with lower GI bleeding are treated endoscopically with methods such as bipolar electrocoagulation and argon-plasma coagulation[11].

The mortality rate is 40% for patients with GI bleeding who are hemodynamically unstable[12]. An accurate diagnosis of upper or lower GI bleeding is important because early endoscopy significantly reduces mortality[13]. When patients present with hematemesis, the diagnosis of upper GI bleeding is readily apparent. Patients presenting with melena (tarry stools) likely have upper GI bleeding, while hematochezia suggests lower GI bleeding[14]. When patients do not display hematemesis, melena, or hematochezia, it is difficult to diagnose upper *vs* lower GI bleeding.

Blood testing is recommended before upper GI endoscopy or colonoscopy is performed because of its low cost and few complications[15]. Therefore, we sought to identify blood test parameters that could be useful in predicting upper *vs* lower GI bleeding.

MATERIALS AND METHODS

Patients

Patient records between April 2011 and March 2014 were analyzed retrospectively. During this time, 3296 patients underwent upper endoscopy and 1520 underwent colonoscopy. The group with upper GI bleeding (Upper group) comprised 50 male (69.2 ± 13.2 years) and 26 female (72.3 ± 10.2 years) patients. The group with lower GI bleeding (Lower group) comprised 35 male (69.4 ± 12.7 years) and 30 female (73.9 ± 10.4 years) patients. The diseases that caused upper GI bleeding are listed in Table 1. Bleeding from a gastric or duodenal ulcer was restricted to a spurting vessel (1a), an oozing vessel (1b), a visible vessel (2a), or a clot (2b), according to the Forrest classification system[16]. Diseases that caused lower GI bleeding are listed in Table 2. Laboratory test parameters were the focus of the present study. Clinical comments are not presented. Our study was reviewed and approved by the National Hospital Organization Shimoshizu Hospital Ethics Committee and was not designated as a clinical trial because it was performed as part of routine clinical practice. Patient anonymity was maintained. All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Upper GI endoscopy and colonoscopy

Patients received upper GI endoscopy for screening, examination of abdominal symptoms, or anemia. The endoscopic devices used were the GIF-N260H, GIF-XP260NS, GIF-PG260, GIF-XQ260, and GIF-Q260 (Olympus, Tokyo, Japan). Colonoscopy was performed in patients with abdominal symptoms, anemia, or a positive fecal occult blood test, or for screening purposes. The devices used were the CF-Q260 and PCF-Q260AI (Olympus).

Blood test variables

The blood test variables analyzed were white blood cell (WBC) count, hemoglobin, C-reactive protein (CRP), platelet count, total protein (TP), albumin, total bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, γ-glutamyl transpeptidase, lactate dehydrogenase (LDH), uric acid, blood urea

Table 1 Diseases that caused upper gastrointestinal bleeding

Diseases	No. of patients
Gastric ulcer	31
Gastric cancer	28
Duodenal ulcer	7
Acute gastric mucosal lesion	4
Esophageal ulcer	2
Esophageal varix	2
Esophagitis	1
Gastric invasion of pancreatic cancer	1
Total	76

Table 2 Diseases that caused lower gastrointestinal bleeding

Diseases	No. of patients
Colorectal cancer	47
Ulcerative colitis	6
Colitis	4
Ulcer	4
Diverticulum	1
Hemorrhoid	1
Proctitis	1
Unknown	1
Total	65

nitrogen (BUN), creatinine, total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, blood glucose, hemoglobin A1c, body mass index, carcinoembryonic antigen, and carbohydrate antigen 19-9.

Statistical analysis

One-way analysis of variance (ANOVA) was performed to reveal differences in the variables between the Upper and Lower groups. Fisher's exact test was used to compare the percentage of patients with cancer in the Upper and Lower groups. Logistic regression analysis was performed to reveal variables that were significantly associated with the diagnosis of upper *vs* lower GI bleeding. Receiver-operator characteristic (ROC) analysis was applied to determine the threshold value that can differentiate between upper and lower GI bleeding. $P < 0.05$ was used to indicate statistical significance. JMP version 10.0.2 (SAS Institute, Cary, NC, United States) was used for statistical analyses. The statistical methods of this study were reviewed by Yasufumi Motoyoshi from National Hospital Organization Shimoshizu Hospital.

RESULTS

To reveal differences in variables between the Upper and Lower groups, ANOVA was performed (Table 3). Hemoglobin ($P = 0.023$), TP ($P = 0.0002$), and LDH ($P = 0.009$) were significantly lower in the Upper group than in the Lower group. BUN was significantly higher in the Upper group than in the Lower group ($P = 0.0065$). Thus, hemoglobin, TP, LDH, and BUN were useful in the diagnosis of upper *vs* lower GI bleeding.

Table 3 Comparison of variables between patients with upper and lower gastrointestinal bleeding

Characteristics	Upper group	Lower group	*P* value
Age (yr)	70.4 ± 12.3	71.5 ± 11.9	0.6016
WBC count ($10^2/\mu L$)	8.792 ± 11.751	7.033 ± 3.438	0.2773
Hb (g/dL)	10.1 ± 3.2	11.3 ± 2.6	0.0230
CRP (mg/dL)	1.4 ± 1.5	2.0 ± 4.0	0.3220
Plt ($10^4/\mu L$)	27.4 ± 11.2	26.5 ± 11.1	0.6633
TP (g/dL)	6.0 ± 0.85	6.7 ± 0.8	0.0002
Alb (g/dL)	3.4 ± 0.6	3.5 ± 0.9	0.5958
T-Bil (mg/dL)	0.69 ± 0.57	0.70 ± 0.38	0.9236
ALP (IU/L)	236 ± 138	306 ± 218	0.2074
AST (IU/L)	25.3 ± 20.7	22.3 ± 10.0	0.3515
ALT (IU/L)	20.2 ± 18.2	16.9 ± 10.9	0.2422
γ-GTP (IU/L)	56.3 ± 88.1	32.1 ± 20.8	0.319
LDH (IU/L)	189.2 ± 51.7	243.7 ± 114.3	0.009
UA (mg/dL)	5.3 ± 2.0	5.8 ± 1.5	0.3541
BUN (mg/dL)	21.1 ± 15.8	14.1 ± 5.5	0.0065
Cre (mg/dL)	0.93 ± 0.35	0.84 ± 0.25	0.1153
T-Chol (mg/dL)	158.4 ± 45.8	175.4 ± 41.9	0.1966
TG (mg/dL)	100.7 ± 49.6	127.0 ± 79.7	0.4095
HDL (mg/dL)	45.4 ± 10.0	55.7 ± 18.2	0.1404
LDL (mg/dL)	90.6 ± 32.2	116.9 ± 40.7	0.1094
BS (mg/dL)	132.8 ± 57.5	117 ± 26.9	0.1979
HbA1c (%)	5.8 ± 0.8	6.6 ± 0.7	0.0379
BMI	21.6 ± 4.2	21.4 ± 3.4	0.9126
CEA (ng/mL)	13.6 ± 25.9	217.5 ± 753.5	0.2884
CA19-9 (U/mL)	784 ± 3.017	159.9 ± 450.5	0.3429

Plt: Platelet; TP: Total protein; Alb: Albumin; T-Bil: Total bilirubin; ALP: Alkaline phosphatase; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; BG: Blood glucose; BMI: Body mass index; BUN: Blood urea nitrogen; CA19-9: Carbohydrate antigen 19-9; CEA: Carcinoembryonic antigen; Cre: Creatinine; CRP: C-reactive protein; γ-GTP: γ-glutamyl transpeptidase; Hb: Hemoglobin; HbA1c: Hemoglobin A1c; HDL: High-density lipoprotein cholesterol; LDH: Lactate dehydrogenase; LDL: Low-density lipoprotein cholesterol; T-Chol: Total cholesterol; TG: Triglyceride; UA: Uric acid; WBC: White blood cell count.

Consequently, further analyses focused on these four variables.

To clarify the strength of the association between the difference in the blood test parameters and diagnosis of upper *vs* lower GI bleeding, logistic regression analysis was performed (Table 4). BUN had the largest χ^2 value and the smallest *P* value, suggesting that BUN was the variable most strongly associated with the diagnosis of upper *vs* lower GI bleeding.

A likelihood analysis was performed to confirm that BUN had the strongest association with the differentiation between upper and lower GI bleeding (Table 5). BUN was the only variable that had $P < 0.05$. These data suggest that BUN was the most useful parameter to distinguish upper from lower GI bleeding.

Threshold values are useful to diagnose upper *vs* lower GI bleeding using blood test parameters. Therefore, ROC analysis was performed to determine the threshold values (Figure 1). The area under the ROC curve (AUC) seemed relatively large for TP and LDH.

The AUC, threshold value, and sensitivity and specificity at the threshold value were calculated and are displayed in Table 6. The sensitivity of each

Table 4 Results of logistic regression analysis

	χ^2	Odds	Odds (95%CI)	*P* value
Hb	0.04	1.045994	0.669624-1.717491	0.8446
TP	1.03	2.221154	0.514904-13.65946	0.3100
LDH	1.05	1.015110	0.987842-1.049002	0.3057
BUN	2.38	0.879782	0.697744-0.980732	0.1232

Hb: Hemoglobin; TP: Total protein; LDH: Lactate dehydrogenase; BUN: Blood urea nitrogen.

Table 5 Results of the likelihood ratio test

	Likelihood χ^2	*P* value
Hb	0.03883460	0.8438
TP	1.12378238	0.2891
LDH	1.13905685	0.2859
BUN	6.62036996	0.0101

Hb: Hemoglobin; TP: Total protein; LDH: Lactate dehydrogenase; BUN: Blood urea nitrogen.

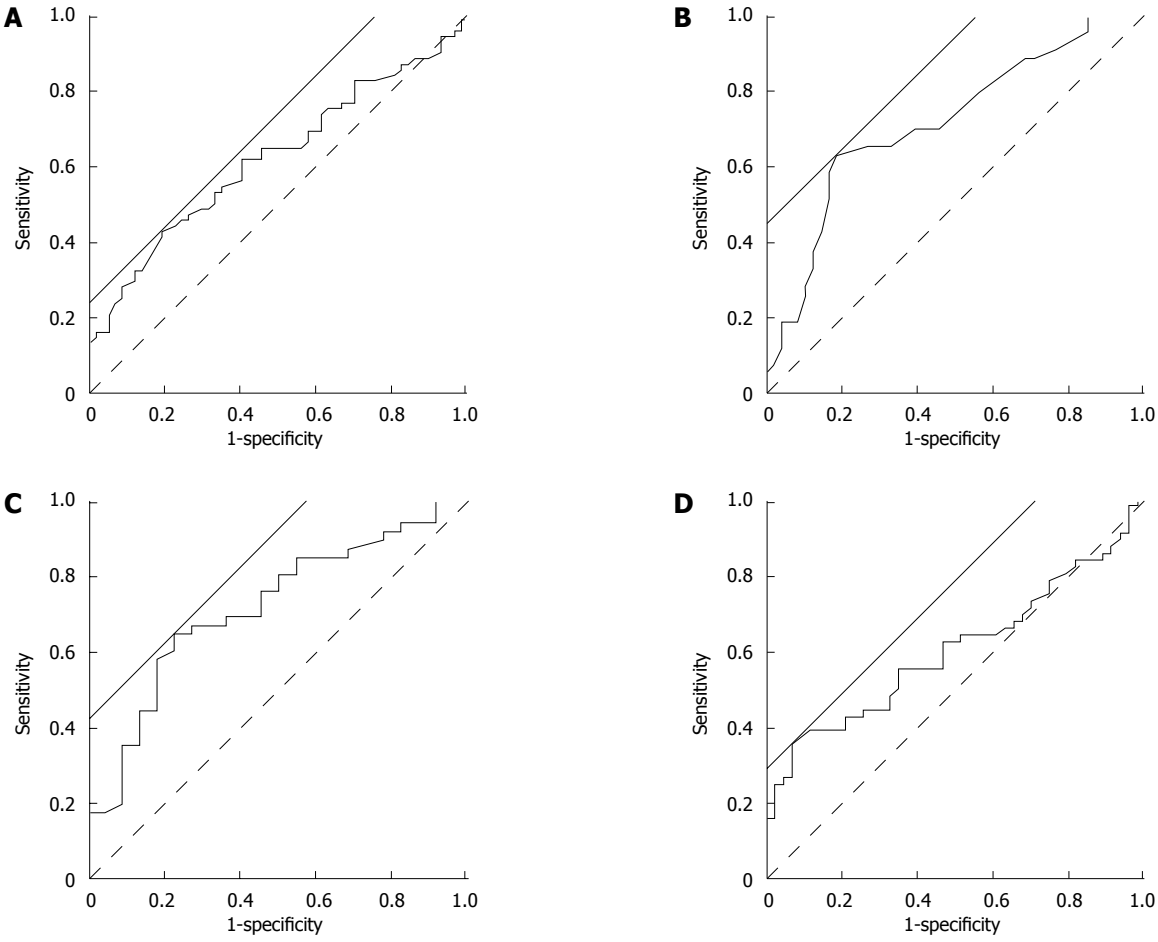

Figure 1 Receiver-operator characteristic analysis. Receiver-operator characteristic analysis was performed to determine the threshold value of hemoglobin (A), total protein (B), lactate dehydrogenase (C), and blood urea nitrogen (D) that could differentiate between upper and lower gastrointestinal bleeding. Solid straight line: a line with a slope of 45° used to calculate the threshold by JMP10.0.2 software, dashed line: reference line.

variable was relatively low. The specificity of BUN was 93.0% at the threshold value of 21.0 mg/dL.

Logistic regression and likelihood analyses revealed that BUN had the strongest association with the diagnosis of upper *vs* lower GI bleeding. Thus, we conclude that BUN was the most useful variable for diagnosing upper *vs* lower GI bleeding, and the threshold value was 21.0 mg/dL, with a specificity of 93.0%.

DISCUSSION

BUN increases after ingestion of a large amount of protein or blood[17]. Thus, it is reasonable to expect that BUN increases following massive upper GI bleeding. The ratio of BUN to creatinine has been used to predict upper GI bleeding. A BUN/creatinine ratio > 30 and hemoglobin level < 8.0 g/dL indicate severe upper GI bleeding[18]. A BUN/creatinine ratio > 36 distinguishes upper from lower GI bleeding[19]. Al-Naamani *et al*[20] reported that BUN alone predicts the severity of upper GI bleeding. All the above-mentioned reports focus on upper GI bleeding. There are no reports on using BUN alone to differentiate between upper and lower GI bleeding. In our study, BUN was able to distinguish upper from lower GI bleeding. This may be explained by blood from upper GI bleeding being digested in the intestine, thereby increasing BUN, with blood in the

Table 6 Results of receiver-operator characteristic analysis

	AUC	Threshold value	Sensitivity	Specificity
Hb	0.61894	8.7 (g/dL)	42.7%	80.7%
TP	0.71197	6.3 (g/dL)	62.8%	81.3%
LDH	0.71515	191 (IU/L)	64.4%	77.32%
BUN	0.61459	21 (mg/dL)	36.4%	93.0%

Hb: Hemoglobin; TP: Total protein; LDH: Lactate dehydrogenase; BUN: Blood urea nitrogen.

colon or rectum due to lower GI bleeding not being digested[17].

In our study, the threshold value of BUN that distinguished upper from lower GI bleeding was 21.0 mg/dL. The sensitivity of the value was low. It is speculated that patients with BUN < 21.0 mg/dL were those with less severe upper GI bleeding or lower GI bleeding. Patients with severe upper GI bleeding had BUN > 21.0 mg/dL. BUN did not increase in patients with lower GI bleeding. Therefore, BUN > 21.0 mg/dL might be specific to patients with upper GI bleeding. When BUN is > 21.0 mg/dL, a clinician would predict upper GI bleeding with sensitivity of 36.4% and specificity of 93.0%. One limitation of the present study was that differentiation would be hard between upper and lower GI bleeding when BUN was < 21.0 mg/dL.

The present study found that hemoglobin, TP, and LDH were lower in patients with upper GI bleeding. Upper GI bleeding is more severe than lower GI bleeding[7]. Hemoglobin clearly decreases in patients with upper GI bleeding[21]. These facts indicate that lower hemoglobin indicates hemodynamic instability. It is reasonable to expect therefore that hemoglobin would be lower in such patients. The reasons for TP and LDH being lower in patients with upper GI bleeding are not clear.

The present study mainly consisted of nonvariceal bleeding, although it included two patients with variceal bleeding. Upper GI bleeding is mainly seen in patients with nonvariceal bleeding[22]. It is recommended that variceal or nonvariceal bleeding be considered regarding management of upper GI bleeding because management of variceal or nonvariceal bleeding is different[8,23].

In conclusion, TP, hemoglobin and LDH were lower, and BUN was higher in patients with upper GI bleeding. The threshold BUN value to distinguish upper from lower GI bleeding was 21.0 mg/dL.

COMMENTS

Background

Upper gastrointestinal (GI) bleeding is defined as bleeding that occurs proximal to the Treitz ligament, and lower GI bleeding occurs distal to the Treitz ligament. The mortality rate is 40% for patients with GI bleeding who are hemodynamically unstable. An accurate diagnosis of upper or lower GI bleeding is important because early endoscopy significantly reduces mortality.

Research frontiers

When patients present with hematemesis, the diagnosis of upper GI bleeding is readily apparent. Patients presenting with melena (tarry stools) likely have upper GI bleeding, while hematochezia suggests lower GI bleeding. When patients do not display hematemesis, melena, or hematochezia, it is difficult to diagnose upper *vs* lower GI bleeding.

Innovations and breakthroughs

Laboratory test variables were investigated for their ability to distinguish upper from lower GI bleeding. Hemoglobin (P = 0.023), total protein (P = 0.0002), and lactate dehydrogenase (P = 0.009) were significantly lower in patients with upper GI bleeding than those with lower. Blood urea nitrogen (BUN) was higher in patients with upper GI bleeding than those with in lower GI bleeding (P = 0.0065). Logistic regression analysis revealed that BUN was most strongly associated with the diagnosis of upper *vs* lower GI bleeding. Receiver-operator characteristics analysis revealed a threshold BUN value of 21.0 mg/dL, with a specificity of 93.0%.

Applications

The threshold BUN value for distinguishing upper from lower GI bleeding was 21.0 mg/dL, with a sensitivity of 36.4% and a specificity of 93.0%.

Peer-review

It was a good idea to differentiate upper or lower GI bleeding with laboratory test variables. It seemed difficult to differentiate upper or lower GI bleeding when BUN < 21.0 mg/dL. Hb was lower in upper GI than lower. The lower Hb indicated severe bleeding, not the site of bleeding. Upper GI bleeding is mainly consisted of non-variceal bleeding. It would be recommended that variceal or non-varicela be considered regarding management of upper GI bleeding because management of variceal or non-variceal bleeding is different. Sensitivity of BUN was low to differentiate upper and lower GI bleeding.

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ORIGINAL ARTICLE

Retrospective Study

Differential diagnosis of benign and malignant branch duct intraductal papillary mucinous neoplasm using contrast-enhanced endoscopic ultrasonography

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Author contributions: Harima H drafted the manuscript; Kaino S designed the study and helped draft the manuscript; Shinoda S, Kawano M and Suenaga S acquired and analyzed the data; and Sakaida I approved the final manuscript.

Ethics approval: This study was reviewed and approved by the Institutional Review Board of Yamaguchi University Graduate School of Medicine.

Informed consent: In this retrospective study, written informed consent was not provided by the participants, but the documents that explain how the data included in this study would be used were published on the internet.

Conflict-of-interest: None of the authors have been funded by any foundation or other nongovernmental source. There are no financial disclosures from any authors.

Data sharing: No additional data are available.

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Abstract

AIM: To elucidate the role of contrast-enhanced endoscopic ultrasonography (CE-EUS) in the diagnosis of branch duct intraductal papillary mucinous neoplasm (BD-IPMN).

METHODS: A total of 50 patients diagnosed with BD-IPMN by computed tomography (CT) and endoscopic ultrasonography (EUS) at our institute were included in this study. CE-EUS was performed when mural lesions were detected by EUS. The diagnostic accuracy for identifying mural nodules (MNs) was evaluated by CT, EUS, and EUS combined with CE-EUS. In the patients who underwent resection, the accuracy of measuring MN height with each imaging modality was compared. The cut-off values to diagnose malignant BD-IPMNs based on MN height for each imaging modality were determined using receiver operating characteristic curve analysis.

RESULTS: Fifteen patients were diagnosed with BD-IPMN with MNs and underwent resection. The remaining 35 patients were diagnosed with BD-IPMN without MNs and underwent follow-up monitoring. The pathological findings revealed 14 cases with MNs and one case without. The accuracy for diagnosing MNs was 92% using CT and 72% using EUS; the diagnostic accuracy increased to 98% when EUS and CE-EUS were combined. The accuracy for measuring MN height significantly improved when using CE-EUS compared with using CT or EUS (median measurement error value, CT: 3.3 mm *vs* CE-EUS: 0.6 mm, $P < 0.05$; EUS: 2.1 mm *vs* CE-EUS: 0.6 mm, $P < 0.01$). A cut-off value of 8.8 mm for MN height as measured by CE-EUS improved the accuracy of diagnosing malignant BD-IPMN to 93%.

CONCLUSION: Using CE-EUS to measure MN height

provides a highly accurate method for differentiating benign from malignant BD-IPMN.

Key words: Contrast-enhanced endoscopic ultrasonography; Endoscopic ultrasonography; Computed tomography; Branch duct intraductal papillary mucinous neoplasm; Mural nodules

Core tip: Both the presence and the height of mural nodules (MNs) are important for differentiating benign from malignant branch duct intraductal papillary mucinous neoplasm (BD-IPMN). However, no studies have determined the ability of contrast-enhanced endoscopic ultrasonography (CE-EUS) to accurately measure MN height. In this study, we demonstrated that CE-EUS is the optimal imaging modality for measuring MN height. Using CE-EUS to measure MN height improved the accuracy of the differential diagnosis of benign *vs* malignant BD-IPMN, therefore enabling patients to avoid unnecessary surgery.

Harima H, Kaino S, Shinoda S, Kawano M, Suenaga S, Sakaida I. Differential diagnosis of benign and malignant branch duct intraductal papillary mucinous neoplasm using contrast-enhanced endoscopic ultrasonography. *World J Gastroenterol* 2015; 21(20): 6252-6260 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6252.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6252

INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) is defined as an intraductal, grossly visible (typically ⩾ 1.0 cm) epithelial neoplasm of mucin-producing cells that arises in the main pancreatic duct (MPD) or its branches. The neoplastic epithelium is usually papillary, and the degrees of mucin secretion, duct dilation (cyst formation), and dysplasia are variable[1]. IPMN can be subdivided into main duct IPMN (MD-IPMN) and branch duct IPMN (BD-IPMN) depending on the location of the primary lesion[2]. Most BD-IPMNs are less invasive and can be monitored; thus, the differential diagnosis of benign and malignant BD-IPMN must be accurate to appropriately indicate surgical resection[3-6]. In 2006, an international panel of experts published the International Consensus Guidelines for the Management of IPMN (ICG2006)[7]. These guidelines were updated in 2012 (ICG2012)[8]. According to the most recent guidelines, all BD-IPMNs diagnosed with mural nodules (MNs) by computed tomography (CT), magnetic resonance imaging (MRI) or endoscopic ultrasound (EUS) are recommended for resection. However, certain studies have indicated that both the presence of MNs and their height may be risk factors for malignancy[9-13]. If MN height is included in the factors used to determine resection, superior criteria for BD-IPMN resection might be created. Therefore, in pre-surgical examinations of BD-IPMN, the identification of MNs as well as accurate measurements of MN height is important.

Recently, many reports have noted the effectiveness of contrast-enhanced EUS (CE-EUS) in the diagnosis of pancreatic tumors[14-17]. With regard to BD-IPMN, it has been reported that CE-EUS is effective at differentiating MNs from mucinous clots[18,19]. Furthermore, CE-EUS is effective at identifying the mucosal fluid attached to MNs, thus enabling more accurate measurements of MN height than when using EUS alone. However, to the best of our knowledge, no study has determined the ability of CE-EUS to accurately measure MN height.

Therefore, in this study, the utility of CE-EUS to accurately evaluate the presence and height of MNs was determined. The purpose of this study was to elucidate the role of CE-EUS in the differential diagnosis of benign and malignant BD-IPMN.

MATERIALS AND METHODS

Study design

This study was approved by the Institutional Review Board of Yamaguchi University Graduate School of Medicine. The clinical records, EUS images, radiologic data, pathology, and surgical reports in this study were all reviewed retrospectively.

Patients

A total of 50 patients diagnosed with BD-IPMN by CT and EUS at our institute between April 2009 and March 2014 were included in this study. Of these, 15 patients diagnosed with MNs underwent resection, and 35 patients diagnosed without MNs were monitored during follow-up. However, two of the 35 patients underwent resection after becoming symptomatic or presenting with an increased MPD diameter despite being diagnosed without MNs. The remaining 33 patients diagnosed without MNs were followed up without intervention.

Definition

According to ICG2012, IPMN can be classified into three types: MD-IPMN, BD-IPMN, and mixed type[8]. According to the ICG2012 criteria, most cases of IPMN are classified as mixed type. Therefore, we defined all cases of IPMN, including mixed type, as BD-IPMN if branch duct dilation was the primary symptom. The pathological results were determined based on the World Health Organization classification system published in 2010[1]. In this study, noninvasive IPMN was considered benign, and only IPMN associated with an invasive carcinoma (IC) was defined as malignant.

Imaging procedure

EUS was performed using an electric radial-type endoscope (GF-UE260-AL5; Olympus, Tokyo, Japan)

and an ultrasound system (ProSound SSD α-10; Aloka, Tokyo, Japan). When mural lesions were detected by EUS, a contrast-enhanced evaluation was conducted. To perform CE-EUS, we used Sonazoid (Daiichi Sankyo, Tokyo, Japan), which is a second-generation ultrasonographic contrast agent composed of perfluorobutane microbubbles with a median diameter of 2-3 μm. After reconstitution with 2 mL of sterile water for injection, 0.5 mL of the agent was administered through a peripheral vein. Each mural lesion was observed for two minutes, during which time the presence or absence of vascularity in the mural lesions was evaluated. Mural lesions that demonstrated vascularity were diagnosed as MNs, and mural lesions without detectable vasculature were diagnosed as mucinous clots. The evaluations were conducted by four or five on-site physicians specializing in biliopancreatic diseases.

Contrast-enhanced CT imaging was performed using a 64-section multidetector CT scanner (Definition and Somatom Sensation 64; Siemens Medical Solutions, Forchheim, Germany). Solid tumors demonstrating contrast effects within the cyst were diagnosed as having MNs. The evaluations were conducted by two or three radiologists specializing in digestive organs.

Variables

(1) In all 50 patients, the ability to diagnose the presence of MNs using each imaging modality (CT, EUS alone, EUS combined with CE-EUS) was calculated. The presence or absence of MNs was ultimately determined based on pathological findings in the 17 patients who underwent a resection. The 33 patients who were followed up without intervention were deemed to have no MNs if obvious malignant findings were not detected during the follow-up periods; (2) in the 15 patients who underwent resection due to being diagnosed with BD-IPMN with MNs, the accuracy of measuring MN height with each imaging modality was compared. In this study, the MN heights measured using CT, EUS, CE-EUS or pathological specimens were expressed as H_{CT}, H_{EUS}, $H_{CE\text{-}EUS}$ or H_{Path}, respectively. H_{CT}, H_{EUS} and $H_{CE\text{-}EUS}$ were compared with H_{Path}, and the absolute differences were calculated ($|H_{CT}-H_{Path}|$, $|H_{EUS}-H_{Path}|$ and $|H_{CE\text{-}EUS}-H_{Path}|$). These numerical values were defined as the measurement error value and were compared; and (3) in the 15 patients who underwent resection after being diagnosed with BD-IPMN with MNs, the cut-off value for MN height as measured using each imaging modality or pathological specimens was established to differentiate between benign and malignant BD-IPMN.

Statistical analysis

Spearman's correlation coefficient was used to identify correlations between MN height as measured by each imaging modality and MN height as measured on pathological specimens. The Wilcoxon *t* test with the Bonferroni correction was used to compare the measurement accuracy of MN height for each imaging modality. A receiver operating characteristic (ROC) curve analysis was used to determine the optimal cut-off value for MN height measured using each imaging modality or pathological specimens to differentiate between benign and malignant BD-IPMN. JMP 9 statistical software (SAS Institute Inc., Cary, North Carolina, United States) was used for the analysis. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

The mean age of the patients was 67.7 ± 9.8 years. The cohort included 29 males and 21 females. The mean cyst diameter was 27.9 ± 10.9 mm, and the mean MPD diameter was 4.6 ± 3.3 mm.

Ability to diagnose the presence of MNs

The flow chart presented in Figure 1 illustrates the clinical course of all the BD-IPMN patients. Of a total of 50 patients, mural lesions were detected by EUS in 28, and all of these patients subsequently underwent CE-EUS. Of these 28 patients, 15 were diagnosed with MNs by CE-EUS; these tumors were then surgically resected. Among these 15 patients, 14 cases were pathologically confirmed as MNs. Using CT, 10 out of the same 50 patients were determined to have MNs; these MNs were all pathologically confirmed. However, CT did not detect the remaining four cases of MNs that were diagnosed by CE-EUS.

Thirteen patients were diagnosed with mucinous clots by CE-EUS, and twenty-two patients were diagnosed as having no mural lesions by EUS. All 35 of these cases were monitored. The clinical features of these cases are presented in Table 1. MNs were not detected during the follow-up period using various imaging modalities. Of these, however, two patients underwent resection due to repeated pancreatitis or an increased MPD diameter to more than 3 mm. MNs could not be confirmed pathologically in either case. The remaining 33 cases were observed for more than 12 mo without obvious malignant findings.

The sensitivity of CT for diagnosing MNs was 71%; the sensitivity of EUS alone for diagnosing MNs was 100%, although the specificity and positive predictive value (PPV) for diagnosing MNs were 61% and 50%, respectively. When EUS was combined with CE-EUS, the specificity and PPV for diagnosing MNs increased to 97% and 93%, respectively. The accuracy of CT, EUS alone and EUS combined with CE-EUS for diagnosing MNs was 92%, 72% and 98%, respectively (Table 2).

Measurement accuracy for MN height

Table 3 shows the clinicopathologic features of the 15 patients who underwent resection after being

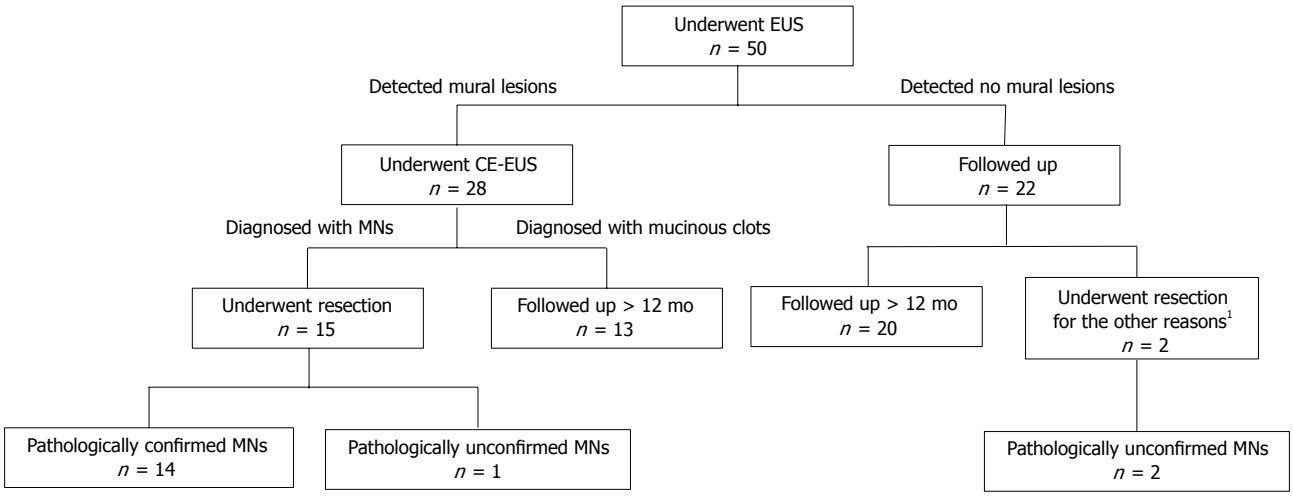

Figure 1 Chart of the clinical course of all the branch duct intraductal papillary mucinous neoplasm patients. [1]These two patients underwent resection after follow-up due to repeated pancreatitis or an increasing main pancreatic duct diameter. BD-IPMN: Branch duct intraductal papillary mucinous neoplasm; MNs: Mural nodules; EUS: Endoscopic ultrasonography; CE-EUS: Contrast-enhanced EUS.

Table 1 Clinical features of the 35 patients who were monitored

	Follow-up cases (n = 35)
Sex, M/F	18/17
Mean age ± SD, yr	67.9 ± 10.2
Mean follow-up period ± SD, mo	27.4 ± 16.7
Cyst size	
Initial examination ± SD, mm	27.0 ± 11.8
Last examination ± SD, mm	30.1 ± 13.1
Changes of the cyst size	
No change	29
Enlarged (≥ 10 mm)	5
Reduced (≥ 10 mm)	1
MPD diameter	
Initial examination ± SD, mm	3.2 ± 1.8
Last examination ± SD, mm	3.5 ± 2.2
Changes in MPD diameter	
No change	26
Enlarged (≥ 1 mm)	7
Reduced (≥ 1 mm)	2
Appearance of MNs during follow-up period	0
Followed up > 12 mo	33
Resected after follow-up	2
Pathological diagnosis	
Low-grade dysplasia	0
Intermediate-grade dysplasia	0
High-grade dysplasia	2
Invasive adenocarcinoma	0

MPD: Mail pancreatic duct; MNs: Mural nodules.

diagnosed with MNs. There were significant positive correlations between MN height as measured by each imaging modality and MN height as measured on pathological specimens (Figure 2). The calculated measurement error values for each imaging modality are presented in Figure 3. The measurement error values for CE-EUS were significantly lower than those for CT or EUS (median measurement error value, CT: 3.3 mm *vs* CE-EUS: 0.6 mm, $P < 0.05$; EUS: 2.1 mm *vs* CE-EUS: 0.6 mm, $P < 0.01$).

Differentiating benign and malignant BD-IPMN using CE-EUS

Of the 15 resected cases that were diagnosed with MNs, a pathological examination revealed that 10 cases were malignant BD-IPMN and five cases were benign BD-IPMN (Table 2). The ROC curve related to the diagnosis of malignant BD-IPMN based on MN height measured using CT, EUS, CE-EUS or pathological specimens yielded area under the curve values of 0.82, 0.87, 0.92 and 0.90, respectively (Table 4). Based on the ROC curve, 8.8 mm was determined to be the optimal threshold value for MN height measured by CE-EUS. With this cut-off value, the diagnosis of malignant BD-IPMN had a sensitivity, specificity, and accuracy of 100%, 86%, and 94%, respectively (Figure 4).

DISCUSSION

Although the frequency of IC in MD-IPMN is high (43.1%; 11%-81%), the frequency of IC in BD-IPMN is relatively low at only 17.7% (1.4%-36.7%). Deliberation is necessary before performing a resection for BD-IPMN because these lesions mostly occur in elderly patients, and the annual malignancy rate is only 2%-3%[8]. ICG2006 suggests that when encountering suspicious findings for malignant BD-IPMN, surgery is recommended in cases with (1) an MPD diameter of 6 mm or greater; (2) a cyst size of 30 mm or greater; or (3) the presence of MNs as determined by diagnostic imaging[7]. However, a recent meta-analysis demonstrated that rather than cyst size and MPD diameter, the presence of MNs is strongly indicative of malignant BD-IPMN[20]. In this study, all BD-IPMN cases in which MNs were pathologically confirmed were ultimately diagnosed with either high-grade dysplasia (HGD) or IC. Along with diagnostic imaging, the methods for diagnosing malignant BD-

Table 2 Ability to diagnose the presence of mural nodules with each imaging modality

	Sensitivity (95%CI)	Specificity (95%CI)	PPV (95%CI)	NPV (95%CI)	Accuracy (95%CI)
CT	71% (0.42-0.92)	100% (0.90-1.00)	100% (0.69-1.00)	90% (0.76-0.98)	92% (0.80-0.98)
EUS alone	100% (0.77-1.00)	61% (0.43-0.77)	50% (0.31-0.70)	100% (0.85-1.00)	72% (0.58-0.84)
EUS combined with CE-EUS	100% (0.76-1.00)	97% (0.85-1.00)	93% (0.66-1.00)	100% (0.90-1.00)	98% (0.89-1.00)

CT: Computed tomography; EUS: Endoscopic ultrasonography; CE-EUS: Contrast-enhanced EUS; PPV: Positive predictive value; NPV: Negative predictive value; CI: Confidence interval.

Table 3 Clinicopathologic features of the 15 patients who underwent a resection

Case	Cyst size (mm)	MPD diameter (mm)	MNs								Pathological diagnosis
			CT		EUS		CE-EUS		Pathology		
			Presence	H_{CT} (mm)	Presence	H_{EUS} (mm)	Presence	$H_{CE\text{-}EUS}$ (mm)	Presence	H_{Path} (mm)	
1	36	12	+	23.4	+	25.2	+	22.2	+	21.2	IC
2	30	12	+	21.3	+	20.1	+	19.2	+	19.8	IC
3	40	12	+	13.9	+	19.1	+	17.6	+	17.2	IC
4	20	8	+	22.1	+	23.6	+	21.5	+	17.2	IC
5	50	2	+	18.9	+	15.2	+	14.3	+	13.1	IC
6	20	6	+	13.1	+	11.5	+	10.4	+	9.8	IC
7	30	9	+	14.4	+	10.6	+	9.5	+	9.3	IC
8	18	8	-	0	+	10.1	+	8.8	+	9.2	IC
9	38	6	-	0	+	10.9	+	9.7	+	9.1	IC
10	27	3	+	5.0	+	12.5	+	10.1	+	7.6	IC
11	25	13	+	11.3	+	13.8	+	10.3	+	10.1	HGD
12	30	6	+	6.4	+	10.1	+	8.5	+	7.3	HGD
13	30	6	-	0	+	10.5	+	8.4	+	5.3	HGD
14	31	12	-	0	+	2.3	+	2.3	+	2.1	HGD
15	28	3	-	0	+	3.9	+	2.7	-	0	ImGD

MPD: Main pancreatic duct; MNs: Mural nodules; CT: Computed tomography; EUS: Endoscopic ultrasonography; CE-EUS: Contrast-enhanced EUS; IC: Invasive carcinoma; HGD: High-grade dysplasia; ImGD: Intermediate-grade dysplasia; H_{CT}: MN height measured by CT; H_{EUS}: MN height measured by EUS; $H_{CE\text{-}EUS}$: MN height measured by CE-EUS; H_{Path}: MN height measured on pathological specimens.

IPMN include pancreatic fluid cell examination and cyst fluid examination; however, both methods have disadvantages. Pancreatic fluid cell examination has the advantage of high specificity, but the sensitivity can vary widely from 11%-92%[21-23], leading to a high risk of false negatives. Although EUS-guided fine needle aspiration cytology and laboratory analysis of cyst fluid have provided excellent results in certain studies[24-27], the safety of this method remains unclear because other reports have indicated the possibility of peritoneal dissemination due to the leakage of the cyst contents[28,29]. Therefore, we believe that diagnosing malignant BD-IPMN by evaluating MNs is both accurate and safe.

CT has been reported as effective for diagnosing MNs in addition to providing the information on BD-IPMN morphology, specifically the location and the presence of any communication with the MPD[30,31]. Nakagawa *et al*[32] reported that using CT to detect MNs in BD-IPMN yielded a sensitivity, specificity and accuracy of 68%, 100%, and 77%, respectively. In our study, using CT to diagnose MNs in BD-IPMN yielded a sensitivity, specificity, and accuracy of 71%, 100%, and 92%, respectively, which were superior values to those reported by Nakagawa *et al*[32] Despite this, out of 14 pathologically confirmed BD-IPMN cases with MNs, CT failed to detect four cases (29%) of MNs during the pre-surgical examination. Furthermore, of these four cases, two were eventually diagnosed with IC based on pathological examination, thus indicating that there are limits to differentiating benign and malignant BD-IPMN using CT alone.

EUS is an invaluable modality for evaluating pancreatic diseases because of its high spatial resolution. Compared with CT and MRI, EUS is excellent at detecting small pancreatic lesions and is also useful for diagnosing IPMN[33]. Thus far, EUS has demonstrated a high level of sensitivity at diagnosing MNs in BD-IPMN in addition to producing very few false negatives[34,35]. In this study, no false negatives were obtained with EUS. The 22 BD-IPMN patients diagnosed without MNs using EUS at the initial examinations did not have detectable MNs during the entire follow-up period. However, because it is difficult to differentiate MNs from mucinous clots using EUS alone, the specificity of this methodology is low, and there is a high risk of false positives[32]. In this study, 13 of the 28 mural lesions (46%) detected by EUS alone were, in fact, mucinous clots. The accuracy of EUS alone (72%) was insufficient. Zhong *et al*[36] reported that ascertaining the features of MNs can improve the ability to

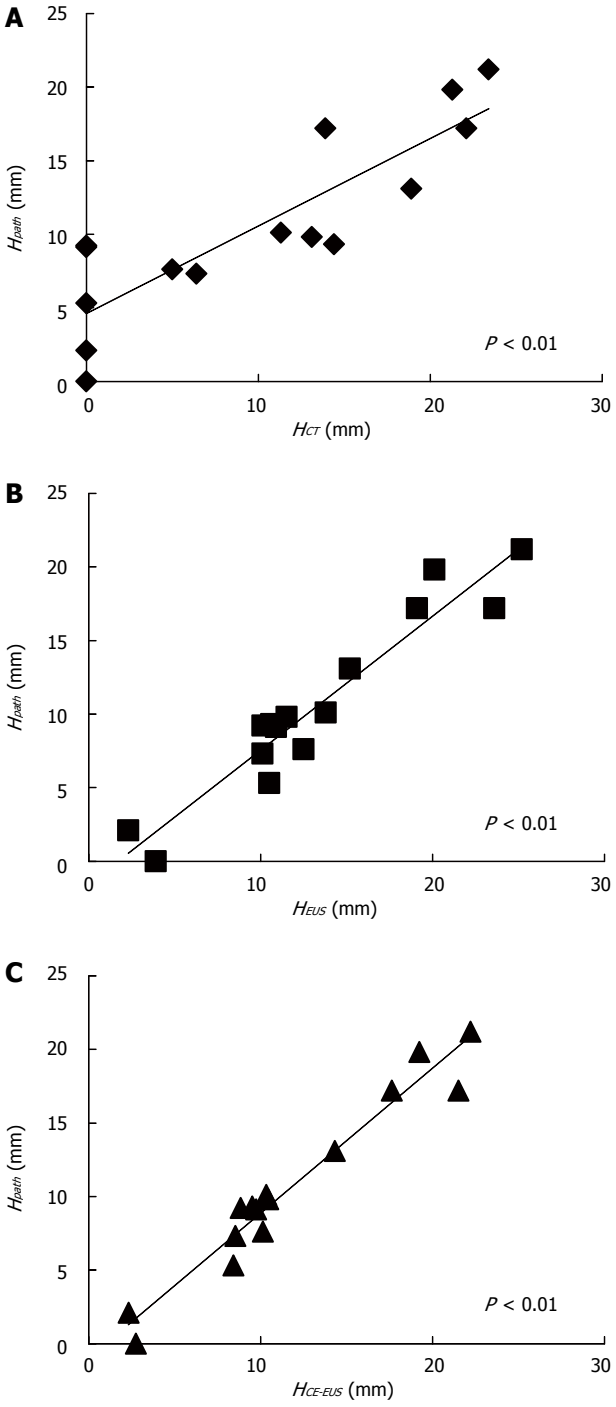

Figure 2 Correlations between mural nodules height measured by each imaging modality and mural nodules height measured on pathological specimens. A: A positive correlation was identified between MN height measured by CT and that measured on pathological specimens; B: A positive correlation was identified between MN height measured by EUS and that measured on pathological specimens; C: A positive correlation was identified between MN height measured by CE-EUS and that measured on pathological specimens. H_{CT}: MN height measured by CT; H_{EUS}: MN height measured by EUS; H_{CE-EUS}: MN height measured by CE-EUS; H_{Path}: MN height measured on pathological specimens; MN: Mural nodule; CT: Computed tomography; EUS: Endoscopic ultrasonography; CE-EUS: Contrast-enhanced EUS.

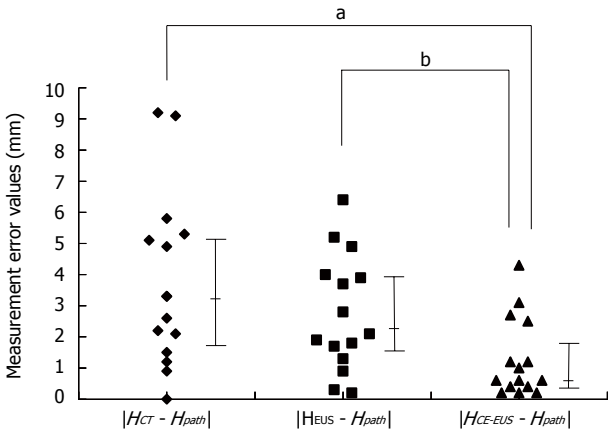

Figure 3 Comparison of measurement error values. The measurement error values for CE-EUS were significantly lower than those for CT or EUS. $|H_{CT}\text{-}H_{Path}|$, measurement error value for CT; $|H_{EUS}\text{-}H_{Path}|$, measurement error value for EUS; $|H_{CE-EUS}\text{-}H_{Path}|$, measurement error value for CE-EUS. Statistically significant, $^{a}P < 0.05$, $|H_{CT}\text{-}H_{Path}|$ *vs* $|H_{CE-EUS}\text{-}H_{Path}|$, statistically significant, $^{b}P < 0.01$, $|H_{EUS}\text{-}H_{Path}|$ *vs* $|H_{CE-EUS}\text{-}H_{Path}|$. CT: Computed tomography; EUS: Endoscopic ultrasonography; CE-EUS: Contrast-enhanced EUS.

differentiate mucinous clots from MNs; however, this only increases the accuracy to 79%, which is not ideal. Therefore, it is difficult to accurately diagnose the presence of MNs using EUS alone.

Originally, a contrast-enhanced imaging technique was not available for EUS because the transducer of the echoendoscope was too small to produce enough acoustic power to perform contrast-enhanced imaging using a first-generation ultrasound contrast agent. A recently developed second-generation contrast agent now allows for the production of harmonic signals, even at lower acoustic power, making CE-EUS available for clinical use[14]. To date, there have been relatively few reports of CE-EUS being used to diagnose pancreatic cystic tumors, and only a few reports exist on the utility of CE-EUS for diagnosing MNs in BD-IPMN[18,19]. In this study, we looked not only at cases in which MNs were diagnosed using CE-EUS and resected but also at cases in which mucinous clots were diagnosed using CE-EUS and followed up. The results indicated that 14 of the 15 cases (93%) diagnosed with MNs using CE-EUS were subsequently pathologically confirmed to have MNs. In addition, the 13 cases diagnosed with mucinous clots by CE-EUS showed no malignant findings during follow-up, and they presented no contradictions regarding the absence of MNs. The accuracy of diagnosing MNs in BD-IPMN increased from 72% to 98% when EUS was combined with CE-EUS. The accuracy of EUS combined with CE-EUS was better than that for CT or EUS alone. As a result, we believe that CE-EUS is the most appropriate method for detecting the presence of MNs in BD-IPMN.

Certain studies have indicated the possibility that MN height is a risk factor for malignant BD-IPMN[9-13]. Therefore, accurately detecting the presence of MNs and accurately measuring MN height may contribute to the differential diagnosis of benign and malignant BD-IPMN. To the best of our knowledge, no previous

Table 4 Optimum cut-off values for mural nodule height to differentiate between benign and malignant branch duct intraductal papillary mucinous neoplasm using mural nodule height as measured using each imaging modality or pathological specimens

	AUC	Cutoff value (mm)	Sensitivity (95%CI)	Specificity (95%CI)	Accuracy (95%CI)
CT	0.82	13.1	70 (0.35-0.93)	100 (0.48-1.00)	80 (0.51-0.96)
EUS	0.87	10.6	90 (0.53-1.00)	80 (0.27-1.00)	87 (0.58-0.99)
CE-EUS	0.92	8.8	100 (0.69-1.00)	80 (0.27-1.00)	93 (0.66-1.00)
Pathological specimens	0.90	7.6	100 (0.69-1.00)	80 (0.27-1.00)	93 (0.66-1.00)

CT: Computed tomography; EUS: Endoscopic ultrasonography; CE-EUS: Contrast-enhanced EUS; AUC: Area under the curve.

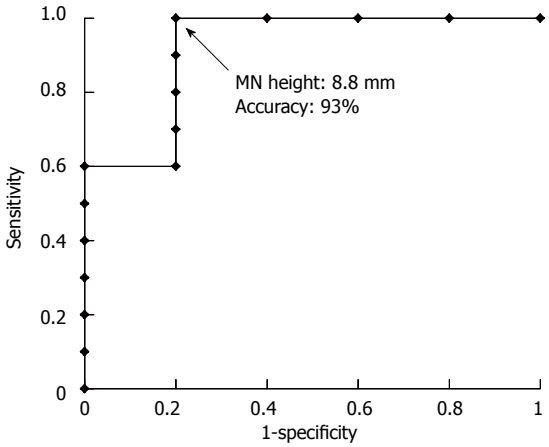

Figure 4 Receiver operating characteristic curve analysis. Based on the receiver operating characteristic curve analysis, when 8.8 mm was utilized as the cut-off value for MN height, as measured by CE-EUS, the diagnosis of malignant BD-IPMN had an accuracy of 94%. BD-IPMN: Branch duct intraductal papillary mucinous neoplasm; MN: Mural nodule; CE-EUS: Contrast-enhanced endoscopic ultrasonography.

studies including CE-EUS have determined the optimal imaging modality for measuring MN height. In this study, the measurement error values, calculated as the absolute values of the difference between the MN height measured by specific imaging modalities and that measured on pathological specimens, were compared. The results indicated that the measurement error values for CE-EUS were significantly lower than those for CT or EUS, supporting our conclusion that CE-EUS is the optimum imaging modality for measuring MN height in BD-IPMN. CE-EUS most likely both differentiates the mucosal fluid attached to MNs and clarifies the structure of the cystic wall, making it possible to accurately measure MN height. The threshold value of 8.8 mm, which was determined from a ROC curve related to the diagnosis of malignant BD-IPMN based on MN height as measured by CE-EUS, gave an excellent accuracy of 93%. CE-EUS accurately measures MN height and has potential applicability in differentiating benign and malignant BD-IPMN.

There were several limitations to our research. Firstly, because this study was a retrospective study with a limited cohort, there is the possibility of selection bias. Secondly, the study involved patients who underwent resection as well as those who were merely monitored. As a result, it was not possible to pathologically confirm whether MNs were actually present in each case. Despite these limitations, CE-EUS demonstrated effectiveness at diagnosing the presence of MNs and in accurately measuring MN height. Recent studies have already begun to adopt MN height to inform treatment policy in BD-IPMN cases[37]. According to our results, CE-EUS is the optimum modality for measuring the height of MNs. If indications of resection in BD-IPMN cases can be determined from both the presence of MNs and their height as measured by CE-EUS, many unnecessary surgeries can be avoided.

In conclusion, CE-EUS offers the potential to accurately diagnose the presence of MNs in BD-IPMN. According to our results, in cases of a diagnosis of BD-IPMN with MNs, it is highly probable that the pathological diagnosis will be HGD or IC, in which case resection is indicated; this is consistent with ICG2012. Furthermore, if the MN height, as measured by CE-EUS, is 8.8 mm or greater, it is highly probable that the pathological diagnosis will be indicative of IC, resulting in a strong positive recommendation for resection. CE-EUS not only diagnoses the presence of MNs but also facilitates the measurement of MN height, thereby playing an important role in differentiating benign and malignant BD-IPMN. CE-EUS should therefore be considered necessary for the accurate pre-surgical evaluation of BD-IPMN.

COMMENTS

Background

Intraductal papillary mucinous neoplasm (IPMN) represents one type of pancreatic tumor. IPMN can be subdivided into main duct IPMN and branch duct IPMN (BD-IPMN). Most BD-IPMNs are less invasive and can be routinely monitored; thus, the differential diagnosis of benign and malignant BD-IPMN must be accurate to indicate surgical resection.

Research frontiers

A meta-analysis demonstrated that the presence of mural nodules (MNs) is a highly suspicious finding for malignant BD-IPMN. Furthermore, certain studies have indicated that both the presence of MNs and their height may be risk factors for malignant BD-IPMN.

Innovations and breakthroughs

This is the first study to demonstrate that contrast-enhanced endoscopic ultrasonography (CE-EUS) is the optimal imaging modality for detecting the presence of MNs and measuring MN height. CE-EUS enabled the detection of MNs with an accuracy of 98%. CE-EUS measured MN height significantly better than computed tomography or endoscopic ultrasonography (EUS). A receiver operating characteristic curve analysis related to the diagnosis of malignant BD-IPMN based on MN height as measured by CE-EUS was performed, and the

cut-off value was determined to be 8.8 mm, which yielded an excellent accuracy of 93%.

Applications

The results of this study suggest that using CE-EUS for the pre-surgical evaluation of BD-IPMN will improve the diagnostic accuracy of malignant BD-IPMN and will help patients avoid unnecessary surgery.

Terminology

Originally, a contrast-enhanced imaging technique was not available for EUS, but a second-generation contrast agent now allows for the clinical use of CE-EUS. Recently, many reports have noted the effectiveness of CE-EUS in the diagnosis of pancreatic lesions.

Peer-review

The authors elucidated the role of CE-EUS in the differential diagnosis of benign and malignant BD-IPMN. And the result of research is inspiring and helpful for clinical practice to diagnose BD-IPMN.

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ORIGINAL ARTICLE

Retrospective Study

Efficacy of cap-assisted colonoscopy according to lesion location and endoscopist training level

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Abstract

AIM: To evaluate the efficacy of cap-assisted colonoscopy (CAC) for detection of colorectal polyps and adenomas according to the lesion location and endoscopist training level.

METHODS: Patients 20 years or older, who underwent their first screening colonoscopy in a single tertiary center from May 2011 to December 2012 were enrolled in this study. All patients underwent either CAC or standard colonoscopy (SC), and all of the procedures were performed by 11 endoscopists (8 trainees and 3 experts). All procedures were performed with high-definition colonoscopes and narrow band imaging. The eight trainees had experiences of performing 150 to 500 colonoscopies, and the three experts had experiences of performing more than 3000 colonoscopies. A 4-mm-long transparent cap was attached to the end of a colonoscope in the CAC group. We retrospectively evaluated the number of polyps and adenomas, polyp detection rate (PDR), and the number of adenomas and adenoma detection rate (ADR) according to the lesion location and endoscopist training level between CAC and SC. We also evaluated the number of polyps and adenomas according to their size between CAC and SC.

RESULTS: Overall, PDR and ADR using CAC were significantly higher than those using SC for both whole

colon (48.5% *vs* 40.7%, *P* = 0.012; 35.7% *vs* 28.3%, *P* = 0.012) and right-side colon (35.3% *vs* 26.6%, *P* = 0.002; 27.0% *vs* 16.9%, *P* < 0.001). The number of polyps and adenomas per patient using CAC was significantly higher than that using SC for both the whole colon (1.07 ± 1.59 *vs* 0.82 ± 1.31, *P* = 0.008; 0.72 ± 1.32 *vs* 0.50 ± 1.01, *P* = 0.003) and right-side colon (0.66 ± 1.18 *vs* 0.41 ± 0.83, *P* < 0.001; 0.46 ± 0.97 *vs* 0.25 ± 0.67, *P* < 0.001). In the trainee group, the PDR and ADR using CAC were significantly higher than those using SC for both the whole colon (46.7% *vs* 39.7%, *P* = 0.040; 33.9% *vs* 26.0%, *P* =0.012) and right-side colon (34.2% *vs* 26.5%, *P* = 0.015; 25.3% *vs* 15.9%, *P* = 0.001). In the expert group, the PDR and ADR using CAC were significantly higher than those using SC only for the right-side colon (42.1% *vs* 27.0%, *P* =0.035; 36.8% *vs* 21.0%, *P* = 0.020).

CONCLUSION: CAC is more effective than SC for detection of colorectal polyps and adenomas, especially when performed by trainees and when the lesions are located in the right-side colon.

Key words: Colonoscopy; Cap-assisted colonoscopy; Colonic polyps; Adenoma; Colorectal neoplasm

Core tip: Missed lesions are the main cause of interval colon cancer. Cap-assisted colonoscopy (CAC) is one of the procedures which can reduce the incidence of missed lesion. Few studies have evaluated the efficacy of CAC based on location and size of lesions or training level of endoscopist. We evaluated the efficacy of CAC, according to the location and size of lesions and the training level of the endoscopists. We suggest that CAC can improve the detection of lesions for trainees in the whole colon and right-side colon, and even for experts in the right-side colon.

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INTRODUCTION

Colonoscopy is an effective procedure for prevention of colorectal cancer (CRC) because it allows for the detection and removal of polyps and adenomas[1]. However, colonoscopy has certain limitations with respect to prevention of CRC, because CRC may be subsequently diagnosed even after negative colonoscopy results have been obtained[2,3]. CRC that has been diagnosed within 6 to 36 mo after colonoscopy is termed interval cancer[4]. Many potential causes of interval cancer have been considered. Among these, missed lesions are considered to be the main cause[5]. A recent systemic review of tandem colonoscopy studies reported that the rate of missed polyps was 19% to 26%[6]. Right-sided lesions, flat lesions, and variable rates of adenoma detection among endoscopists were considered to be the main causes of missed lesions and interval cancer[7]. Additionally, the adenoma detection rate (ADR) is reportedly an independent predictor of the risk of interval CRC[8]. Therefore, several new technologies such as chromoendoscopy, narrow band imaging (NBI), high-definition (HD) colonoscopy, wide-angle colonoscopy, retrograde-viewing device, and cap-assisted colonoscopy (CAC) have been developed to improve polyp and adenoma detection[9]. Among these technologies, CAC is particularly useful because the cap can depress the semilunar folds, allowing the endoscopist to inspect the blind mucosal area[10]. Although CAC can reduce the blind mucosal area, and was originally expected to increase the rates of polyp and adenoma detection, many studies have produced conflicting results, raising doubt regarding the effectiveness of CAC for polyp and adenoma detection. Several studies have reported that CAC did not improve the ADR[11-14]. Furthermore, one study even showed that CAC was associated with a lower ADR[15]. In contrast, other studies have reported that CAC improved the ADR[10,16-22]. Therefore, we aimed to investigate the efficacy of CAC for polyp and adenoma detection according to the size and location of the polyps and adenomas, and the training level of the endoscopists.

MATERIALS AND METHODS

Study design

This single-center, retrospective case-control study was conducted at Pusan National University Yangsan Hospital (PNUYH). The study protocol was approved by the Institutional Review Board of PNUYH (IRB No. 05-2014-050).

Study population

A total of 1134 patients underwent their first colonoscopy at PNUYH from May 2011 to December 2012. Of these patients, 1023 were enrolled in the present study. Standard colonoscopy (SC) was performed in 508 patients from May 2011 to December 2011, and CAC was performed in 515 patients from May 2012 to December 2012. The inclusion criteria were an age of ⩾ 20 years and screening or evaluation of mild symptoms as the reason for examination. The exclusion criteria were a history of abdominal surgery (excluding appendectomy), a history of colonoscopy, active gastrointestinal bleeding, severe enterocolitis, and a history of inflammatory bowel disease. Bowel

preparation was evaluated and graded by the Aronchick scale[23]. Patients with a poor or inadequate rating on the Aronchick scale were also excluded to eliminate the influence of bowel preparation on the ability to detect polyps and adenomas.

Colonoscopy procedure

All patients underwent either CAC or SC, and all colonoscopies were performed by 11 endoscopists (8 trainees and 3 experts). The eight trainees had experience performing 150 to 500 colonoscopies, and three experts had experience performing more than 3000 colonoscopies. All trainees were endoscopists who had performed more than 150 colonoscopies, because technical competence in screening and diagnostic colonoscopy generally requires experience performing more than 150 procedures[24]. HD colonoscopes and NBI were used in all examinations (CF-H260AI; Olympus Optical Co., Ltd., Tokyo, Japan). Moderate sedation was induced with a combination of intravenous midazolam and meperidine. Cecal intubation was attempted in all cases. If the trainees failed to accomplish cecal intubation within 10 min or the patients complained of intolerable pain, the experts attempted the intubation instead of the trainees. When the experts successfully reached the cecum with the colonoscope, the trainees operated the colonoscope during the withdrawal phase. As soon as the colonoscope reached the cecum, the withdrawal time was measured with a stopwatch. All endoscopists were aware that the withdrawal time was recorded during the procedures. The withdrawal time included not only the time for inspection of the mucosa, but also time for fluid suction, colonic mucosa cleansing, and polyp removal. If possible, a retroflexion technique was implemented in the rectum. The retroflexion technique was not performed in the ascending colon.

Cap-assisted colonoscopy

In the CAC group, a 4-mm-long transparent cap (D-201-14304; Olympus Optical Co., Ltd., Tokyo, Japan) was attached to the end of a colonoscope.

Polyps

Polyps that were detected during the examination were evaluated with respect to size, morphology, and location. Their sizes were estimated by the thickness of a forcep. The polyps were removed by cold forcep biopsy or hot snare polypectomy and pathologically evaluated. Diminutive polyps were defined as $\leqslant$ 5-mm polyps, and all such polyps were removed by cold forcep biopsy. As recommended in another study, multiple diminutive serrated-appearing lesions of the sigmoid colon and rectum were not removed[25]. All > 5-mm polyps were removed by hot snare polypectomy.

Outcome variables

The primary endpoint of this study was comparison of the polyp detection rate (PDR) and ADR in the whole colon and right-side colon between the CAC and SC groups. In this study, the right-side colon included the cecum, ascending colon, hepatic flexure, and transverse colon. The PDR and ADR constituted the proportion of patients with at least one polyp and at least one adenoma, respectively. The secondary endpoint was comparison of the ADR and PDR in the whole colon and right-side colon using the two examination methods (CAC and SC) between experts and trainees.

Statistical analysis

Continuous variables are presented as mean ± SD. The Student's *t*-test was used to compare the means of continuous variables between the CAC and SC groups. The χ^2 test was used to compare categorical variables between the two groups. The Mann-Whitney *U* test was used to compare ordered categorical variables, such as bowel preparation between the two groups. The Mann-Whitney *U* test was also used to compare continuous variables with non-normal distribution, such as number of detected lesions of each colon segments in age $\geqslant$ 76 years. *P* values of < 0.05 were considered to indicate a statistically significant difference. All statistical analyses were performed using PASW Statistics 18.0 for Windows (SPSS Inc., Chicago, IL, United States).

The statistical methods of this study were reviewed by Junhee Han from Research and Statistical Support, Research Institute for Convergence of Biomedical Science and Technology, Pusan National University Yangsan Hospital.

RESULTS

Baseline characteristics

A total of 1134 patients underwent their first colonoscopy at PNUYH from May 2011 to August 2012. Of these patients, 111 were excluded owing to poor or inadequate bowel preparation (*n* = 96), active gastrointestinal bleeding (*n* = 5), history of abdominal surgery (*n* = 6) or diagnosis of inflammatory bowel disease (*n* = 4). Therefore, 1023 patients were enrolled in this study. CAC was performed in 515 patients, and SC was performed in the remaining 508 patients. In the CAC group, 76 patients underwent CAC by experts and 439 patients underwent CAC by trainees. In the SC group, 100 patients underwent SC by experts and 408 patients underwent SC by trainees (Figure 1). Evaluation of the baseline characteristics of the patients showed no significant differences in age, sex, or bowel preparation between the two groups. The combined withdrawal time of both therapeutic and non therapeutic (no biopsy or polypectomy) colonoscopies was significantly longer in the CAC group than in the SC group (14.67 ± 7.70 min *vs* 12.97 ± 7.20 min, *P* < 0.001). However, the withdrawal time of only non therapeutic colonoscopies in the CAC was not

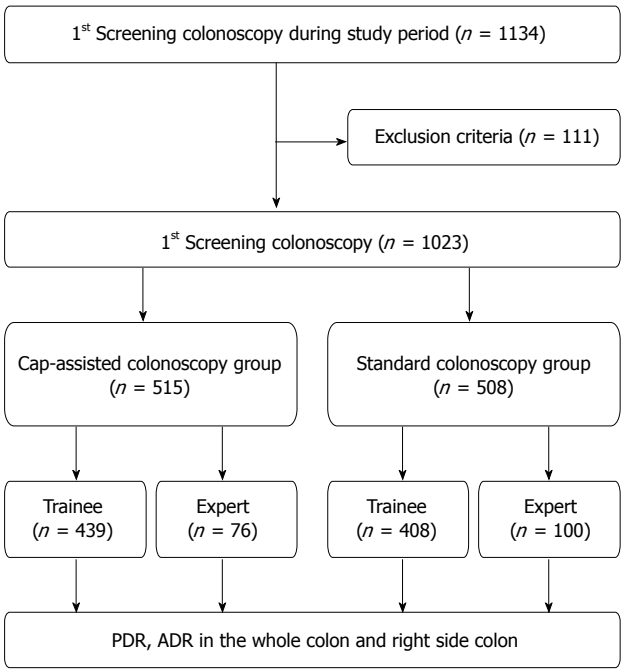

Figure 1 Patient enrollment. PDR: Polyp detection rate; ADR: Adenoma detection rate.

significantly different than that in the SC (10.68 ± 3.09 min *vs* 10.33 ± 4.24 min, $P = 0.272$) (Table 1).

Polyp detection

In total, 967 polyps were detected in the whole colon and 547 polyps were detected in the right-side colon. The total number of polyps and the PDR in the whole colon were significantly higher in the CAC group than in the SC group (549 *vs* 418, $P = 0.008$ and 48.5% *vs* 40.7%, $P = 0.012$). The number of polyps per patient in the whole colon was significantly higher in the CAC group than in the SC group (1.07 ± 1.59 *vs* 0.82 ± 1.31, $P = 0.008$). The total number of polyps and the PDR in the right-side colon were also significantly higher in the CAC group than in the SC group (339 *vs* 208, $P < 0.001$ and 35.3% *vs* 26.6%, $P = 0.002$). The number of polyps per patient in the right-side colon was significantly higher in the CAC group than in the SC group (0.66 ± 1.18 *vs* 0.41 ± 0.83, $P < 0.001$). When the polyps were classified by location, the numbers of polyps in the ascending colon, hepatic flexure, and splenic flexure were significantly higher in the CAC group than in the SC group (179 *vs* 87, $P < 0.001$; 56 *vs* 24, $P = 0.001$; and 24 *vs* 6, $P = 0.001$) (Table 2).

Adenoma detection

In total, 623 adenomas were detected in the whole colon and 365 adenomas were detected in the right-side colon. The total number of adenomas and the ADR in the whole colon were significantly higher in the CAC group than in the SC group (370 *vs* 253, $P = 0.003$ and 35.7% *vs* 28.3%, $P = 0.011$). The number of adenomas per patient in the whole colon was significantly higher in the CAC group than in the SC group (0.72 ± 1.32 *vs* 0.50 ± 1.01, $P = 0.003$). The total number of adenomas and the ADR in the right-side colon were also significantly higher in the CAC group than in the SC group (236 *vs* 129, $P < 0.001$ and 27.0% *vs* 16.9%, $P < 0.001$). The number of adenomas per patient in the right-side colon was significantly higher in the CAC group than in the SC group (0.46 ± 0.97 *vs* 0.25 ± 0.67, $P < 0.001$). When the adenomas were classified by location, the numbers of adenomas in the ascending colon, hepatic flexure, and splenic flexure were significantly higher in the CAC group than in the SC group (129 *vs* 50, $P < 0.001$; 44 *vs* 18, $P = 0.002$ and 22 *vs* 6, $P = 0.003$) (Table 3).

Table 1 Baseline characteristics of enrolled patients *n* (%)

Characteristics	Total	CAC	SC	*P* value
Patients	1023	515	508	
mean age ± SD	54.75 ± 10.52	55.06 ± 10.29	54.44 ± 10.74	0.342
Gender				0.650
Male	549 (53.7)	280 (54.4)	269 (53.0)	
Female	474 (46.3)	235 (45.6)	239 (47.0)	
Withdrawal time of total colonoscopies (min)	13.83 ± 7.50	14.67 ± 7.70	12.97 ± 7.20	< 0.001
No. of patients of non therapeutic colonoscopies	566	265	301	
Withdrawal time of non therapeutic colonoscopies (min)	10.50 ± 3.75	10.68 ± 3.09	10.33 ± 4.24	0.272
Bowel preparation (Aronchick scale)				0.244
Excellent	38 (3.7)	19 (3.7)	19 (3.7)	
Good	646 (63.1)	316 (61.4)	330 (65.0)	
Fair	339 (33.1)	180 (35.0)	159 (31.3)	

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy.

Total number of polyps and adenomas according to size

The total number of polyps and adenomas in the CAC and SC groups were evaluated according to size (Table 4). Overall, 698 diminutive polyps were detected. The total number of diminutive polyps in the CAC group was significantly higher than that in the SC group (398 *vs* 300, $P = 0.011$). The total number of diminutive adenomas in the CAC group was also significantly higher than that in the SC group (253 *vs* 165, $P = 0.003$). On the other hand, the total numbers of larger polyps and adenomas in the CAC groups were not significantly higher than those in the SC group (151 *vs* 118, $P = 0.168$ and 117 *vs* 88, $P = 0.178$).

PDR, ADR, and the number of polyps and adenomas per patient based on age

We evaluated the PDR, ADR, and the number of polyps and adenomas per patient of whole colon (Table 5) and

Table 2 Polyp detection with cap-assisted colonoscopy *vs* standard colonoscopy on the all ages and both genders *n* (%)

Whole colon	Total (n = 1023)	CAC (n = 515)	SC (n = 508)	P value
Total polyps	967	549	418	0.008
Polyps per patient	0.95 ± 1.46	1.07 ± 1.59	0.82 ± 1.31	0.008
PDR	457 (44.7)	250 (48.5)	207 (40.7)	0.012
Right-side colon	Total (n = 1023)	CAC (n = 515)	SC (n = 508)	P value
Total polyps	547	339	208	< 0.001
Polyps per patient	0.53 ± 1.03	0.66 ± 1.18	0.41 ± 0.83	< 0.001
PDR	317 (31.0)	182 (35.3)	135 (26.6)	0.002
Polyps of segment	Total (n = 1023)	CAC (n = 515)	SC (n = 508)	P value
Cecum	85	42	43	0.888
Ascending colon	266	179	87	< 0.001
Hepatic flexure	80	56	24	0.001
Transverse colon	116	62	54	0.560
Splenic flexure	30	24	6	0.001
Descending colon	65	31	34	0.683
Sigmoid colon	223	105	118	0.406
Rectum	102	50	52	0.804

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy; PDR: Polyp detection rate.

Table 3 Adenoma detection with cap-assisted colonoscopy *vs* standard colonoscopy on the all ages and both genders *n* (%)

Whole colon	Total (n = 1023)	CAC (n = 515)	SC (n = 508)	P value
Total adenomas	623	370	253	0.003
Adenomas per patient	0.61 ± 1.18	0.72 ± 1.32	0.50 ± 1.01	0.003
ADR	328 (32.1)	184 (35.7)	144 (28.3)	0.011
Right-side colon	Total (n = 1023)	CAC (n = 515)	SC (n = 508)	P value
Total adenomas	365	236	129	< 0.001
Adenomas per patient	0.36 ± 0.84	0.46 ± 0.97	0.25 ± 0.67	< 0.001
ADR	225 (22.0)	139 (27.0)	86 (16.9)	< 0.001
Adenomas of segment	Total (n = 1023)	CAC (n = 515)	SC (n = 508)	P value
Cecum	45	20	25	0.490
Ascending colon	179	129	50	< 0.001
Hepatic flexure	62	44	18	0.002
Transverse colon	79	43	36	0.529
Splenic flexure	28	22	6	0.003
Descending colon	46	23	23	0.966
Sigmoid colon	130	61	69	0.490
Rectum	54	28	26	0.829

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy; ADR: Adenoma detection rate.

right-side colon (Table 6) based on age (20-49 years, 50-65 years, 66-75 years, > 76 years). In summary, the number of polyps and adenomas per patient of right-side colon in the CAC group was significantly higher than that in the SC group, on all ages except ⩾ 75 years. On the age ⩾ 75 years, none of PDR, ADR and the number of polyps and adenomas per patient in the CAC group was significantly higher than those in the SC group, perhaps because the sample size of the age ⩾ 75 years was too small. When we analyzed the number of polyps and adenomas per patient based on each colon segment, which of ascending colon in

Table 4 Total number of polyps and adenomas according to size

Total number	Total (n = 1023)	CAC (n = 515)	SC (n = 508)	P value
Polyps < 5 mm, n	698	398	300	0.011
Per patient	0.68 ± 1.15	0.77 ± 1.27	0.59 ± 1.00	0.011
Adenomas < 5 mm, n	418	253	165	0.003
per Patient	0.41 ± 0.89	0.49 ± 1.03	0.32 ± 0.71	0.003
Polyps ⩾ 5 mm, n	269	151	118	0.168
per Patient	0.26 ± 0.70	0.29 ± 0.76	0.23 ± 0.63	0.168
Adenomas ⩾ 5 mm, n	205	117	88	0.178
Per patient	0.20 ± 0.64	0.23 ± 0.70	0.17 ± 0.56	0.178

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy.

Table 5 Polyp detection rate, adenoma detection rate, and the number of polyps and adenomas of whole colon based on age *n* (%)

	Whole colon			
Age (yr)	Total	CAC	SC	P value
20-49	308	147	161	
Polyps per patient	0.51 ± 1.07	0.63 ± 1.32	0.41 ± 0.77	0.078
PDR	94 (30.5)	51 (34.7)	43 (26.7)	0.128
Adenomas per patient	0.26 ± 0.77	0.37 ± 1.02	0.16 ± 0.41	0.013
ADR,	57 (18.5)	35 (23.8)	22 (13.7)	0.022
50-65	544	293	251	
Polyps per patient	1.01 ± 1.47	1.09 ± 1.57	0.92 ± 1.34	0.194
PDR	260 (47.8)	146 (49.8)	114 (45.4)	0.305
Adenomas per patient	0.67 ± 1.22	0.76 ± 1.34	0.57 ± 1.04	0.068
ADR	190 (34.9)	107 (36.5)	83 (33.1)	0.400
66-75	154	68	86	
Polyps per patient	1.55 ± 1.84	1.93 ± 1.91	1.26 ± 1.74	0.024
PDR	91 (59.1)	47 (69.1)	44 (51.2)	0.024
Adenomas per patient	1.04 ± 1.53	1.28 ± 1.62	0.85 ± 1.43	0.084
ADR	71 (46.1)	38 (55.9)	33 (38.4)	0.030
⩾ 76	17	7	10	
Polyps per patient	1.24 ± 1.20	1.14 ± 0.90	1.30 ± 1.41	0.959
PDR	12 (70.6)	6 (85.7)	6 (60.0)	0.338
Adenomas per patient	1.00 ± 1.11	0.71 ± 0.75	1.20 ± 1.31	0.502
ADR	10 (58.8)	4 (57.1)	6 (60.0)	1.000

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy; PDR: Polyp detection rate; ADR: Adenoma detection rate.

the CAC group was significantly higher than that in the SC group on the all ages except ⩾ 75. The number of polyps and adenomas per patient of transverse and descending colon in the CAC was not significantly different than that in the SC group on the all ages.

PDR, ADR, and the number of polyps and adenomas per patient based on gender

We evaluated the PDR, ADR, and the number of polyps and adenomas per patient of whole colon and right-side colon based on gender (Table 7). The number of adenomas per patient and ADR of right-side colon in the CAC group were significantly higher than those in the SC group, based on the both genders. When we analyzed the number of polyps and adenomas per patient based on each colon segment, which of ascending colon in the CAC group was significantly

Table 6 Polyp detection rate, adenoma detection rate, and the number of polyps and adenomas of right-side colon based on age *n* (%)

	Right-side colon			
Age (yr)	Total	CAC	SC	*P* value
20-49	308	147	161	
Polyps per patient	0.25 ± 0.82	0.35 ± 1.09	0.15 ± 0.45	0.030
PDR	49 (15.9)	30 (20.4)	19 (11.8)	0.039
Adenomas per patient	0.13 ± 0.61	0.20 ± 0.83	0.06 ± 0.25	0.033
ADR	28 (9.1)	20 (13.6)	8 (5.0)	0.008
50-65	544	293	251	
Polyps per patient	0.59 ± 1.00	0.68 ± 1.13	0.49 ± 0.82	0.025
PDR	194 (35.7)	110 (37.5)	84 (33.5)	0.322
Adenomas per patient	0.41 ± 0.86	0.50 ± 1.00	0.31 ± 0.66	0.009
ADR	138 (25.4)	82 (28.0)	56 (22.3)	0.129
66-75	154	68	86	
Polyps per patient	0.91 ± 1.31	1.21 ± 1.38	0.67 ± 1.21	0.012
PDR	86 (55.8)	38 (55.9)	30 (34.9)	0.009
Adenomas per patient	0.62 ± 1.04	0.81 ± 0.99	0.47 ± 1.07	0.043
ADR	54 (35.1)	34 (50.0)	20 (23.3)	0.001
⩾ 76	17	7	10	
Polyps per patient	0.59 ± 1.00	0.86 ± 1.06	0.40 ± 0.96	0.167
PDR	6 (35.3)	4 (57.1)	2 (20.0)	0.162
Adenomas per patient	0.41 ± 0.71	0.57 ± 0.78	0.30 ± 0.67	0.362
ADR	5 (29.4)	3 (42.9)	2 (20.0)	0.593

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy; PDR: Polyp detection rate; ADR: Adenoma detection rate.

Table 7 Polyp detection rate, adenoma detection rate, and the number of polyps and adenomas based on gender *n* (%)

Gender	Total	CAC	SC	*P* value
	Whole colon			
Male, *n*	549	280	269	
Polyps per patient	1.25 ± 1.67	1.37 ± 1.84	1.12 ± 1.47	0.082
PDR	300 (54.6)	158 (56.4)	142 (52.8)	0.392
Adenomas per patient	0.81 ± 1.38	0.90 ± 1.53	0.71 ± 1.20	0.109
ADR	219 (39.9)	114 (40.7)	105 (39.0)	0.688
Female	474	235	239	
Polyps per patient	0.60 ± 1.08	0.71 ± 1.14	0.49 ± 1.00	0.029
PDR	157 (33.1)	92 (39.1)	65 (27.2)	0.006
Adenomas per patient	0.38 ± 0.84	0.50 ± 0.98	0.26 ± 0.67	0.002
ADR	109 (23.0)	70 (29.8)	39 (16.3)	< 0.001
	Right-side colon			
Male	549	280	269	
Polyps per patient	0.70 ± 1.20	0.87 ± 1.39	0.52 ± 0.94	0.001
PDR	205 (37.3)	118 (42.1)	87 (32.3)	0.018
Adenomas per patient	0.49 ± 1.00	0.60 ± 1.14	0.37 ± 0.82	0.007
ADR	153 (27.9)	90 (32.1)	63 (23.4)	0.023
Female	474	235	239	
Polyps per patient	0.35 ± 0.73	0.41 ± 0.78	0.28 ± 0.67	0.066
PDR	112 (23.6)	64 (27.2)	48 (20.1)	0.067
Adenomas per patient	0.21 ± 0.56	0.29 ± 0.67	0.13 ± 0.42	0.002
ADR	72 (15.2)	49 (20.9)	23 (9.6)	0.001

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy; PDR: Polyp detection rate; ADR: Adenoma detection rate.

higher than that in the SC group on the both genders. The number of polyps and adenomas per patient of transverse and descending colon in the CAC was not significantly different than that in the SC group on both genders.

PDR and ADR of the whole colon and right-side colon between trainees and experts

The PDR and ADR were evaluated according to the endoscopists' training level. When the procedures were performed by the trainees, the PDR and ADR of the whole colon in the CAC group were significantly higher than those in the SC groups (46.7% *vs* 39.7%, $P = 0.040$ and 33.9% *vs* 26.0%, $P = 0.012$). The PDR and ADR of the right-side colon in the CAC group were significantly higher than those in the SC group (34.2% *vs* 26.5%, $P = 0.015$ and 25.3% *vs* 15.9%, $P = 0.001$) (Table 8). When the procedures were performed by the experts, the PDR and ADR of the whole colon in the CAC group were not significantly different from those in the SC group (59.2% *vs* 45.0%, $P = 0.062$ and 46.1% *vs* 38.1%, $P = 0.283$). However, the PDR and ADR of the right-side colon in the CAC group were significantly higher than those in the SC group (42.1% *vs* 27.0%, $P = 0.035$ and 36.8% *vs* 21.0%, $P = 0.020$) (Table 9).

Complications

No significant complications such as perforation or massive bleeding occurred in either group during the study period.

DISCUSSION

Colonoscopy is one of the most effective procedures for prevention of CRC. However, colonoscopy has certain limitations. According to two previous population-based studies, interval cancer may develop after colonoscopy[2,3]. New-onset CRC, incomplete polyp resection, and missed lesions are considered to be among the causes of interval cancer development; of these, missed lesions are considered to be the main cause[5]. Additionally, interval colon cancers are considered to be associated with localization in the right-side colon, microsatellite instability, and CpG island methylator phenotype-high[26,27]. Moreover, Rex *et al*[25] suggested that it is important to reduce the rate of both missed serrated lesions and missed adenomas to prevent right-side colon cancer. Therefore, increases in both the PDR and ADR are thought to be critical for the prevention of interval cancer.

Several new technologies have been developed to reduce the incidence of missed lesions and improve the PDR and ADR, including chromoendoscopy, NBI, HD colonoscopy, wide-angle colonoscopy, retrograde-viewing device, and CAC[9]. Most of these techniques are associated with increased procedure times and higher costs. In contrast, CAC can be easily implemented by simply attaching a transparent rubber cap to the tip of the colonoscope, can reduce the cecal intubation time, and is not associated with a high cost[10,12,14,15]. Additionally, the cap maintains an appropriate distance between the colonic mucosa

Table 8 Polyp detection rate and adenoma detection rate of trainees *n* (%)

Trainees	Total (*n* = 847)	CAC (*n* = 439)	SC (*n* = 408)	*P* value
Whole colon				
PDR	367 (43.3)	205 (46.7)	162 (39.7)	0.040
ADR	255 (30.1)	149 (33.9)	106 (26.0)	0.012
Right-side colon				
PDR	258 (30.5)	150 (34.2)	108 (26.5)	0.015
ADR	176 (20.8)	111 (25.3)	65 (15.9)	0.001

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy; PDR: Polyp detection rate; ADR: Adenoma detection rate.

Table 9 Polyp detection rate and adenoma detection rate of experts *n* (%)

Experts	Total (*n* = 176)	CAC (*n* = 76)	SC (*n* = 100)	*P* value
Whole colon				
PDR	90 (51.1)	45 (59.2)	45 (45.0)	0.062
ADR	73 (41.5)	35 (46.1)	38 (38.0)	0.283
Right-side colon				
PDR	59 (33.5)	32 (42.1)	27 (27.0)	0.035
ADR	49 (27.8)	28 (36.8)	21 (21.0)	0.020

CAC: Cap-assisted colonoscopy; SC: Standard colonoscopy; PDR: Polyp detection rate; ADR: Adenoma detection rate.

and lens of the colonoscope; this helps to prevent red-out (the duration of time during which the lens of colonoscope is obscured by contact with the mucosa), improve the orientation of the lumen, and allows the endoscopist to advance the colonoscope with less air insufflation. All of these factors contribute to shortening of the cecal intubation time[28].

The cap not only keeps the tip of the colonoscope an adequate distance away from the colonic mucosa, but also separates and depresses the semilunar folds. Thus, the cap allows the endoscopist to maintain a continuous visual field around the colonic bends and to thoroughly inspect the blind mucosa (Figure 2), such as the proximal aspect of ileocecal valve, flexures, haustral folds, and rectal valves[10,18,28]. These advantages of CAC allow for improvement in the ADR and PDR in the proximal colon, flexures, and whole colon. The cap can also stretch or splay the colonic mucosa, further contributing to improved detection rates[19].

Eight previous studies reported that CAC was better able to detect polyps or adenomas than was SC[10,16-22]. However, our study differed from these previous studies in several aspects. Three of these previous studies did not evaluate bowel preparation between the CAC and SC groups[10,16,17]. None of the remaining five studies evaluated the efficacy of CAC according to the training level of the endoscopists. In the present study, we evaluated the grade of bowel preparation in both the CAC and SC groups. We then confirmed that there were no significant differences in bowel preparation between the CAC and SC groups, and we excluded patients with poor or inadequate bowel preparation. We evaluated the location and size of the detected polyps and adenomas, and we compared the PDR and ADR obtained by both experienced and inexperienced endoscopists.

Despite the benefits of CAC, there are conflicting results regarding its PDR and ADR. Several studies have reported that CAC did not improve polyp and adenoma detection[11-14]. However, no studies have evaluated polyp and adenoma detection according to the training level of the endoscopists with a large sample size while eliminating the influence of bowel preparation and patient characteristics. Harada *et al*[14] did not evaluate the bowel preparation in the CAC or SC group, and Tee *et al*[13] included patients with poor bowel preparation and evaluated a small sample. Although Dai *et al*[11] reported that CAC conducted by trainees did not improve the PDR, their study evaluated a small sample and patients in the SC group were older than those in the CAC group, especially in the trainee group. Finally, de Wijkerslooth *et al*[12] did not evaluate the PDR and ADR associated with CAC performed by trainees.

One study reported that CAC may decrease the PDR and ADR. Lee *et al*[15] reported that the ADR in the CAC group was lower than that in the SC group. However, the bowel preparation was significantly less satisfactory in the CAC group, the withdrawal time was shorter in the CAC group, and the procedure was performed with a mucosectomy cap. Mucosectomy caps are longer at 10 mm in length; this increased length may make them difficult to clean, and they may impair the endoscopist's vision because fecal matter can more easily adhere to the longer cap. For these reasons, their study showed a lower ADR in the CAC group.

In contrast to previous studies, many of which reported conflicting results, we excluded the influence of bowel preparation and patients' baseline characteristics and evaluated the efficacy of CAC according to lesion size and location and endoscopists' training level. Our sample was also sufficiently large. We found that CAC can improve the PDR and ADR in the ascending colon, hepatic flexure, splenic flexure, and whole colorectum (Tables 2 and 3). The results of our study provide evidence that CAC allows endoscopists to inspect the blind mucosal surfaces of the flexures and haustral folds. Additionally, the detection rates of diminutive polyps and adenomas were higher in the CAC group than in the SC group (Table 4). We found that CAC performed by trainees was associated with a higher PDR and ADR in the whole colorectum and right-side colon, although CAC performed by experts was associated with a higher PDR and ADR only in the right-side colon (Tables 5 and 6). We assume that experts can inspect the blind mucosa without the cap to some extent, while trainees have difficulty performing this inspection without the cap. Furthermore, even when

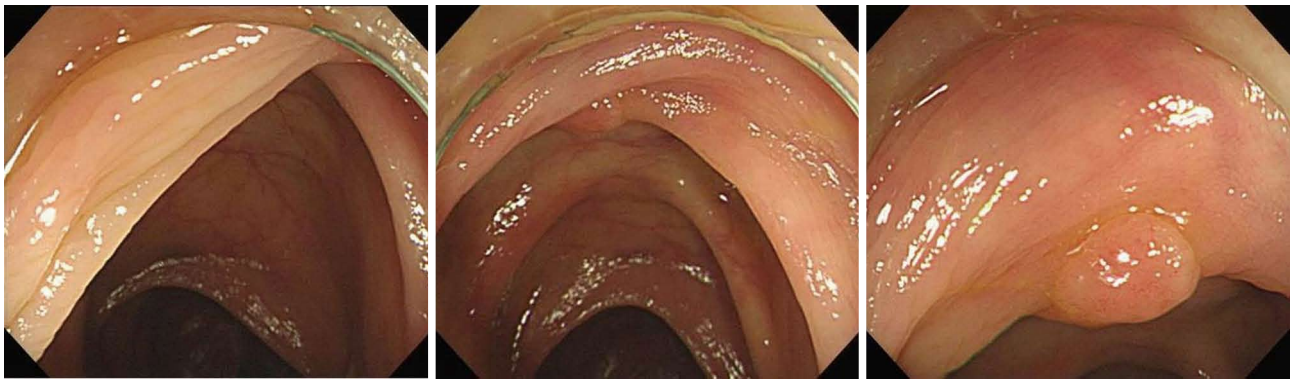

Figure 2 Images of cap-assisted colonoscopy. A lesion was located in the proximal aspect of a haustral fold. The lesion was not observed before cap-assisted colonoscopy depressed the haustral fold. When a cap depressed the haustral fold, the lesion was able to be observed.

experts perform the colonoscopy, we believe that the cap would help to detect polyps and adenomas in the right-side colon.

Rex *et al*[29] suggested that ADR must be at least 25% for male and at least 15% for female, and ADR is known to vary widely among providers in both academic and community settings[30]. Our study reported higher PDR (40.7%) and high ADR (28.3%) in SC group than previous studies. However, recent studies reported the high PDR and ADR with long withdrawal times, self-recording of withdrawal time, HD colonoscope, fair bowel preparation and an academic setting of improving ADR[31-35]. In our study, the minimal withdrawal time of most procedures was at least 7 min, all endoscopists were aware of self-recording of withdrawal time and all procedures were performed with HD colonoscopies and NBI, and optimal or fair bowel preparation. Furthermore, quality improvement program of colonoscopy was performed at PNUYH every month. These are the reason that our PDR and ADR in SC group were higher than those of previous studies.

This study has several limitations. First, this study was a single-center, retrospective, case-controlled study. Second, four of the trainees performed the SC from May 2011 to December 2011, and the other four trainees performed CAC from May 2012 to December 2012. Thus, there is a potential for selection bias, and the sample performed by experts was relatively small. Third, the combined withdrawal time of both therapeutic and non therapeutic (no biopsy or polypectomy) colonoscopies in the CAC group was significantly longer than that in the SC group, indicating that the withdrawal time is associated with increase in PDR and ADR. However, although the combined withdrawal time of both colonoscopies in the CAC group was significantly longer than that in the SC group, the withdrawal time of only non therapeutic colonoscopies was not significantly different between the CAC group and SC group. Moreover, more polyps and adenomas were detected in CAC than SC. Therefore, we concluded that the withdrawal time of CAC was longer than that of SC because more lesions were detected in CAC than SC, and more lesion removal time, such as cold forcep biopsy or hot snare polypectomy, was needed in CAC than SC. We believe that the inspection time of CAC was not longer than that of SC, and the higher ADR of CAC was not associated with the longer withdrawal time of CAC.

In conclusion, we believe that CAC can be very helpful for trainees to detect lesions in the whole colon and even for experts to detect lesions in the right-side colon. Additionally, CAC can be very useful to prevent interval colon cancer, especially when performed by inexperienced endoscopists in patients with satisfactory bowel preparation. We recommend the routine use of CAC for screening colonoscopy.

COMMENTS

Background

Colonoscopy is one of the most effective procedure for prevention of colorectal cancer. However, colorectal cancer can be subsequently diagnosed after negative colonoscopy, and it is called the interval cancer. The interval cancer is known to be associated with missed lesions and right-side colon. Therefore, to reduce the incidence of missed lesion and improve polyp detection rate (PDR) and adenoma detection rate (ADR) are important to prevent the interval cancer, especially in the right-side colon. Several new technologies have been developed to improve the PDR and ADR, and cap-assisted colonoscopy (CAC) is an inexpensive and simple method among these technologies. The authors aimed to evaluate the efficacy of CAC based on location of lesions and training level of endoscopists.

Research frontiers

This study did not only aim to evaluate the efficacy of CAC in the whole colon, but also based on location and size of lesions, and training level of endoscopists.

Innovations and breakthroughs

According to this study, CAC is more effective for experts than standard colonoscopy for detection of lesions in the right-side colon. And CAC is also more effective for trainees in the whole colon and right-side colon.

Applications

CAC is an effective procedure to reduce the incidence of missed lesion and improve the detection rate of lesions for the trainees, and even for the experts.

Terminology

CAC: Cap-assisted colonoscopy. A 4-mm-long transparent cap is attached to the end of a colonoscope, and the cap is able to separate and depress the semilunar folds. Therefore, CAC allows the endoscopists to inspect the blind mucosa of colon. SC: Standard colonoscopy. PDR: Polyp detection rate. PDR

is usually defined as the proportion of patients in whom at least one polyp was identified. ADR: adenoma detection rate. ADR is usually defined as the proportion of patients in whom at least one adenoma was identified. ADR is known as a quality indicator of colonoscopy.

Peer-review

This study aimed to evaluate the efficacy of CAC based on location of lesions and training level of endoscopists. CAC can be helpful to improve the detection rate of lesions for trainees and even experts.

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ORIGINAL ARTICLE

Retrospective Study

Relationship between expression of NADPH oxidase 2 and invasion and prognosis of human gastric cancer

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Author contributions: Wang P participated in the design of the study, performed the majority of experiments and wrote this article; Shi Q participated in the design of this study, performed the Western blot and helped to write the paper; Wang P and Shi Q contributed equally to this work and should be considered co-first authors; Deng WH participated in the design of the study and collected the clinical samples; Yu J participated in the design of the study, collected the clinical samples and did the work of follow-up visit; Zuo T and Mei FC participated in the design of the study and did the work of follow-up visit; Wang WX participated in the design of this study and collected all the human material in addition to providing financial support for this work.

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Abstract

AIM: To assess the expression and prognostic value of nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) in gastric cancer, and its correlation with vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR).

METHODS: Tumor and adjacent tissues were obtained from 123 patients who underwent radical surgery for gastric cancer at Renmin Hospital of Wuhan University from 2008-2009. The expression of NOX2, VEGF, EGFR and CD68 in tumor tissues was detected by immunohistochemistry. The expression of NOX2 in gastric cancer and adjacent tissues was detected by Western blot analysis. Spearman's correlation was performed to elucidate the relationship of NOX2 with VEGF and EGFR. The Kaplan-Meier method was used to calculate survival time, and the log-rank test was used to evaluate differences in survival. Cox's proportional hazards regression model was applied in a stepwise manner to analyze the independent prognostic factors.

RESULTS: NOX2 exhibited positive expression in 47.2% (58/123) of the gastric cancer tissues. Western blot analysis revealed that NOX2 was up-regulated in tumor tissues compared to the adjacent tissue [39.0% (48/123)]. Immunohistochemistry staining revealed that CD68, which is a specific marker of macrophages, and NOX expression presented a similar localization and staining intensity. The expression of NOX2 was

positively correlated with that of VEGF and EGFR. Comparison of the 5-year survival rates of the NOX2 positive and NOX2 negative groups showed that the NOX2 positive group presented a poor prognosis.

CONCLUSION: NOX2 positively correlates with the levels of VEGF and EGFR. NOX2 may be used as a new biomarker and a potential therapeutic target for gastric cancer.

Key words: NADPH oxidase 2; Macrophages; Vascular endothelial growth factor; Epidermal growth factor receptor; Prognosis; Survival time

Core tip: In this article, the authors used immunohistochemistry to analyze the expression of nicotinamide adenine dinucleotide phosphate oxidase 2 (NOX2) in formalin-fixed paraffin-embedded tissues from 123 gastric cancer patients. Western blot analysis was employed to examine NOX2 expression in tumor and adjacent tissues. NOX2 was up-regulated in tumor tissues. NOX2 was positively correlated with vascular endothelial growth factor and epidermal growth factor receptor. The results also showed that patients who were NOX2 positive clearly presented worse outcomes than NOX2-negative patients. The expression of NOX2 could be used as a prognostic biomarker for gastric cancer.

Wang P, Shi Q, Deng WH, Yu J, Zuo T, Mei FC, Wang WX. Relationship between expression of NADPH oxidase 2 and invasion and prognosis of human gastric cancer. *World J Gastroenterol* 2015; 21(20): 6271-6279 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6271.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6271

INTRODUCTION

Reactive oxygen species (ROS) are considered toxic by-products of various types of cellular aerobic respirations. The role of ROS *in vivo* is to modulate various cell functions, such as cell migration, proliferation and phagocytosis[1]. Recent studies have shown that ROS are implicated in many human diseases, including cancer, but the precise mechanisms for ROS to regulate cancer remain elusive. ROS and oxidative stress have been linked to cancer initiation and progression through their propensity to induce DNA mutations or DNA damage, genome instability and cell proliferation[2], therefore, the chromosomal imbalance theory and the DNA, RNA, or protein damage theory are thought to be the key words. Thus, ROS are believed to play an important role in the activation of oncogene products[3,4]. Enzymes are the major sources of ROS generation[5], and among all the resources of ROS the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family is the most important[6]. The NADPH oxidase (NOX) homologues include NOX1 to NOX5, DUOX1 and DUOX2. These enzymes can transfer electrons from NADPH to molecular oxygen, and in this process, form ROS. Additionally, the NOX family can promote angiogenesis and has been confirmed to be associated with the metastatic potential of many solid tumor[7]. Within the NOX family, the NOX2 isoform can maintain the cytoskeletal structure of the cell and prevents cell apoptosis[8], in addition to potentially influencing tumor proliferation.

Gastric cancer is the fourth most common type of malignant tumor worldwide and the second most common cause of cancer-related death[9]. For advanced gastric cancer patients, radical gastrostomy and lymph node dissection with chemotherapy are the common therapeutic methods[10]. However, the prognosis of gastric cancer patients is still poor. The 5-year survival rate for advanced gastric cancer is approximately 5%-20%, and the median overall survival is less than 1 year[11,12]. The reason for the poor outcome of gastric cancer remains elusive, though the eutrophic condition of gastric tumors has recently become a research hot spot. Tumor angiogenesis is a key point in the progression of solid tumors including gastric cancer. Angiogenesis is a vital step in the metastasis of tumors[13]. Vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen, can activate vascular endothelial growth factor receptor 2 (VEGFR2), a receptor tyrosine kinase (PTK) in endothelial cells to promote proliferation and migration. Thus, VEGF represents a key molecule in the angiogenesis of tumors[7]. The angiogenic effect of VEGF can stimulate ROS such as superoxides[14]. The major source of ROS is the NOX family of enzymes, and NOX2 is related to tumor-associated macrophages which are the key modulators of tumor angiogenesis[15]. Thus, NOX2 may stimulate tumor angiogenesis through VEGF.

Epidermal growth factor receptor (EGFR) belongs to the receptor tyrosine kinase family which plays an important role in cellular processes, such as cell proliferation, apoptosis, angiogenesis and metastasis[16]. In gastric cancer a high level of EGFR expression is always associated with an increased risk of invasion or metastasis, and EGFR positive gastric cancer patients exhibit a worse prognosis[17]. NOX2 also plays an important role in cellular processes and can stimulate angiogenesis. NOX2 has been found to be highly expressed in prostate cancer cells and tissues, and a high level of NOX2 is correlated with the progression of prostate cancer[18]. However, whether NOX2 is expressed in gastric cancer and its correlation with the expression of VEGF and EGFR in gastric cancer remain unexplored.

In this study, we examined the expression of NOX2 and its role in the processes of gastric cancer. Then, we evaluated the prognostic value of NOX2 in gastric

cancer.

MATERIALS AND METHODS

Patients and clinical specimens

A total of 123 patients who underwent a radical operation for gastric cancer at Renmin Hospital of Wuhan University from July 2008 to July 2009 were included in this study. None of these patients received preoperative and intraoperative chemotherapy or radiotherapy. The gastric cancer tissues and adjacent tissues were divided into two groups, one was used to produce the paraffin-embedded tissue sections, and the other was placed in liquid nitrogen for Western blot analysis. Clinical-pathological data were collected from all of the patients to analyze the relationship between the expression of NOX2 and the clinical-pathological data.

Follow-up of patients

No patients died during their hospital stay. All patients were followed until July 2014 or death. The methods employed for follow-up were out-patient review and a telephone follow-up study.

Immunohistochemsitry staining analysis

Paraffin-embedded tissues were obtained from Renmin Hospital of Wuhan University and were sectioned into 3 μm thick tissue sections. Immunohistochemical analyses of NOX2, CD68, VEGF and EGFR were performed using a standard strepavidin-peroxidase (SP) method[19]. Minor steps were changed as follows: sections were deparaffinized and dehydrated using a graded series of ethanol solutions. The slides were immersed in 10 mmol/L citrate buffer (pH 6.0) and boiled for 15 min in the microwave oven for the antigen retrieval. Then the slides were allowed to cool at room temperature. Hydrogen peroxidase (0.3%) was used to halt the endogenous peroxidase activity for 15 min at the room temperature. Non-specific binding was blocked by the goat serum (5%) for 10 min. The primary antibody was a rabbit antibody to NOX2 (1:100, abcam, United Kingdom), mouse antibody to CD68 (1:100, abcam, United Kingdom), rabbit antibody to VEGF (1:75, ZSGB-BIO, China), or rabbit antibody to EGFR (1:100, ZSGB-BIO, China). Sections were incubated with the primary antibody overnight at 4 ℃, then incubated with a second antibody. The staining results was visualized using the 3,5-diaminobenzidine. In each immunohistochemistry staining analysis, PBS instead of the primary antibody was used as the negative control.

Evaluation of immunohistochemistry staining results

Two independent observers who were experienced in examining immunohistochemistry results evaluated the results. Both were blinded to the clinical data of the patients. An OLYMPUS BX53 upright microscope was used to obtain photographs of the immunohistochemistry staining results. Positive expression of NOX2 was observed as yellow or brown staining in the cytoplasm. Image-pro Plus 6.0 was used to judge the area of staining and to obtain the density and optical density values for the Immunohistochemsitry (IHC) sections. According to the observed staining intensity, the IHC results can be defined as 0 points, no staining; 1 point, pale yellow staining; 2 points, yellow staining; 3 points, brown staining. The percentage of positive tumor cells was determined in at least 5 areas under × 200 magnification and then averaged. The mean percentage was subsequently divided into four categories: 0, < 5%; 1, 5%-25%; 2, 26%-50%; and 3, 51%-100%. Finally, we combined the results for the staining intensity and the percentage of positive tumor cells as follows: 0-2 was defined as negative expression, and 3-6 as positive expression[20].

Western blot analysis

The fresh gastric cancer tissue and the adjacent tissue were homogenized in ice-cold lysis buffer in the presence of protease inhibitor cocktail. Concentrations of protein in the samples were determined using the Bradford method with bovine serum albumin as a standard. In brief, an equal amount of protein samples were separated on 10% sodium dodecyl sulfatepolyacrylamide gels and then transferred to a PVDF membrane. The membrane was blocked with 5% skim milk in the TBST buffer (TBS containing 0.1% Tween-20) at room temperature for 2 h and then incubated with the rabbit polyclonal anti-NOX2 antibody (1:1000, Abcam, United Kingdom) at 4 ℃ overnight. After extensive rinsing with TBST, the blots were incubated with IRDye 800CW goat anti-rabbit secondary antibody (926-32211 LI-COR, United States 1:10000) at room temperature for 1.5 h, and the expression of NOX2 was detected with the Odyssey imaging system.

Statistical analysis

The data were analyzed with SPSS 19.0 statistical software. The χ^2 test and Fisher's exact test were used to assess the association of NOX2 expression with the clinical-pathological characters. The Kaplan-Meier method was employed to calculate the survival curve, and the log-rank test was employed to evaluate the differences in survival. Cox's regression model was applied in a stepwise manner to analyze the independent prognostic factors. The correlations of NOX2 with VEGF and EGFR were evaluated *via* Spearman's rank correlation. A *P*-value less than 0.05 was considered to indicate statistical significance.

RESULTS

Immunohistochemical staining results for NOX2

NOX2 was overexpressed in the gastric cancer tissues compared with the adjacent tissue. NOX2 expression was observed in the gastric cancer cytoplasm. Among

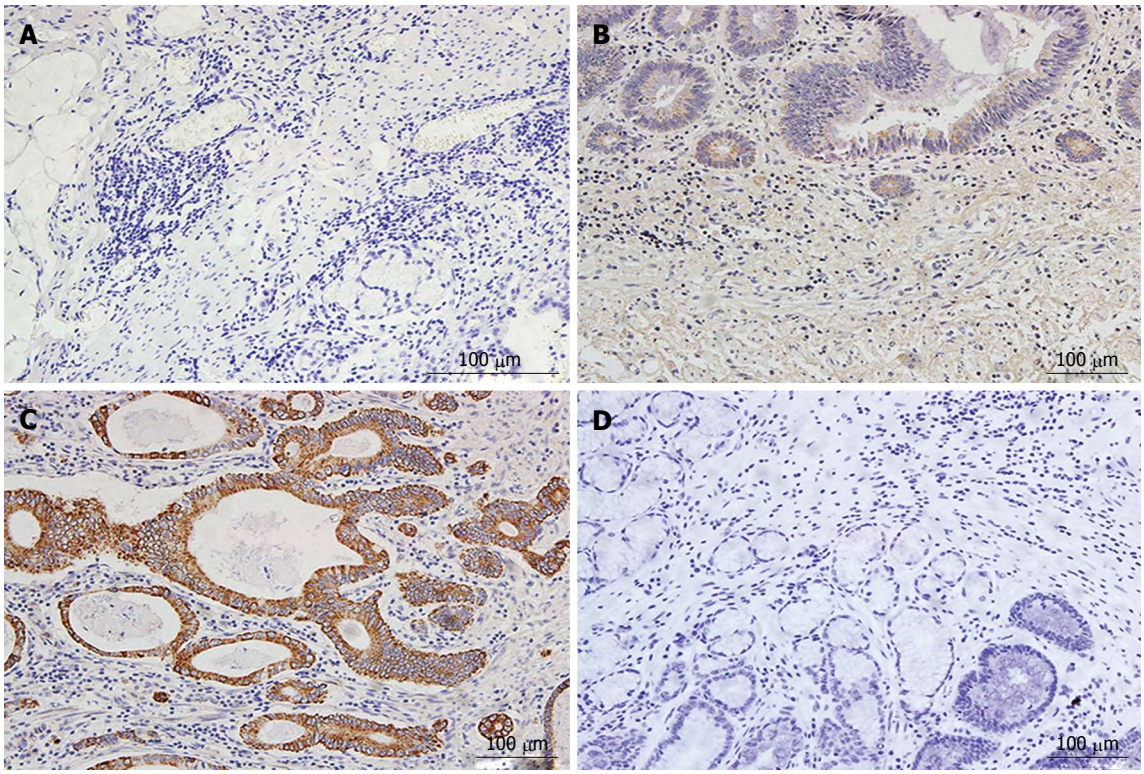

Figure 1 Immunohistochemical staining results for NADPH oxidase 2. A: Negative PBS control in gastric cancer; B: Weak expression of NADPH oxidase 2 (NOX2) in gastric cancer, C: Strongly positive expression of NOX2 in gastric cancer, D: Negative expression of NOX2 in adjacent noncancerous tissue (× 200).

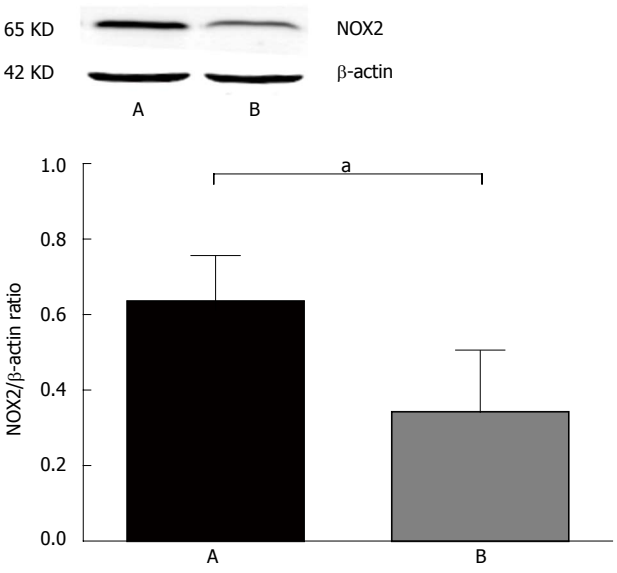

Figure 2 Western blot results for NADPH oxidase 2. A: Gastric cancer tissue; B: Adjacent noncancerous tissue. $^{a}P < 0.05$ *vs* group A.

the gastric cancer tissues, 47.2% (58/123) were positive for NOX2 expression. The rate of NOX2 expression in the adjacent tissue was 44.7% (55/123), and the staining density in the adjacent tissue was weaker than in the GC tissues (Figure 1).

Western blot results for NOX2

The rate of NOX2 up-regulation in gastric cancer was 39.0% (48/123), and the expression of NOX2 in gastric cancer tissues was higher than in the adjacent cancer tissues (Figure 2).

Correlation between NOX2 expression and clinical-pathological characters in gastric cancer

The histological types of the gastric cancer tissues from all the 123 patients were adenocarcinomas. The adenocarcinomas were divided into highly differentiated (22 patients), moderately differentiated (34 patients), poorly differentiated (62 patients) and undifferentiated (5 patients). The pathological TNM staging of gastric cancer revealed stages Ⅰ + Ⅱ in 35 patients and stages Ⅲ + Ⅳ in 70 patients. In the patients showing a poor histological grade and tumor stage, NOX2 expression was higher than in those with a good grade, and NOX2 expression showed no significant correlation with age, sex or tumor size. Information about the relationship between NOX2 expression and clinical-pathological character is shown in Table 1.

Localization of NOX2 and CD68 staining

The IHC staining results presented in Figure 3 show that CD68 and the NOX expression presented similar locations and staining intensities**.**

IHC staining results for VEGF and EGFR in gastric cancer

VEGF and EGFR expression was observed in gastric

Table 1 Correlation between NADPH oxidase 2 expression and clinical-pathological characters in gastric cancer *n* (%)

Clinical feature	Total number (*n* = 123)	NOX2 positivity negativity	χ^2-value	*P*-value
Gender				
Male	69	30 (43.5) 39 (56.5)	0.852	0.229
Female	54	28 (51.9) 26 (48.1)		
Age (yr)				
< 59	55	27 (49.1) 28 (50.9)	0.150	0.419
≥ 59	68	31 (45.6) 37 (54.4)		
Position				
Cardia and fundus	30	8 (26.6) 22 (73.3)	6.683	0.008
Body and antrum	93	50 (53.8) 43 (46.2)		
Differentiation type				
High	22	6 (27.3) 16 (72.7)	5.062	0.042
Moderate	34	16 (47.1) 18 (52.9)		
Poor	62	34 (54.8) 28 (45.2)		
Undifferentiation	5	2 (40) 3 (60)		
Lymph node metastasis				
Yes	53	34 (64.2) 19 (35.8)	10.796	0.001
No	70	24 (34.3) 46 (65.7)		
Vascular invasion				
Yes	60	35 (58.3) 25 (41.7)	5.875	0.012
No	63	23 (36.5) 40 (63.5)		
Clinical stage				
Early stage	32	9(28.1) 23(71.9)	6.285	0.010
Advanced stage	91	49(53.8) 42(46.2)		
TNM				
Ⅰ + Ⅱ	35	10(28.6) 25(71.4)	6.779	0.008
Ⅲ + Ⅳ	88	48(68.6) 40(31.4)		

NOX2: NADPH oxidase 2.

cancer. Positive expression of VEGF and EGFR was detected in the cytoplasm of the tumor tissues. The expression of VEGF and EGFR in the cancers was correlated with the clinical-pathological features. In patients with a poor tumor stage and histological grade the immunohistochemistry staining score was higher than in those presenting a good grade and stage. In Figure 4 strong positive expression of VEGF and weak expression of VEGF are shown.

Expression of NOX2 is positively correlated with VEGF and EGFR in gastric cancer

The mean IOD values of NOX2, VEGF and EGFR in the gastric cancer tissues were measured. Based on determination of the spearman rank correlation coefficient, as shown in Figure 5, we found that the expression of NOX2 was positively correlated with VEGF ($r = 0.763$, $P < 0.05$) and EGFR ($r = 0.710$, $P < 0.05$).

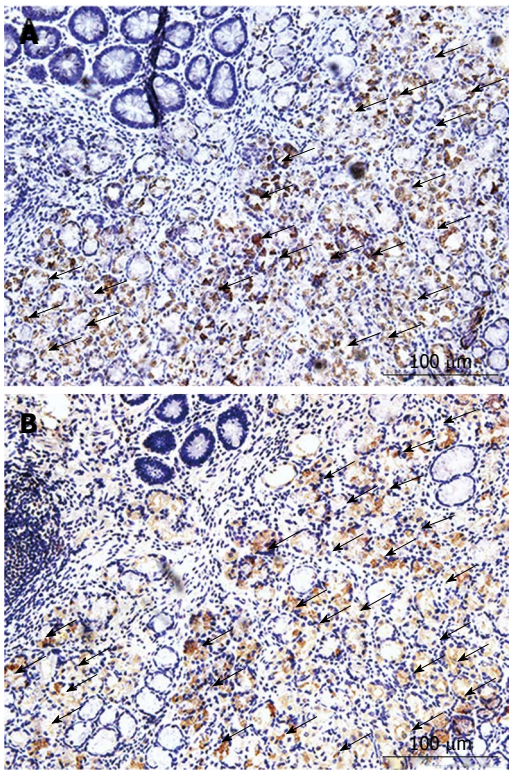

Figure 3 Localization of NOX2 and CD68 staining. A: NOX2 expression in gastric cancer; B: CD68 expression in gastric cancer (× 200).

Follow-up outcomes

No patients died during their hospital stay. All patients were followed until July 1, 2014 or death. The follow-up investigation of the 5-year survival study showed that the patients who were NOX2 positive clearly presented a worse outcome than the NOX2 negative patients (Figure 6). Thus NOX2 may be a novel biomarker of gastric cancer for estimating its prognosis. Based on the univariate analysis of the clinical-pathological features related to prognosis in the 123 gastric cancer patients presented in Table 2, overall survival was significantly correlated with clinical stage, lymph node metastasis, vascular invasion, TNM stage, and NOX2 positivity. Furthermore, the association between NOX2 positivity and survival was still significant after controlling for other prognostic markers in the multivariate analysis, while in the outcome of the multivariate analysis, it was shown that the differentiation type, lymph node metastasis, vascular invasion and TNM stage were independent prognostic factors as well (Table 3).

DISCUSSION

Oxidative stress has been demonstrated to play a key role in many clinical phenomena, such as the

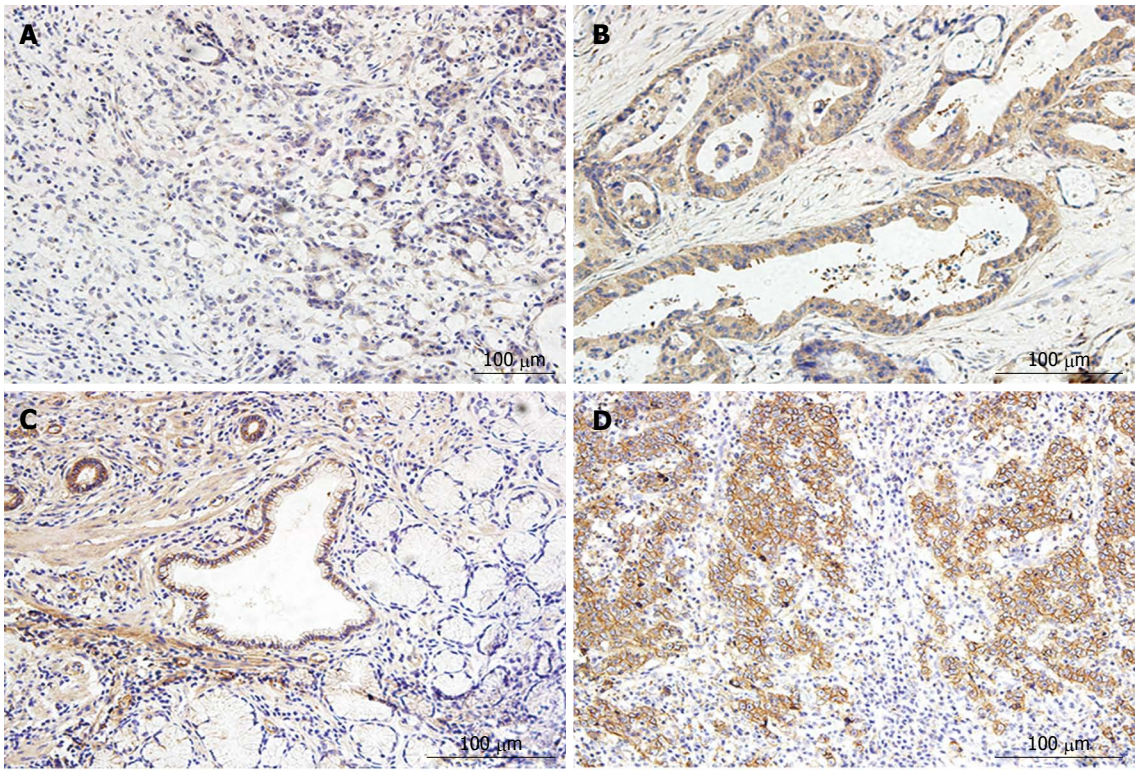

Figure 4 Immuohistochemistry staining results for vascular endothelial growth factor and epidermal growth factor receptor in gastric cancer. A: Weakly positive expression of vascular endothelial growth factor (VEGF) in gastric cancer; B: Strongly positive expression of VEGF in gastric cancer; C: Weakly positive expression of epidermal growth factor receptor (EGFR) in gastric cancer; D: Strongly positive expression of EGFR in gastric cancer (× 200).

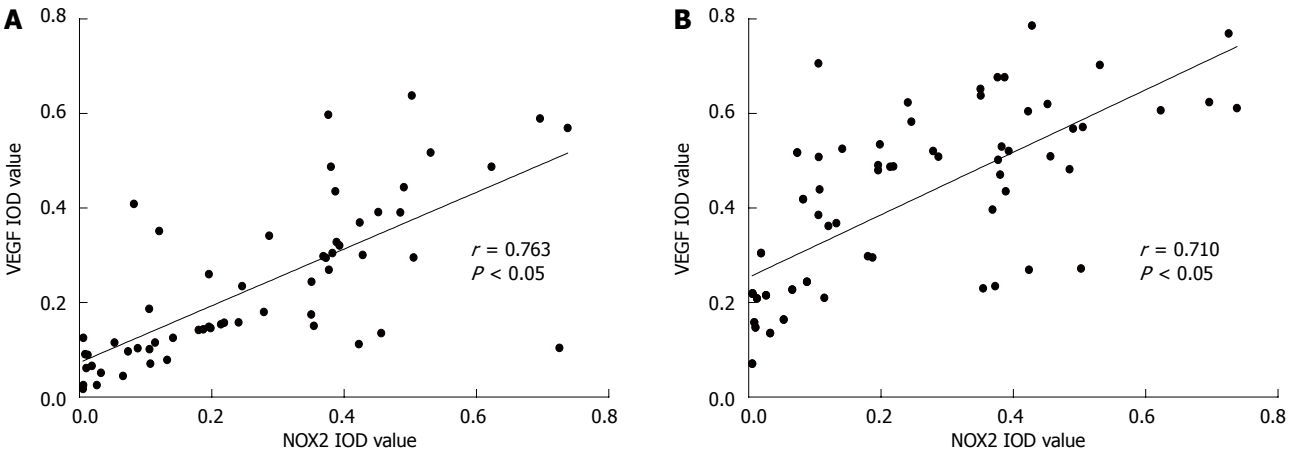

Figure 5 Expression of NADPH oxidase 2 was positively correlated with vascular endothelial growth factor and epidermal growth factor receptor in gastric cancer. A: Spearman correlation between NOX2 and vascular endothelial growth factor; B: Spearman correlation between NOX2 and epidermal growth factor receptor. VEGF: Vascular endothelial growth factor; EGFR: Epidermal growth factor receptor.

inflammatory response and the ageing process[21]. Recent studies have shown that the expression of oxidative stress is higher in many malignancies, including breast cancer, colon cancer and head neck neoplasms[22]. Oxidative stress is the major cause of enhanced cell migration, and it can induce the expression of oncogenes and suppress the activity of anti-survival molecules[23]. The level of oxidative stress is associated with the intracellular ROS level, and NADPH oxidase is a major source of the ROS. NOX2 was the first identified member of the NADPH oxidase family[24], and its role in the progression of malignancies may be correlated with the promotion of angiogenesis[7]. In tumor progression, NADPH oxidases can play the role of a mediator, and may damage DNA within cells and activate oncogenic transformation[25]. In recent studies, it has been shown that ROS can also induce apoptosis of tumor cells[26]. This repeated apoptosis of tumor cells may promote the ability to resist apoptosis in tumor cells, which

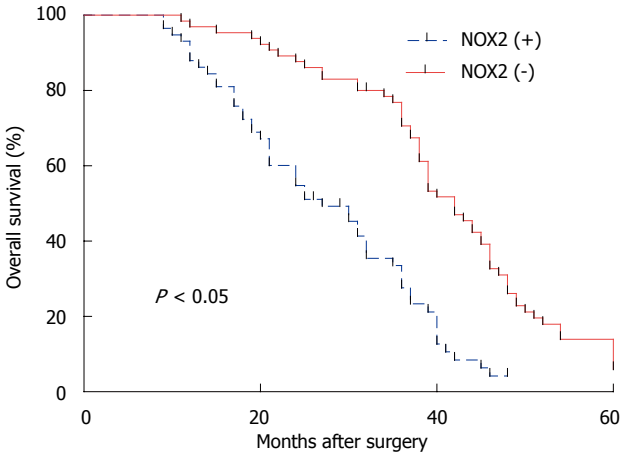

Figure 6 Follow-up investigation of the 5-year survival study showed that the patients who were NADPH oxidase 2 positive clearly presented a worse outcome than the NADPH oxidase 2 negative patients.

could introduce mutations in these cells. Therefore, in the present study, we examined the expression of NOX2 in gastric cancer and its correlation with clinical-pathological characters. We included CD68 in analyses because macrophages may one of the source of NOX2. IHC staining for CD68 and NOX2 in the same tissue sections showed that CD68 and NOX2 share the same positive sites. Therefore, we deduced that macrophages may be one of the sources of NOX2. We determined VEGF and EGFR levels in different clinical-pathological gastric tumor tissues. Then, we studied the correlation of NOX2 with VEGF and EGFR through Spearman's rank correlation. The Spearman's rank correlation coefficients for NOX2 with VEGF and EGFR were 0.763 and 0.710, respectively, and NOX2 was positively correlated with the levels of VEGF and EGFR in gastric cancer. Thus, sections with a high level of NOX2 always show high expression of VEGF and EGFR in gastric cancer. NOX2 may play a key role in the progression of gastric cancer.

This study reports the expression of NOX2, which is the central component of NADPH oxidase in gastric cancer for the first time. Based on the results of IHC staining, it can be observed that the staining of NOX2 occurs at similar sites to CD68 (Figure 2). This finding indicates that NOX2 is closely associated with inflammatory cells such as macrophages. Additionally, macrophages could be a critical determinant of angiogenesis and tumor progression[15]. Thus, it follows that NOX2 may also be associated with angiogenesis and tumor progression, and a recent study implied that NOX2 does, in fact, stimulate angiogenesis in mouse models[27]. Angiogenesis is widely accepted as an important indicator of tumor metastasis because without a blood supply the tumor itself may degenerate. Thus, regulation of angiogenesis through certain signaling pathways may promote the treatment of tumors[28]. Angiogenesis represents a key point in the tumor blood supply. Therefore, if we can control

Table 2 Univariate analysis of clinicopathological features for the prognosis in 123 gastric cancer patients

Variable	Coef	SE	Wald	RR value (95%CI)	*P*-value
Clinical stage	0.531	0.221	5.7970	1.701 (1.104-2.620)	0.016
Differentiation type	0.363	0.193	3.5250	1.438 (0.984-2.101)	0.060
Lymph node metastasis	0.971	0.213	20.857	0.379 (0.250-0.575)	0.000
Vascular invasion	0.926	0.198	21.854	0.396 (0.269-0.584)	0.000
TNM	0.638	0.214	8.9030	1.892 (1.245-2.876)	0.003
NOX2	1.080	0.212	26.004	0.339 (0.224-0.514)	0.000

NOX2: NADPH oxidase 2.

Table 3 Multivariate analysis of clinicopathological features for the prognosis in 123 gastric cancer patients

Variable	Coef	SE	Wald	RR value (95%CI)	*P*-value
Clinical stage	0.670	0.236	8.0800	1.955 (1.230-3.103)	0.004
Differentiation type	0.176	0.201	0.7710	1.193 (0.805-1.769)	0.380
Lymph node metastasis	0.760	0.232	10.725	0.468 (0.297-0.737)	0.001
Vascular invasion	1.061	0.220	23.330	0.346 (0.225-0.532)	0.000
TNM	0.519	0.229	5.1360	0.637 (0.406-0.999)	0.049
NOX2	0.824	0.236	12.153	0.439 (0.276-0.697)	0.000

NOX2: NADPH oxidase 2.

angiogenesis, we can control the blood supply to the tumor. ROS are the major cause of revascularization, and NOX2 can stimulate the human body to produce ROS. Increasing evidence has shown that ROS are implicated in tumor metastasis[29]. ROS can cause the release of VEGF[30], which is important for the angiogenesis of the tumor, and ROS can also stimulate VEGF expression in vascular smooth muscle cells and endothelial cells[31]. EGFR, which is a major treatment bio-target for gastric cancer, is an important biomarker for the prognosis of gastric cancer. A high expression level of EGFR may predict poor survival in gastric cancer. Inhibition of EGFR may suppress the growth, angiogenesis, and metastasis of gastric cancer cells. In this study, we chose to use EGFR as a biomarker for gastric cancer, and we subsequently applied Spearman correlation to analyze the relationship between NOX2 and EGFR levels in gastric cancer. The result of Spearman correlation showed that NOX2 was positively correlated with the level of EGFR. Therefore, as the major source of ROS, NOX2 may also play an important role in the progression of tumor and cardiovascular diseases.

Gastric cancer is a common digestive system malignancy, and although the diagnosis of and therapeutic strategies for gastric cancer have improved, it remains one of the most dangerous human malignancies[32]. Gastric cancer is the second most common cause of cancer-related death[9]. The classification of advanced gastric cancer is important for the prognosis of patients. The classification determines the growth and metastasis of the tumor cells and, thus, affects

the prognosis of gastric cancer. Blood vessel and lymphatic invasion are also important phenomena regarding the prognosis of gastric cancer[33], and among the factors related to blood vessel invasion, VEGF is a core promoter. VEGF represents a key point in the angiogenesis, which is the foundation for the growth and metastasis of tumors, and anti-VEGF therapy has been applied in clinical research[34]. In the present study, we examined VEGF expression in 123 patients with different clinical-pathological features. Our data showed that positive expression of VEGF occurred in 73.4% of the total gastric cancer cases, and the immunohistochemical scores for patients presenting a poor clinical-pathology were higher than for those showing a good clinical-pathology. The correlation between the expression of NOX2 and VEGF was positive, with a correlation coefficient of 0.330. This result indicated that NOX2 could be used as a new prognostic bio-marker of gastric cancer, and anti-oxidative stress may be a new way to control the initiation and progression of gastric cancer.

In summary, the present study demonstrated that the expression level of NOX2 was increased in gastric cancer tissue compared with para-carcinoma tissue, and the obtained IHC scores were higher in the poor clinical-pathology group compared with the good clinical-pathology group. Based on these findings, NOX2 may be used as a new bio-marker of gastric cancer. As a key promoter of angiogenesis and tumorigenesis, NOX2 may promote pro-tumor processes. However, given the current paucity from the field of this research in gastric cancer, further studies must apply a logical and unifying approach to identify cell-specific pathways influenced by NOX2 and the underlying molecular mechanism in the progression and prognosis of gastric cancer.

COMMENTS

Background

Gastric cancer (GC) is one of the most common types of malignant tumors in the digestive system. Despite the progress in the diagnosis and treatment of GC, the outcome of GC is still poor. Exploring a novel biomarker of GC will aid in establishing early diagnoses, improving treatment regimens and determining the prognosis for GC patients.

Research frontiers

Reactive oxygen species (ROS) represent a hot spot in the field of tumor research. The functions of ROS and oxidative stress *in vivo* are complicated, but in tumor studies, they have been demonstrated to be important participants in the initiation and progression of solid tumors. NADPH oxidase 2 (NOX2), as a main source of ROS, has been reported to be associated with the metastatic potential of many solid tumors and shows a close correlation with the angiogenesis of tumors. However, the expression of NOX2 in GC and its correlation with vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) have not been studied previously. In this study, the authors observed up-regulated expression of NOX2 in GC, and the expression of GC was positively correlated with the levels of VEGF and EGFR.

Innovations and breakthroughs

The results of this research showed that NOX2 expression was positively correlated with the levels of VEGF and EGFR. Patients with higher expression of NOX2 presented a worse outcome. NOX2 may be used as a novel indicator of a poor prognosis in patients with GC.

Applications

By studying the association of NOX2 with VEGF and EGFR and the prognosis of GC, this work may stimulate other researchers to pay special attention to the new biomarker NOX2. NOX2 could be included in the assessment of the prognosis of GC patients.

Terminology

NOX2 belongs to the NADPH oxidase family. It was the first identified member of the NADPH oxidase family, and its role in the progression of malignancy may be correlated with the promotion of angiogenesis. VEGF represents a key point in angiogenesis, which is the foundation for the growth and metastasis of tumors. EGFR, which is a major treatment bio-target for many cancers, is an important biomarker for the prognosis of tumors. A high expression level of EGFR may predict poor survival in GC.

Peer-review

Through the immunohistochemical and western blot analyses of NOX2 in 123 GC tissues and corresponding adjacent tissues, the authors found that NOX2 was up-regulated in the GC tissues compared with the adjacent tissues. The levels of NOX2 were positively correlated with VEGF and EGFR levels. The relationship of NOX2 expression with the clinicopathological features and prognosis of GC patients showed that NOX2 may present potential value in the prognosis of GC.

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ORIGINAL ARTICLE

Retrospective Study

Significance of the preoperative neutrophil-to-lymphocyte ratio in the prognosis of patients with gastric cancer

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Abstract

AIM: To investigate the significance of the preoperative neutrophil-to-lymphocyte ratio (NLR) in the prognosis of patients with gastric cancer (GC).

METHODS: The clinical data of 291 GC patients were analysed retrospectively; these patients were divided into two groups according to their preoperative NLR: a high-NLR group (NLR $\geqslant$ 3.5, 131 cases) and a low-NLR group (NLR < 3.5, 160 cases). The clinicopathological characteristics and five-year survival rates of the two groups were compared. The NLR and other clinicopathological factors were subjected to univariate and multivariate survival analysis to evaluate the effects of the NLR on the prognosis of GC patients.

RESULTS: The lowest preoperative NLR among the 291 patients was 0.56, whereas the highest preoperative NLR was 74.5. The mean preoperative NLR was 5.99 ± 8.98. Age, tumour size, T staging, tumour-node-metastasis (TNM) staging and platelet count were significantly different between the high- and low-NLR groups (P < 0.05). The five-year survival rate of the high-NLR group was 17.0%, which was significantly lower than that of the low-NLR group (43.6%; 17.0% *vs* 43.6%, P < 0.05). The univariate analysis results showed that the five-year survival rate was related to age, tumour size, T staging, N staging, TNM staging, carcinoembryonic antigen value and NLR (P < 0.05). Multivariate analysis results showed that the NLR was an independent risk factor that likely affected the five-year survival rate of GC patients (P = 0.003, HR = 0.626, 95%CI: 0.460-0.852).

CONCLUSION: The preoperative NLR could be used as a prognostic factor for GC patients; in particular, a high NLR corresponded to poor prognosis of GC patients.

Key words: Gastric cancer; Neutrophil-to-lymphocyte ratio; Prognosis; Inflammation; Survival rate

Core tip: This research preliminarily investigated the relationship between the preoperative neutrophil-to-lymphocyte ratio (NLR) and gastric cancer. The results revealed that a high NLR corresponded to poor prognosis of gastric cancer patients. Furthermore, preoperative NLR could be used as a prognostic factor for these patients.

Yu L, Lv CY, Yuan AH, Chen W, Wu AW. Significance of the preoperative neutrophil-to-lymphocyte ratio in the prognosis of patients with gastric cancer. *World J Gastroenterol* 2015; 21(20): 6280-6286 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6280.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6280

INTRODUCTION

Gastric cancer (GC) is one of the most common types of gastrointestinal cancer; the mortality of GC ranks second among all malignancies[1]. Although the incidence of GC declined in recent years, prognosis has not greatly improved, and the five-year accumulative survival rate remains at approximately 25%[2]. GC is mainly treated by radical surgery; thus, factors associated with the prognosis of GC should be determined to effectively assist intervention therapy and to improve patient outcomes. The body's inflammatory response plays an important role in tumour occurrence and development[3]. Inflammatory responses can inhibit apoptosis, promote angiogenesis and damage DNA, thereby promoting tumour growth and proliferation[4,5]. In cancer patients who are in the aggressive phase, inflammatory response indicators, such as C-reactive protein levels and platelet count, are usually higher and are related to poor prognosis[6,7]. Similarly, the body's inflammatory response can cause changes in the peripheral white blood cell count, which is reflected as an increased neutrophil count and reduced lymphocyte count[8]. Therefore, NLR could be used as a good indicator of the systemic inflammatory state of cancer patients. NLR is closely related to the prognosis of various malignant tumours, such as liver cancer, colorectal cancer, breast cancer, bladder cancer and non-small cell lung cancer[9-14]. However, few studies have investigated the relationships of NLR and prognosis of GC patients[15-17]. This study aimed to investigate the effects of preoperative NLR in the prognosis of GC patients; our study also provided a reference for diagnostic and treatment strategies for GC.

MATERIALS AND METHODS

General information

A total of 291 GC cases treated and subjected to radical surgery in the Department of General Surgery, Nanjing First Hospital, China, from January 2005 to December 2009 were selected. These patients were not subjected to preoperative chemotherapy and were not affected by infectious diseases. The intraoperative situation confirmed that no distant metastasis was present. The patients' clinical and pathological data were collected (Table 1). Postoperative regular telephone or outpatient follow up was performed for six months to five years; the follow-up rate was 91.1%. The clinicopathological staging of this research was in accordance with the criteria of American Joint Committee on Cancer Staging (7th edition)[18].

Blood sampling

Neutrophil, lymphocyte and platelet counts and carcinoembryonic antigen (CEA) values of the patients were collected one week before these patients underwent surgery. NLR was then calculated, and 3.5 was set as a critical value. The patients were then divided into two groups: high-NLR group (NLR $\geqslant$ 3.5) with 131 cases and low-NLR group (NLR < 3.5) with 160 cases.

Statistical analysis

Data were statistically analysed using SPSS 20.0 statistical software. Counted data were subjected to a χ^2 test. Variables likely to affect NLR were evaluated by logistic regression. The survival rate was calculated according to the Kaplan-Meier method. Survival rates were then compared by performing log-rank tests. Univariate and multivariate survival analyses were also conducted using a Cox proportional hazards model, in which $P < 0.05$ was considered statistically significant.

RESULTS

Relationships of preoperative NLR and other clinicopathological factors

The lowest preoperative NLR of the 291 patients was 0.56, whereas the highest NLR was 74.5. The mean NLR was 5.99 ± 8.98. The distributions of NLR were listed as follows: NLR < 1.5, 35 cases; 1.5 $\leqslant$ NLR < 2.5, 27 cases; 2.5 $\leqslant$ NLR < 3.5, 53 cases; 3.5 $\leqslant$ NLR < 4.5, 42 cases; 4.5 $\leqslant$ NLR < 5.5, 15 cases; and NLR $\geqslant$ 5.5, 74 cases. The compared *P* values among different survival-rate groups were as follows (Figure 1A): $P = 0.953$ (NLR < 1.5 and 1.5 $\leqslant$ NLR < 2.5); $P = 0.066$ (1.5 $\leqslant$ NLR < 2.5 and 2.5 $\leqslant$ NLR < 3.5); $P = 0.010$ (2.5 $\leqslant$ NLR < 3.5 and 3.5 $\leqslant$ NLR < 4.5); $P = 0.703$ (3.5 $\leqslant$ NLR < 4.5 and 4.5 $\leqslant$ NLR < 5.5); and $P = 0.852$ (4.5 $\leqslant$ NLR < 5.5 and NLR $\geqslant$ 5.5). On the basis of these results ($P = 0.010$; 2.5 $\leqslant$ NLR < 3.5 and 3.5 $\leqslant$ NLR < 4.5), we selected NLR = 3.5 as the threshold. The patients were then divided into a high-NLR group (NLR $\geqslant$ 3.5) and a low-NLR group (NLR < 3.5).

Age, tumour size, T staging, tumour-node-metastasis (TNM) staging and platelet count significantly differed between high- and low-NLR groups ($P < 0.05$).

Table 1 Comparison of clinicopathological characteristics between high- and low-neutrophil-to-lymphocyte groups *n* (%)

Clinicopathological feature	*n*	High-NLR group	Low-NLR group	χ^2	*P* value
Gender				0.163	0.686
Male	210	93 (44.3)	117 (55.7)		
Female	81	38 (46.9)	43 (53.1)		
Age				12.377	0.000
< 65 yr	142	49 (34.5)	93 (65.5)		
⩾ 65 yr	149	82 (55.0)	67 (45.0)		
Tumor size				20.852	0.000
< 5 cm	143	45 (31.5)	98 (68.5)		
⩾ 5 cm	148	86 (58.1)	62 (41.9)		
Differentiation degree					
Middle and high differentiation	130	59 (45.4)	71 (54.6)	0.013	0.910
Low differentiation	161	72 (44.7)	89 (55.3)		
T staging					
T1	20	2 (10.0)	18 (90.0)	20.731	0.000
T2	29	13 (41.4)	16 (58.6)		
T3	177	74 (41.8)	103 (58.2)		
T4	65	42 (64.6)	23 (35.4)		
N staging				4.185	0.242
N0	55	18 (32.7)	37 (67.3)		
N1	127	60 (47.2)	67 (52.8)		
N2	78	38 (48.7)	40 (51.3)		
N3	31	15 (48.4)	16 (51.6)		
TNM staging				11.363	0.003
Stage Ⅰ	32	8 (25.0)	24 (75.0)		
Stage Ⅱ	123	49 (39.8)	74 (60.2)		
Stage Ⅲ	136	74 (54.4)	62 (45.6)		
platelet counting				9.672	0.002
< 300×10^9/L	253	105 (41.5)	148 (58.5)		
⩾ 300×10^9/L	38	26 (68.4)	12 (31.6)		
CEA				2.972	0.085
< 5 ng/mL	178	73 (41.0)	105 (59.0)		
⩾ 5 ng/mL	113	58 (51.3)	55 (48.7)		

NLR: Neutrophil-to-lymphocyte ratio; CEA: Carcinoembryonic antigen.

By contrast, gender, differentiation degree, N staging and CEA values were not significantly different ($P > 0.05$). As the tumour invasion depth increased and clinicopathological staging progressed, the proportion of patients with high NLR correspondingly increased. The patients in the high-NLR group were older and exhibited larger tumours and high platelet counts (Table 1).

Logistic regression analysis was performed to evaluate the clinicopathological factors that likely caused the increased NLR. The results showed that age and tumour size were independent risk factors that possibly increased the NLR ($P < 0.05$; Table 2).

Effects of NLR on the prognosis of GC patients

The five-year survival rate of the high-NLR group was 17.0%, which was significantly lower than that of the low-NLR group (43.6%; $\chi^2 = 32.818$, $P < 0.001$, Figure 1B). The univariate analysis results showed that the five-year survival rate was related to age, tumour size, T staging, N staging, TNM staging, CEA value and NLR ($P < 0.001$). These parameters were then subjected to multivariate analysis. The results showed that TNM staging and NLR were independent prognostic factors for the five-year survival rate of patients ($P < 0.05$; Table 3).

Our data were subjected to further stratification analysis. Our results showed that the five-year survival rates of high- and low-NLR groups of stage Ⅰ patients were not significantly different ($\chi^2 = 0.732$, $P = 0.392$; Figure 1C). By contrast, the five-year survival rate of the high-NLR group of stage Ⅱ and stage Ⅲ patients was significantly lower than that of the low-NLR group ($\chi^2 = 12.299$, $P < 0.001$; $\chi^2 = 7.507$, $P = 0.006$; Figure 1D and E).

DISCUSSION

Abnormal phenotypes of malignant cancer cells likely stimulate the accumulation of inflammatory cells and destroy the tumour-surrounding tissues, thereby causing a series of non-specific inflammatory responses. As a tumour grows, these inflammatory responses likely increase the peripheral blood neutrophil count and decrease the lymphocyte count; as a result, NLR increases. This result is consistent with those of previous studies[15,16,19]. Our study further found that the proportion of patients in the high-NLR group increased as the tumour invasion depth increased and the disease progressed; this finding is

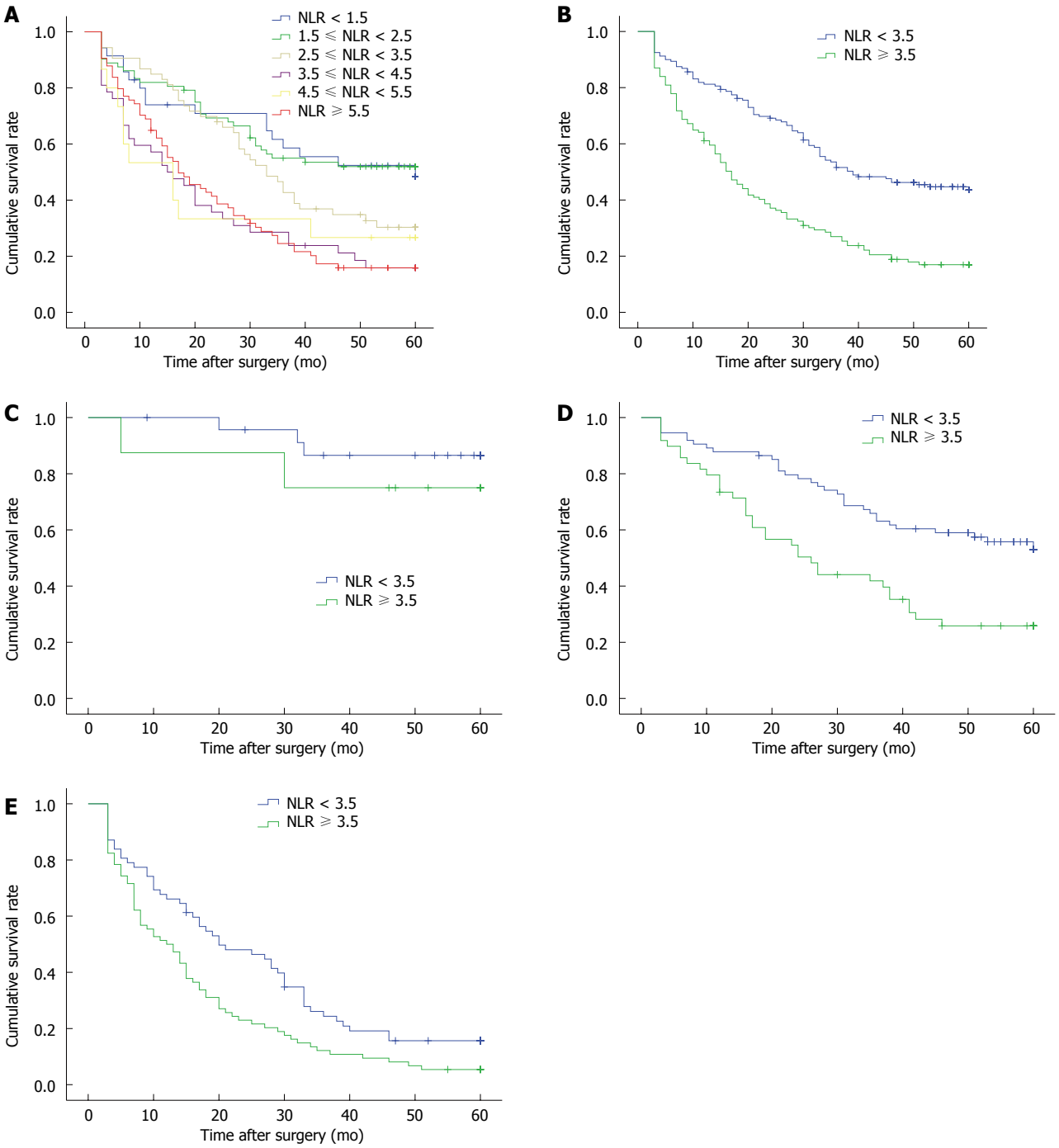

Figure 1 Five-year survival curves. A: All patients; B: High- and low-neutrophil-to-lymphocyte ratio groups; C: Stage Ⅰ patients; D: Stage Ⅱ patients; E: Stage Ⅲ patients. NLR: Neutrophil-to-lymphocyte ratio.

also consistent with those in previous studies. Shimada *et al*[15] performed a logistical regression analysis of clinicopathological factors that likely influence the increase in NLR and found that old age and high blood platelet count are independent risk factors of high NLR; the data of the present study showed that age and tumour size were independent risk factors that likely affected the increase in NLR.

High NLR is related to poor prognosis of patients with various malignant tumours[9,10,12,14]. Hirashima *et al*[17] revealed that NLR is related to the prognosis of patients with GC in the early stage; however, they did not further analyse whether NLR is an independent factor affecting the prognosis of GC patients. Jung *et al*[16] investigated patients with stage Ⅲ and Ⅳ GC and found that the overall survival rate of the high-NLR group (⩾ 2.0) was significantly lower than that of the low-NLR group. Indeed, NLR is an independent factor affecting patient's overall survival rate. Shimada *et al*[15] studied 1028 GC cases subjected to radical

Table 2 Multivariate analysis of neutrophil-to-lymphocyte ratio-associated risk factors

Clinicopathological feature	HR	95%CI	*P* value
Gender (M/F)	1.219	0.693-2.146	0.492
Age (yr)	1.036	1.014-1.059	0.002
Tumor size	2.690	1.584-4.565	0.000
Differentiation degree	0.966	0.575-1.622	0.895
T staging	1.269	0.705-2.287	0.427
N staging	0.743	0.453-1.219	0.239
TNM staging	1.732	0.680-4.409	0.249
Platelet counting	0.999	0.996-1.002	0.395
CEA	1.001	0.998-1.003	0.630

M/F: Male/female; CEA: Carcinoembryonic antigen; HR: Hazard ratio.

surgery and found that the five-year survival rate of patients with high NLR (≥ 4.0) was significantly lower than that of patients with low NLR. Similarly, Shimada *et al*[15] found that NLR is an independent factor affecting patient's five-year survival rate. Other scholars[20,21] also investigated patients with advanced GC treated with chemotherapy and found that high NLR is an independent risk factor influencing patient's disease-free survival period and overall survival rate. In our study, the effect on five-year survival rate of the patients with NLR ≥ 3.5 was apparent compared with that of patients with NLR < 3.5 possibly because NLR was related to the development of GC. Multivariate analysis results showed that NLR was an independent factor that likely affected the patient's five-year survival rate. Therefore, high preoperative NLR is an indicator of the poor prognosis of patients with GC.

Several explanations have been provided regarding the relationship of high NLR and poor prognosis. For instance, high NLR corresponds to an enhanced response of neutrophils to tumour inflammation; neutrophils secrete angiogenic factors, such as vascular endothelial growth factor, thereby stimulating angiogenesis and promoting tumour growth and metastasis[22]. Alternatively, peripheral blood lymphocytes are decreased, leading to reduced lymphocyte-mediated anti-tumour immune responses, which would accelerate disease progression. Furthermore, systemic inflammation is closely related to nutritional status and decreased organ function in cancer patients; thus, poor prognosis is observed[23].

High preoperative NLR indicated poor cancer prognosis; this result is very significant for cancer prevention and treatment. Moreover, the effects of anti-inflammatory drugs on tumour occurrence and development have been investigated extensively. For example, the prophylactic application of non-steroidal anti-inflammatory drugs (NSAIDs) can reduce the incidence of colon cancer by 40% to 50%; NSAIDs elicit the same preventive effects on lung cancer, oesophageal cancer and stomach cancer[24,25]. In addition, vaccination has been administered to promote an immune response of lymphocytes against

Table 3 Univariate and multivariate survival analysis results

Clinicopathological feature	Univariate-analyzed *P* value	Multivariate analysis	
		P value	HR (95%CI)
Gender	0.611		
Male			
Female			
Age	0.000	0.096	
< 65 yr			0.774 (0.573-1.046)
≥ 65 yr			1.000
Tumor size	0.000	0.122	
< 5 cm			0.784 (0.576-1.067)
≥ 5 cm			1.000
Differentiation degree	0.108		
Middle and high differentiation			
Low differentiation			
T staging	0.000	0.583	
T1		0.898	0.879 (0.122-6.338)
T2		0.579	0.797 (0.358-1.776)
T3		0.170	0.739 (0.480-1.138)
T4			1.000
N staging	0.000	0.080	
N0		0.303	0.622 (0.252-1.535)
N1		0.910	1.037 (0.556-1.934)
N2		0.091	0.678 (0.433-1.063)
N3			1.000
TNM staging	0.000	0.008	
Stage Ⅰ		0.030	0.134 (0.022-0.822)
Stage Ⅱ		0.004	0.387 (0.204-0.735)
Stage Ⅲ			1.000
Platelet counting	0.382		
< 300 × 10^9/L			
≥ 300 × 10^9/L			
CEA	0.000	0.547	
< 5 ng/mL			0.912 (0.675-1.231)
≥ 5 ng/mL			1.000
NLR	0.000	0.003	
< 3.5			0.626 (0.460-0.852)
≥ 3.5			1.000

NLR: Neutrophil-to-lymphocyte ratio; CEA: Carcinoembryonic antigen; HR: Hazard ratio.

tumours, thereby improving patient prognosis[26]. Indeed, patients with high preoperative NLR should be considered as high-risk patients who should be integrated with multi-mode anti-tumour therapies, such as chemotherapy, radiotherapy and immune therapy.

In summary, preoperative NLR was closely related to the prognosis of GC; in particular, a high NLR was an indicator that could be used to determine the poor prognosis of patients with GC. NLR could be determined using a simple, rapid and cost-effective detection technique; this technique could be applied efficiently to predict the prognosis of GC patients and to provide a reference for the integrated treatment of GC for broad applications.

COMMENTS

Background

Gastric cancer (GC) is one of the most common types of gastrointestinal

cancers; however, the prognosis of GC is poor. The body's inflammatory response plays an important role in tumour development. The neutrophil-to-lymphocyte ratio (NLR), which indicates the systemic inflammatory state of the body, is closely related to the prognosis of GC.

Research frontiers

NLR is closely related to the prognosis of various malignant tumours, such as liver cancer, colorectal cancer, breast cancer, bladder cancer and non-small cell lung cancer. However, few studies have investigated the relationships of NLR and prognosis of GC patients.

Innovations and breakthroughs

This study revealed that NLR was an independent risk factor that likely affected the five-year survival rate of GC patients.

Applications

A high NLR was one indicator that could be used to evaluate the poor prognosis of patients with GC. This finding suggested that NLR might provide a reference of the integrated treatment for patients with GC. NLR could be determined using a simple, rapid and cost-effective technique; thus, this technique could be used to predict the prognosis of patients with GC.

Terminology

The neutrophil-to-lymphocyte ratio, calculated as neutrophil counts divided by lymphocyte counts, is a possible marker of general immune responses to various stress stimuli.

Peer-review

This study investigated the significance of NLR retrospectively in patients who received surgical therapy to treat GC. The results are significant and applicable to clinical practices and studies.

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ORIGINAL ARTICLE

Observational Study

Cholecystectomy is independently associated with nonalcoholic fatty liver disease in an Asian population

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Data sharing: The technical appendix, statistical code and dataset are available from the corresponding author at messmd@chol.com. No additional data are available.

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Abstract

AIM: To investigate the relationship between gallstone disease and nonalcoholic fatty liver disease (NAFLD) in a large Asian population.

METHODS: A cross-sectional study including 17612 subjects recruited from general health check-ups at the Seoul National University Hospital, Healthcare System Gangnam Center between January 2010 and December 2010 was conducted. NAFLD and gallstone disease were diagnosed based on typical ultrasonographic findings. Subjects who were positive for hepatitis B or C, or who had a history of heavy alcohol consumption (> 30 g/d for men and > 20 g/d for women) or another type of hepatitis were excluded. Gallstone disease was defined as either the presence of gallstones or previous cholecystectomy, and these two entities (gallstones and cholecystectomy) were analyzed separately. Clinical parameters including body mass index, waist circumference, hypertension, diabetes, smoking status, and regular physical activity were reviewed. Laboratory parameters, including serum levels of gamma-glutamyl transpeptidase, alanine aminotransferase, aspartate aminotransferase, fasting glucose, fasting insulin, total cholesterol, triglycerides, and high-density lipoprotein, were also reviewed.

RESULTS: The mean age of the subjects was 48.5

± 11.3 years, and 49.3% were male. Approximately 30.3% and 6.1% of the subjects had NAFLD and gallstone disease, respectively. The prevalence of gallstone disease (8.3% *vs* 5.1%, $P < 0.001$), including both the presence of gallstones (5.5% *vs* 3.4%, $P < 0.001$) and a history of cholecystectomy (2.8% *vs* 1.7%, $P < 0.001$), was significantly increased in the NAFLD group. In the same manner, the prevalence of NAFLD increased with the presence of gallstone disease (41.3% *vs* 29.6%, $P < 0.001$). Multivariate regression analysis showed that cholecystectomy was associated with NAFLD (OR = 1.35, 95%CI: 1.03-1.77, $P = 0.028$). However, gallstones were not associated with NAFLD (OR = 1.15, 95%CI: 0.95-1.39, $P = 0.153$). The independent association between cholecystectomy and NAFLD was still significant after additional adjustment for insulin resistance (OR = 1.45, 95%CI: 1.01-2.08, $P = 0.045$).

CONCLUSION: This study shows that cholecystectomy, but not gallstones, is independently associated with NAFLD after adjustment for metabolic risk factors. These data suggest that cholecystectomy may be an independent risk factor for NAFLD.

Key words: Fatty liver; Hepatic steatosis; Gallbladder; Cholelithiasis; Gallbladder removal

Core tip: The relationship between gallstone disease (gallstones and cholecystectomy, separately) and ultrasonographically diagnosed nonalcoholic fatty liver disease (NAFLD) was analyzed in a large Asian population. The prevalence of gallstone disease increased with the presence of NAFLD, and the prevalence of NAFLD increased with the presence of gallstone disease. Multivariate regression analysis showed that cholecystectomy was associated with NAFLD. However, gallstones were not associated with NAFLD. The independent association between cholecystectomy and NAFLD was still significant after additional adjustment for insulin resistance. This study showed that cholecystectomy, but not gallstones, is independently associated with NAFLD after adjustment for metabolic risk factors.

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is one of the most common liver diseases, with a prevalence of 20%-35% in the general population[1,2]. NAFLD includes a spectrum of liver diseases, from simple steatosis to nonalcoholic steatohepatitis, cirrhosis, and hepatocellular carcinoma[3,4]. As obesity, type 2 diabetes, dyslipidemia, and insulin resistance are the underlying metabolic conditions that favor the occurrence of NAFLD, NAFLD is regarded as the hepatic manifestation of metabolic syndrome[5]. Gallstone disease is also common, and the prevalence of gallstones varies between 5% and 25%[6,7]. Increased age, female sex, obesity, metabolic syndrome, hypertriglyceridemia, diabetes, and insulin resistance are considered to be the major risk factors for gallstones[8,9].

As mentioned above, gallstone disease and NAFLD are both prevalent in the general population and share the same risk factors, including obesity and insulin resistance. Therefore, several studies have investigated the association between gallstone disease and NAFLD and have demonstrated an independent association between them[8,10]. One study demonstrated a dose-dependent association between the severity of hepatic inflammation or fibrosis and the prevalence of gallstone disease[11]. On the contrary, another study showed no association between gallstone disease and the severity of fibrosis in NAFLD patients[12]. Recently, a population-based study using the National Health and Nutrition Examination Survey Ⅲ, evaluated gallstone disease by separating patients according to the presence of either gallstones or a history of cholecystectomy. This study revealed an association between cholecystectomy and NAFLD, but no association between gallstones and NAFLD, suggesting that cholecystectomy has metabolic consequences[13]. As discussed above, previous studies showed inconsistent results regarding the association between gallstone disease and NAFLD.

Therefore, the purpose of this study was to investigate the relationship between gallstone disease (including gallstones and cholecystectomy) and NAFLD in a large Asian population.

MATERIALS AND METHODS

Study population

Subjects who voluntarily visited the Seoul National University Hospital, Healthcare System Gangnam Center, for a health check-up between January 2010 and December 2010 were initially enrolled. Most of the screenees routinely underwent hepatic ultrasonography and blood sampling as part of their health care program. Of the 24550 initially enrolled subjects, patients with other causes of chronic liver disease were excluded as follows: 267 for hepatitis C (diagnosed by a positive hepatitis C antibody); 1186 for hepatitis B (diagnosed by a positive hepatitis B surface antigen); 3926 for excessive alcohol consumption (defined as > 30 g/d for men and > 20 g/d for women); and 105 for

a history of other liver diseases (*e.g.*, Wilson's disease, autoimmune hepatitis, primary biliary cirrhosis, and hemochromatosis). We also excluded 697 subjects who had taken drugs that can cause fatty liver within the past year. Two subjects who were found to have gallbladder cancer on abdominal ultrasonography were also excluded. Additionally, 755 subjects who did not answer the questionnaire regarding alcohol drinking, smoking status, exercise, and past medical history were excluded. Therefore, 17612 subjects were finally included in this analysis. The study was approved by the Institutional Review Board of Seoul National University Hospital (H1309-019-518) and was performed according to the ethical guidelines of the 1975 Declaration of Helsinki and its later amendments. The need to obtain informed consent from the subjects was waived by the Institutional Review Board of Seoul National University Hospital. A corresponding author and all the co-authors had access to the full data of this study and reviewed and approved the manuscript.

Definition of NAFLD by ultrasonographic examination

Hepatic ultrasonography was performed by experienced radiologists. At the time of the procedure, the radiologists were blinded to the laboratory and clinical data of the subjects. Fatty liver was diagnosed by ultrasonographic findings (Acuson, Sequoia 512, Siemens, Mountain View, CA, United States), based on liver brightness, hepatorenal echo contrast, vascular blurring, and deep attenuation[14].

NAFLD was defined as the presence of fatty liver by ultrasonography without the presence of the following other possible causes of chronic liver disease: (1) excessive alcohol consumption (defined as > 30 g/d for men and > 20 g/d for women); (2) positivity for antibodies against the hepatitis C virus or the hepatitis B surface antigen; (3) other known causes of chronic liver disease; and (4) the use of drugs that can cause fatty liver.

Definition of gallstone disease

Gallstone disease was diagnosed by experienced radiologists using ultrasonography (Acuson, Sequoia 512, Siemens, Mountain View, CA, United States) after the subjects had fasted for at least 8 h. Gallstone disease was defined as the ultrasonographic presence of gallstones or absence of the gallbladder on ultrasonography due to a previous history of cholecystectomy. Gallstones were diagnosed based on the presence of movable hyper-echoic foci with acoustic shadows.

Clinical and laboratory assessments

Each subject answered a questionnaire regarding past medical history, including previous history of cholecystectomy. Anthropometric measurements and laboratory tests were performed on the same day. Waist circumference was measured by a trained nurse using a tape placed at the midpoint between the iliac crest and the lower costal margin. Height and weight were measured using a digital scale, and body mass index (BMI) was calculated using the following formula: BMI = weight (kg)/height squared (m^2). Systolic and diastolic blood pressures were checked twice, and the mean values of the two measurements were used. Hypertension was defined as the current use of anti-hypertensive drugs, a systolic blood pressure over 140 mmHg, or a diastolic blood pressure over 90 mmHg. The presence of diabetes was defined as the current use of anti-diabetic drugs or a fasting glucose level greater than or equal to 126 mg/dL. Current smokers were defined as subjects who had smoked at least 100 cigarettes in their lifetime and who smoked either every day or on some days during the previous year. Ex-smokers were defined as subjects who reported smoking at least 100 cigarettes in their lifetime and who had not smoked during the previous year. Regular physical activity was defined as regularly exercising more than once per week.

Laboratory examinations included serum gamma-glutamyl transpeptidase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol, triglycerides, high-density lipoprotein (HDL), fasting glucose, HbA1c, fasting insulin, antibodies against the hepatitis C virus, and hepatitis B surface antigen. Blood sampling was performed before 10 am after an overnight fast. All the biochemical examinations were performed in the same laboratory according to standard laboratory methods. The homeostasis model assessment-estimated insulin resistance (HOMA-IR) was used to assess insulin resistance as follows: HOMA-IR = fasting plasma glucose (mmol/L) × fasting plasma insulin (μIU/mL)/22.5[15].

Statistical analysis

To compare the variables between subjects according to gallstone disease status (control, gallstones, cholecystectomy) and between subjects with and without NAFLD, the Student's *t*-test was used for continuous variables and the χ^2-test was used for categorical variables. Multivariate logistic regression analysis was performed including previously established risk factors and variables with a *P* value < 0.05 in the unadjusted analyses. SPSS 19 (SPSS Inc., Chicago, IL, United States) software was used. A two-tailed *P* value < 0.05 was considered statistically significant.

The statistical methods of this study were reviewed by Seung-sik Hwang from Inje University School of Medicine.

RESULTS

A total of 17612 individuals (8682 males and 8930 females, mean age, 48.5 years) were ultimately

Table 1 Baseline characteristics of control and nonalcoholic fatty liver disease subjects

	Control (P = 12275)	NAFLD (P = 5337)	P value
Age (yr)	47.4 ± 11.4	50.9 ± 10.5	< 0.001
Male	4839 (39.4)	3843 (72.0)	< 0.001
Waist circumference (cm)	80.3 ± 7.2	89.2 ± 6.9	< 0.001
Body mass index (kg/m^2)	22.0 ± 2.6	25.4 ± 2.7	< 0.001
Systolic blood pressure (mmHg)	111.3 ± 14.3	119.3 ± 13.2	< 0.001
Diastolic blood pressure (mmHg)	71.4 ± 11.0	78.1 ± 10.5	< 0.001
Hypertension	1570 (12.8)	1451 (27.2)	< 0.001
Diabetes	373 (3.0)	498 (9.3)	< 0.001
Gamma-glutamyl transpeptidase (IU/L)	24.7 ± 31.3	42.7 ± 39.8	< 0.001
Alanine aminotransferase (IU/L)	19.1 ± 16.1	33.0 ± 23.5	< 0.001
Aspartate aminotransferase (IU/L)	20.7 ± 11.5	25.6 ± 12.8	< 0.001
Total cholesterol (mg/dL)	191.6 ± 32.9	200.5 ± 34.9	< 0.001
Triglycerides (mg/dL)	85.9 ± 50.0	144.8 ± 84.1	< 0.001
HDL-cholesterol (mg/dL)	57.6 ± 12.3	48.9 ± 9.6	< 0.001
Fasting glucose (mg/dL)	92.4 ± 12.7	102.5 ± 19.8	< 0.001
HbA1c (%)	5.7 ± 0.4	6.0 ± 0.7	< 0.001
Fasting insulin, μIU/mL (n = 8622)	5.8 ± 3.4	9.6 ± 5.5	< 0.001
HOMA-IR index (n = 8622)	1.4 ± 1.0	2.5 ± 1.6	< 0.001
Gallstones	421 (3.4)	292 (5.5)	< 0.001
Cholecystectomy	207 (1.7)	149 (2.8)	< 0.001
Gallstone disease	628 (5.1)	441 (8.3)	< 0.001
Smoking			
Never-smoker	8300 (67.6)	2365 (44.3)	< 0.001
Current smoker	1419 (11.6)	1114 (20.9)	
Ex-smoker	2556 (20.8)	1858 (34.8)	
Regular physical activity	8112 (66.1)	3496 (65.5)	0.455

Data are presented as the mean ± SD or n (%). NAFLD: Nonalcoholic fatty liver disease; IR: Insulin resistance.

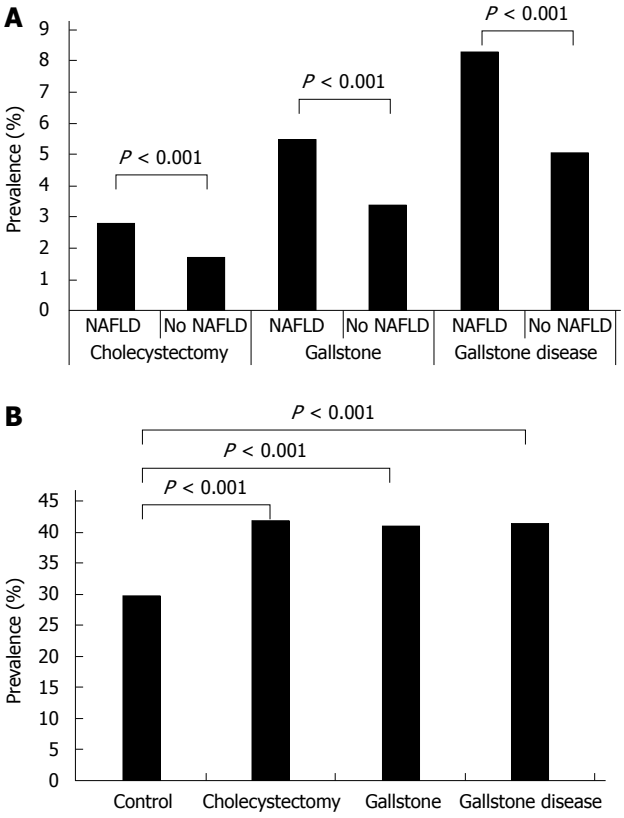

Figure 1 Prevalence of disease in subjects with or without nonalcoholic fatty liver disease. A: Prevalence of cholecystectomy, gallstones, and gallstone disease in subjects with and without nonalcoholic fatty liver disease (NAFLD). Cholecystectomy, gallstones, and gallstone disease were more commonly observed in subjects with NAFLD compared with subjects without NAFLD (P < 0.001 for all); B: The prevalence of NAFLD in the control, cholecystectomy, gallstone, and gallstone disease groups. NAFLD was significantly more likely in the cholecystectomy, gallstone and gallstone disease groups compared with the control group (all P < 0.001). NAFLD: Nonalcoholic fatty liver disease.

analyzed in this study. Of these, 5337 (30.3%) had NAFLD. Table 1 shows the baseline characteristics of the controls and subjects with NAFLD. The following factors were significantly associated with NAFLD: increased age; male sex; larger waist circumference; higher BMI; higher blood pressure; the presence of hypertension and diabetes; elevated levels of GGT, ALT, AST, cholesterol, triglycerides, fasting glucose, HbA1c, and HOMA-IR; and lower levels of HDL cholesterol (all P < 0.001). The presence of gallstones (5.5% *vs* 3.4%, P < 0.001) and a history of cholecystectomy (2.8% *vs* 1.7%, P < 0.001) were both significantly increased in the NAFLD group (Figure 1A).

Table 2 shows the baseline characteristics of the subjects according to their gallstone disease status (control, gallstones, cholecystectomy, and gallstone disease). Approximately 6.1% of subjects (n = 1069) had gallstone disease. Compared with the control group, subjects in the gallstone disease group were older. They also had larger waist circumference, a higher BMI, and elevated levels of GGT, ALT, AST, triglycerides, fasting glucose, HbA1c, and HOMA-IR, as well as lower levels of HDL cholesterol (all P < 0.001). There were more subjects with diabetes and hypertension in the gallstone disease group (P < 0.001). Figure 1B shows that the rate of NAFLD was increased in the gallstone (41.0% *vs* 29.6%, P < 0.001), cholecystectomy (41.9% *vs* 29.6%, P < 0.001), and gallstone disease (41.3% *vs* 29.6%, P < 0.001) groups.

When we analyzed the association between gallstone disease and NAFLD, gallstone disease (OR = 1.67, 95%CI: 1.47-1.90, P < 0.001), cholecystectomy (OR = 1.67, 95%CI: 1.35-2.07, P < 0.001), and gallstones (OR = 1.65, 95%CI: 1.42-1.92, P < 0.001) were significantly associated with NAFLD in the unadjusted analyses. Gallstone disease was independently associated with NAFLD after adjustment for age and sex (OR = 1.47, 95%CI: 1.28-1.68, P < 0.001). Cholecystectomy (OR = 1.46, 95%CI: 1.16-1.83, P = 0.001) and gallstones (OR = 1.46, 95%CI: 1.24-1.72, P < 0.001) were also independently associated with NAFLD in the age- and sex-adjusted model. In the multivariate model, after adjusting for other covariates such as BMI, smoking, physical activity, hypertension, diabetes, total cholesterol,

Table 2 Baseline characteristics of the subjects according to gallstone disease status

	Control (n = 16543)	Gallstone disease (n = 1069)	Gallstone disease (n = 1069)	
			Gallstones (n = 713)	Cholecystectomy (n = 356)
Age (yr)	48.1 ± 11.1	54.5 ± 11.4[a]	53.8 ± 11.2[b]	55.8 ± 11.5[c]
Male	8122 (49.1)	560 (52.4)[a]	377 (52.9)[b]	183 (51.4)
Waist circumference (cm)	82.8 ± 8.2	86.4 ± 8.5[a]	86.3 ± 8.2[b]	86.3 ± 7.7[c]
Body mass index (kg/m^2)	23.0 ± 3.1	24.1 ± 3.2[a]	24.0 ± 3.1[b]	23.9 ± 2.9[c]
Systolic blood pressure (mmHg)	113.5 ± 14.4	116.3 ± 15.2[a]	117.0 ± 15.0[b]	118.4 ± 14.5[c]
Diastolic blood pressure (mmHg)	73.3 ± 11.3	75.2 ± 11.4[a]	75.2 ± 11.2[b]	75.2 ± 10.6[c]
Hypertension	2710 (16.4)	311 (29.1)[a]	205 (28.8)[b]	106 (29.8)[c]
Diabetes	771 (4.7)	100 (9.4)[a]	67 (9.4)[b]	33 (9.3)[c]
Gamma-glutamyl transpeptidase (IU/L)	29.8 ± 32.9	34.6 ± 48.8[a]	35.7 ± 58.8[b]	38.0 ± 75.0[c]
Alanine aminotransferase (IU/L)	23.1 ± 19.8	25.9 ± 18.6[a]	26.0 ± 17.9[b]	26.1 ± 16.4[c]
Aspartate aminotransferase (IU/L)	22.1 ± 12.2	23.2 ± 11.1[a]	23.6 ± 10.7[b]	24.5 ± 9.9[c]
Total cholesterol (mg/dL)	194.4 ± 33.7	193.4 ± 33.7	192.5 ± 33.6	190.6 ± 33.4[c]
Triglycerides (mg/dL)	103.2 ± 68.0	111.1 ± 62.3[a]	112.9 ± 65.9[b]	116.4 ± 72.5[c]
HDL-cholesterol (mg/dL)	55.1 ± 12.2	53.2 ± 12.1[a]	53.3 ± 12.2[b]	53.7 ± 12.6[c]
Fasting glucose (mg/dL)	95.2 ± 15.5	99.6 ± 20.4[a]	99.6 ± 20.8[b]	99.5 ± 17.0[c]
HbA1c (%)	5.8 ± 0.5	5.9 ± 0.7[a]	5.9 ± 0.6[b]	6.0 ± 0.5[c]
Fasting insulin, μIU/mL (n = 8622)	6.9 ± 4.4	8.3 ± 6.2[a]	8.3 ± 6.0[b]	8.3 ± 5.7[c]
HOMA- IR index (n = 8622)	1.7 ± 1.3	2.1 ± 1.7[a]	2.1 ± 1.7[b]	2.2 ± 1.7[c]
NAFLD	4896 (29.6)	441 (41.3)[a]	292 (41.0)[b]	149 (41.9)[c]
Smoking				
Never-smoker	10042 (60.7)	623 (58.3)[a]	419 (58.8)	204 (57.3)[c]
Current smoker	2398 (14.5)	135 (12.6)[a]	95 (13.3)	40 (11.2)[c]
Ex-smoker	4103 (24.8)	311 (29.1)[a]	199 (27.9)	112 (31.5)[c]
Regular physical activity	10835 (65.5)	773 (72.3)[a]	521 (73.1)[b]	252 (70.8)[c]

Data are presented as the mean ± SD or n (%). [a]P < 0.05, control *vs* gallstone disease; [b]P < 0.05, control *vs* gallstone; [c]P < 0.05, control *vs* cholecystectomy. NAFLD: Nonalcoholic fatty liver disease; GSD: Gallstone disease; IR: Insulin resistance.

triglycerides, and HDL cholesterol, in addition to age and sex, subjects with gallstone disease had an increased risk of NAFLD (OR = 1.22, 95%CI: 1.04-1.43, P = 0.016). When cholecystectomy and gallstones were analyzed separately, cholecystectomy was independently associated with NAFLD in the multivariate model (OR = 1.35, 95%CI: 1.03-1.77, P = 0.028). In other words, subjects who underwent cholecystectomy had a 35% higher risk of NAFLD compared with subjects who had not undergone cholecystectomy. However, the presence of gallstones was not independently associated with NAFLD after adjusting for known metabolic risk factors (OR = 1.15, 95%CI: 0.95-1.39, P = 0.153) (Table 3). Similarly, when waist circumference, which is a surrogate marker for visceral obesity, was additionally accounted for in the multivariate analysis, cholecystectomy, but not gallstones, was independently associated with NAFLD (data not shown).

A subgroup analysis was conducted in 8622 subjects in whom fasting insulin was examined. The baseline characteristics of subjects with or without fasting insulin measurements are shown in Table 4. In the group whose fasting insulin levels were checked, there were more older subjects and male subjects with hypertension or diabetes. Gallstone disease was associated with NAFLD (at a marginal level of significance) after adjusting for insulin resistance in addition to metabolic risk factors (OR = 1.25, 95%CI: 1.00-1.55, P = 0.052) (Table 5). Cholecystectomy was independently associated with NAFLD in the multivariate model (OR = 1.45, 95%CI: 1.01-2.08, P = 0.045), but gallstones were not (OR = 1.14, 95%CI: 0.87-1.50, P = 0.336).

DISCUSSION

This study demonstrated that gallstone disease is associated with NAFLD, independent of well-established, common metabolic risk factors. This association was mainly attributable to a history of cholecystectomy, not the presence of gallstones. Subjects who underwent cholecystectomy had a 35% higher prevalence of NAFLD. However, gallstones were not independently associated with NAFLD.

The association between gallstone disease and NAFLD has been evaluated in several studies; however, most previous studies did not differentiate between gallstones and cholecystectomy[8,11,16]. Therefore, the effect of cholecystectomy itself has not been fully investigated. An *in vivo* study suggested that cholecystectomy has metabolic consequences by demonstrating that cholecystectomized mice had increased levels of hepatic and serum triglycerides and very low-density lipoprotein[17]. Recently, Ruhl *et al*[13] reported an independent association between NAFLD and cholecystectomy, but not between NAFLD and gallstones, indicating cholecystectomy *per se* is a risk factor for NAFLD in the United States. However, it is hard to apply this result directly to Asian populations

Table 3 Univariate and multivariate analyses for the presence of nonalcoholic fatty liver disease according to gallstone disease status

Variable	Univariate model		Age, sex-adjusted model		Multivariate model[1]	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Gallstone disease						
Control (*n* = 16543)	1		1		1	
Gallstone disease (*n* = 1069)	1.67 (1.47-1.90)	< 0.001	1.47 (1.28-1.68)	< 0.001	1.22 (1.04-1.43)	0.016
Cholecystectomy						
Control	1		1		1	
Cholecystectomy (*n* = 356)	1.67 (1.35-2.07)	< 0.001	1.46 (1.16-1.83)	0.001	1.35 (1.03-1.77)	0.028
Gallstones						
Control	1		1		1	
Gallstones (*n* = 713)	1.65 (1.42-1.92)	< 0.001	1.46 (1.24-1.72)	< 0.001	1.15 (0.95-1.39)	0.153

[1]The multivariate model was adjusted for age, sex, hypertension, diabetes, body mass index, smoking, physical activity, total cholesterol, triglycerides and HDL cholesterol. NAFLD: Nonalcoholic fatty liver disease; OR: Odds ratio.

Table 4 Comparison of characteristics between subjects who underwent fasting insulin testing and those who did not

	Fasting insulin absent (*n* = 8990)	Fasting insulin available (*n* = 8622)	*P* value
Age (yr)	45.8 ± 11.8	51.2 ± 9.9	< 0.001
Male	4808 (46.5)	4748 (55.1)	< 0.001
Waist circumference (cm)	82.8 ± 8.3	83.3 ± 8.1	< 0.001
Body mass index (kg/m^2)	23.0 ± 3.1	23.1 ± 3.0	0.167
Systolic blood pressure (mmHg)	113.2 ± 14.2	114.3 ± 14.6	< 0.001
Diastolic blood pressure (mmHg)	73.3 ± 11.3	73.9 ± 11.3	0.020
Hypertension	1300 (14.5)	1721 (20.0)	< 0.001
Diabetes	356 (4.0)	515 (6.0)	< 0.001
Gamma-glutamyl transpeptidase (IU/L)	30.1 ± 30.7	30.3 ± 39.0	0.732
Alanine aminotransferase (IU/L)	23.1 ± 19.4	23.5 ± 20.0	0.142
Aspartate aminotransferase (IU/L)	21.8 ± 12.4	22.6 ± 11.8	< 0.001
Total cholesterol (mg/dL)	193.0 ± 33.3	195.6 ± 34.1	< 0.001
Triglycerides (mg/dL)	105.0 ± 70.2	102.5 ± 65.5	0.017
HDL-cholesterol (mg/dL)	55.0 ± 12.0	55.0 ± 12.5	0.693
Fasting glucose (mg/dL)	95.4 ± 14.9	95.6 ± 16.9	0.606
HbA1c (%)	5.77 ± 0.51	5.84 ± 0.56	< 0.001
NAFLD	2619 (29.1)	2718 (31.5)	0.001
Gallstone	336 (3.7)	377 (4.4)	0.033
Cholecystectomy	160 (1.8)	196 (2.3)	0.020
Gallstone disease	496 (5.5)	573 (6.6)	0.002
Smoking			< 0.001
Never-smoker	5300 (59.0)	5365 (62.2)	
Current smoker	1440 (16.0)	1093 (12.7)	
Ex-smoker	2250 (25.0)	2164 (25.1)	
Regular physical activity	5559 (61.8)	6049 (70.2)	< 0.001

Data are presented as the mean ± SD or *n* (%). NAFLD: Nonalcoholic fatty liver disease; IR: Insulin resistance.

because the prevalence of and risk factors for gallstone disease vary across ethnicities[9,18,19]. In general, BMI, one of the risk factors for gallstones, is lower in Asians compared with Western populations; however, Asians have a higher risk of visceral obesity than Caucasian populations with the same BMI[20,21]. Thus, differences in general obesity, as assessed by BMI and visceral obesity, may have some effect on the association between gallstone disease and NAFLD. To date, studies evaluating the association between gallstone disease and NAFLD in Asian populations are scarce. This is the largest study confirming the independent association between cholecystectomy, but not gallstones, and NAFLD in an Asian population. This study supports the idea that cholecystectomy may have some effect on the development of NAFLD.

There are several possible mechanisms for this relationship: (1) Because the gallbladder regulates bile acid homeostasis, alterations in bile acid metabolism after cholecystectomy may alter glucose and lipid metabolism, causing NAFLD[22,23]. Bile acids exercise their action by binding to nuclear receptors, such as the farnesoid X receptor and TGR5, leading to gene expression changes in the liver[24,25]. The farnesoid X receptor plays an important role not only in maintaining cholesterol and bile acid homeostasis, but also in the regulation of many metabolic enzymes and transporters[26]. TGR5 also plays crucial roles in lipid metabolism, glucose homeostasis, and energy expenditure[27]. Thus, it can be inferred that

Table 5 Univariate and multivariate analyses for the presence of nonalcoholic fatty liver disease according to gallstone disease status in subjects in whom fasting insulin testing was performed

Variable	Univariate model		Age, sex-adjusted model		Multivariate model 1[1]		Multivariate model 2[2]	
	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value	OR (95%CI)	*P* value
Gallstone disease								
Control (*n* = 8049)	1		1		1		1	
Gallstone disease (*n* = 573)	1.67 (1.41-1.99)	< 0.001	1.51 (1.26-1.82)	< 0.001	1.27 (1.03-1.58)	0.029	1.25 (1.00-1.55)	0.052
Cholecystectomy								
Control	1		1		1		1	
Cholecystectomy (*n* = 196)	1.76 (1.32-2.35)	< 0.001	1.59 (1.17-2.16)	0.003	1.45 (1.01-2.07)	0.044	1.45 (1.01-2.08)	0.045
Gallstones								
Control	1		1		1		1	
Gallstones (*n* = 377)	1.63 (1.32-2.01)	< 0.001	1.48 (1.18-1.85)	0.001	1.18 (0.91-1.54)	0.21	1.14 (0.87-1.50)	0.336

[1]The multivariate model 1 was adjusted for age, sex, hypertension, diabetes, body mass index, smoking, physical activity, total cholesterol, triglycerides and high-density lipoprotein cholesterol; [2]The multivariate model 2 was adjusted for multivariate model 1 in addition to insulin resistance as assessed by HOMA-IR. NAFLD: Nonalcoholic fatty liver disease; IR: Insulin resistance; OR: Odds ratio; HOMA-IR: Homeostasis model assessment-estimated insulin resistance.

cholecystectomy may alter the circulation of bile acid, the activation of bile acid receptors, and the downstream signaling pathways related to hepatic lipid and glucose metabolism, thereby contributing to the development of NAFLD; (2) Gallbladder-related hormonal effects represent another plausible explanation, whereby fibroblast growth factor 19 (FGF 19), which is secreted from the gallbladder mucosa and regulates the synthesis of bile salts, has a beneficial effect on the metabolic syndrome[28]. *In vitro* and *in vivo* studies have demonstrated the inhibitory effect of FGF-19 on hepatic fatty acid synthesis[29,30]. Lower serum FGF-19 levels were reported in NAFLD patients[31], and cholecystectomy reduces FGF-19 levels[32]. Therefore, it can be inferred that decreased FGF-19 levels after cholecystectomy may increase the hepatic triglyceride content, thereby exerting some effect on the development of NAFLD[13,33]. Although insulin resistance is a well-known risk factor for both gallstones and NAFLD, the association between NAFLD and cholecystectomy persisted with only a minimal change after additional adjustment for insulin resistance. This is similar to the results of previous studies[13,34]; and (3) There may be an association between pain or inflammatory symptoms, which are associated with gallbladder pathology, before cholecystectomy and the occurrence of NAFLD[35]. A recent population-based study[13] demonstrated that cholecystectomy in subjects with pain had a lower OR for NAFLD than cholecystectomy in subjects without pain. As we did not have any data concerning abdominal pain or biliary colic due to our study design, we could not evaluate the effect of pain on the association between cholecystectomy and NAFLD. Further prospective studies should be conducted to investigate the exact mechanism of the association between cholecystectomy and NAFLD.

In contrast to our study, several previous studies have demonstrated an independent association between gallstones and NAFLD[8,10,36]. This contradiction may be due to different definitions of NAFLD or insufficient adjustment for NAFLD risk factors. In other studies, NAFLD was defined by AST/ALT levels, which often underestimate and misclassify NAFLD. In our study, we defined NAFLD by ultrasonography and sufficiently adjusted for metabolic risk factors, including insulin resistance. Another plausible explanation is that ethnic differences may affect the association between gallstones and NAFLD. Ethnic differences are observed in the prevalence of gallstone disease; specifically, the prevalence of gallstones is reported to be as high as 60% to 70% in American Indians, 25% to 30% in Hispanic populations in Central and South America, and 10% to 15% in Caucasian adults in developed countries. A low prevalence is reported in African Americans and East Asians[9,37]. In Asia, the prevalence of gallstone disease was reported to be 3.2% in Japan, 3.1% in India, and 10.7% in Taiwan[10,38,39]. Similar to other Asian countries, the prevalence of gallstone disease in this Korean population was 6.1%.

This study has strengths compared with previous studies. First, the subjects in this study were representative of the general population, considering the nature of the health screenings. Thus, due to the sufficiently large sample size, we could provide more definitive evidence for an independent association between cholecystectomy itself and NAFLD, consistent with a previous report[13]. Second, this study confirmed the independent association between cholecystectomy and NAFLD in an Asian population, which may have different characteristics to a Western population.

This study also has several limitations. First, it was a cross-sectional study; thus, the temporal relationship between cholecystectomy and NAFLD could not be evaluated. Second, we diagnosed NAFLD by ultrasonography without histological confirmation, which is considered the gold standard for diagnosing NAFLD. However, histological diagnosis of NAFLD is difficult to accomplish in a large general population and creates the risk of certain complications. Third,

insulin resistance was not evaluated in all patients due to the retrospective design of our study. Fourth, the type of cholecystectomy (open or laparoscopic) and the conversion rate of cholecystectomy (laparoscopic to open), which may have affected its association with NAFLD, were not reviewed in this study, because the previous history of cholecystectomy, not the type of cholecystectomy were only available due to our study design[40].

In conclusion, this study showed an independent association between previous cholecystectomy and NAFLD, independent of other established metabolic risk factors, in a large Asian population. However, gallstones were not independently associated with NAFLD. This result suggests that cholecystectomy may increase the risk of NAFLD. Further prospective studies are warranted to confirm these observations.

COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) and gallstone disease are both prevalent diseases which share the same risk factors, including insulin resistance and obesity. However, the association between gallstone disease and NAFLD has not been definitively established.

Research frontiers

This study investigated the relationship between gallstone disease (presence of gallstones or previous cholecystectomy) and NAFLD in a large Asian population.

Innovations and breakthroughs

This study showed that cholecystectomy, but not gallstones, is independently associated with NAFLD after adjustment for other established metabolic risk factors in a large Asian population.

Applications

Clinicians may be more alert to the risk of NAFLD in patients with a history of cholecystectomy.

Terminology

Cholecystectomy is the surgical removal of the gallbladder for symptomatic gallstones or other gallbladder conditions.

Peer-review

This study is original and interesting and includes a large population. This study showed an independent association between cholecystectomy and NAFLD in the Asian population. Further prospective studies are warranted to confirm these associations.

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ORIGINAL ARTICLE

Observational Study

Outcomes of liver transplantation for end-stage biliary disease: A comparative study with end-stage liver disease

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Abstract

AIM: To evaluate the outcomes of patients with end-stage biliary disease (ESBD) who underwent liver transplantation, to define the concept of ESBD, the criteria for patient selection and the optimal operation for decision-making.

METHODS: Between June 2002 and June 2014, 43 patients with ESBD from two Chinese organ transplantation centres were evaluated for liver transplantation. The causes of liver disease were primary biliary cirrhosis (n = 8), cholelithiasis (n = 8), congenital biliary atresia (n = 2), graft-related cholangiopathy (n = 18), Caroli's disease (n = 2), iatrogenic bile duct injury (n = 2), primary sclerosing cholangitis (n = 1), intrahepatic bile duct paucity (n = 1) and Alagille's syndrome (n = 1). The patients with ESBD were compared with an end-stage liver disease (ESLD) case control group during the same period, and the potential prognostic values of multiple demographic and clinical variables were assessed. The examined variables included recipient age, sex, pre-transplant clinical status, pre-transplant laboratory values, operation condition and postoperative complications, as well as patient and allograft survival rates. Survival analysis was performed using Kaplan-Meier curves, and the rates were compared using log-rank tests. All variables identified by univariate analysis with P values < 0.100 were subjected to multivariate analysis. A Cox proportional hazard regression model was used to determine the effect of the study variables on outcomes in the study group.

RESULTS: Patients in the ESBD group had lower model for end-stage liver disease (MELD)/paediatric end-stage liver disease (PELD) scores and a higher frequency of previous abdominal surgery compared to patients in the ESLD group (19.2 ± 6.6 *vs* 22.0 ± 6.5, P = 0.023 and 1.8 ± 1.3 *vs* 0.1 ± 0.2, P = 0.000). Moreover, the

operation time and the time spent in intensive care were significantly higher in the ESBD group than in the ESLD group (527.4 ± 98.8 *vs* 443.0 ± 101.0, P = 0.000, and 12.74 ± 6.6 *vs* 10.0 ± 7.5, P = 0.000). The patient survival rate in the ESBD group was not significantly different from that of the ESBD group at 1, 3 and 5 years (ESBD: 90.7%, 88.4%, 79.4% *vs* ESLD: 84.9%, 80.92%, 79.0%, χ^2 = 0.194, P = 0.660). The graft-survival rates were also similar between the two groups at 1, 3 and 5 years (ESBD: 90.7%, 85.2%, 72.7% *vs* ESLD: 84.9%, 81.0%, 77.5%, χ^2 = 0.003, P = 0.958). Univariate analysis identified MELD/PELD score (HR = 1.213, 95%CI: 1.081-1.362, P = 0.001) and bleeding volume (HR = 0.103, 95%CI: 0.020-0.538, P = 0.007) as significant factors affecting the outcomes of patients in the ESBD group. However, multivariate analysis revealed that MELD/PELD score (HR = 1.132, 95%CI: 1.005-1.275, P = 0.041) was the only negative factor that was associated with short survival time.

CONCLUSION: MELD/PELD criteria do not adequately measure the clinical characteristics and staging of ESBD. The allocation system based on MELD/PELD criteria should be re-evaluated for patients with ESBD.

Key words: Liver transplantation; End-stage biliary disease; Model for end-stage liver disease; Paediatric end-stage liver disease; Complication

Core tip: In this work, we evaluated the clinical characteristics of end-stage biliary disease (ESBD) and demonstrated that ESBD comprises a subset of disease that significantly differs from end-stage liver disease (ESLD), which is caused by hepatitis and cirrhosis. However, previous research on ESBD has been classified within the category of ESLD. The model for end-stage liver disease (MELD) does not adequately measure the clinical characteristics and stages of patients with ESBD before liver transplantation. Patients with ESBD would be less likely to receive priority for liver transplantation, and thus, the allocation system based on the MELD score is inappropriate and should be re-evaluated for patients with ESBD. In addition, the concept of ESBD and the indications for liver transplantation are established in this paper.

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INTRODUCTION

Liver transplantation (LT) has rapidly evolved from a high-risk experimental procedure to a mainstream therapy for patients with end-stage liver disease (ESLD)[1]. Currently, LT is primarily performed in patients with benign ESLD and hepatocellular carcinoma (HCC). However, LT for biliary diseases is not rare[2]. In 1963, Starzl *et al*[3] completed the first clinical LT, for an indication of congenital biliary atresia. However, equitable organ allocation to patients with biliary disease awaiting LT has been controversial in the model for end-stage liver disease (MELD) era[4]. Huang *et al*[5] proposed the concept of end-stage biliary disease (ESBD) in 2002 to distinguish ESBD-associated diseases from ESLD caused by hepatocyte damage due to hepatitis and liver cirrhosis. Currently, the indications for LT, the optimal timing of the operation, the surgical method, and the clinical outcomes for the concept of ESBD remain ambiguous. Therefore, in this study, data of 43 patients with ESBD who underwent LT from June 2002 to June 2014 in two LT centres in China were retrospectively analyzed. This study aimed to evaluate the clinical characteristics of ESBD and the efficacy of the LT treatment modality through a retrospective analysis of a multi-institutional cohort. The outcomes of this cohort were also compared with those of a group of patients with ESLD who underwent LT.

MATERIALS AND METHODS

Between June 2002 and June 2014, 43 patients with ESBD caused by primary or secondary biliary disease underwent LT at the Department of Liver Transplantation Centre of PLA General Hospital in Beijing and at Southwest Hospital in Chongqin. All operations were performed by two surgical teams under the direction of the same senior surgeon (Dong JH).

The data that were obtained from the two transplant centres were combined in a retrospective cohort study. Patients with ESBD who underwent LT were compared with the case controls with ESLD during the same time. Two controls were randomly selected and matched by sex, age (± 5 years), allograft type, and transplant year (± 5 years). Similar exclusions were used for the case-control group. The demographic distributions of the two patient groups at the two centres were similar. Patients were excluded if they had concomitant liver malignancies, had undergone multi-organ transplantation, or were lost to follow-up. Complications were analysed using the Clavien-Dindo classification[6]. Patients with complications of degree Ⅲ and higher were enrolled in this study. The examined variables included recipient age, sex, pre-

Table 1 Aetiologies of liver transplantation in the two groups

Aetiology of liver transplant	Number
ESBD	43
Primary sclerosing cholangitis	1
Intrahepatic bile duct paucity	1
Alagille's syndrome	1
Congenital biliary atresia	2
Caroli's disease	2
Iatrogenic bile duct injury	2
Primary biliary cirrhosis	8
Cholelithiasis	8
Graft cholangiopathy	18
ESLD	86
Autoimmune cirrhosis	3
Drug-related cirrhosis	3
Hepatitis C virus-related cirrhosis	3
Wilson's disease	4
Alcoholic cirrhosis	6
Hepatitis B virus-related cirrhosis	67

ESBD: End-stage biliary disease; ESLD: End-stage liver disease.

transplant clinical status, and pre-transplant laboratory values, as well as the survival rates of the patients and allografts.

The patients were assessed regularly by specialists at the two centre registries. The database prospectively collected pre-transplant data, transplant, and follow-up data for all individuals considered for LT at the two centres. All demographic data for the recipients, the statuses of the patients and their laboratory values, and the survival data for the patients and allografts were obtained from the database.

Statistical analysis

Continuous variables were compared as means by using Student's *t*-test or the Mann-Whitney non-parametric test. Categorical variables were compared using Pearson's χ^2 test. Survival analysis was performed using Kaplan-Meier curves, and the different survival rates were compared using log-rank tests. A Cox proportional hazard regression model was used to determine the effects of the study variables on the outcomes in the study group. All variables identified through the univariate analysis with *P* values < 0.100 were subjected to multivariate analysis. SPSS 18.0 (SPSS Corp., Chicago, IL, United States) was used for all statistical analyses. Statistical significance was defined as a $P < 0.05$ for all tests.

The statistical methods of this study were reviewed by Xin-Yuan Tong from Chinese PLA General Hospital, Chinese PLA Postgraduate Medical School.

RESULTS

A total of 129 patients were enrolled in this study. The aetiologies for LT are presented in Table 1. Based on the study design, the following recipient variables were similar in the two groups: age, sex, allograft type, and transplant year. Patients in the ESBD group had a significantly higher rate of previous surgery than patients in ESLD group ($P < 0.05$). The pre-transplant laboratory values for both groups are summarised in Table 2. Patients in the ESBD group had significantly higher platelet (PLT) counts, total bilirubin (TBIL) levels, glutamyl transpeptidase (r-GT) levels, and serum albumin (Alb), whereas serum creatinine levels, MELD/Paediatric End-Stage Liver Disease (PELD) scores, and international normalised ratios (INR) were significantly lower in patients in the ESBD group compared with patients in the ESLD group ($P < 0.05$).

Table 2 Comparison of patient characteristics before surgery in the two groups

Index	ESBD group (n = 43)	ESLD group (n = 86)	*P* value
Age (yr)[1]	40.2 ± 15.5 (0.5-65)	43.2 ± 15.3 (5-65)	0.105
Gender (M:F)[2]	28:15 (65%:35%)	56:30 (65%:35%)	1.000
WBC ($\times 10^9$/L)[1]	6.7 ± 4.3 (1.1-23.4)	6.1 ± 4.5 (1.3-24.5)	0.178
Hb (g/L)[1]	103.3 ± 22.6 (47.2-149)	102.3 ± 20.1 (55-154)	0.798
Plt ($\times 10^9$/L)[1]	126.0 ± 59.6 (37-247)	91.0 ± 74. 6 (11-379)	0.000
TBIL (mg/dL)[1]	17.0 ± 13.8 (1.29-47.57)	9.4 ± 5.4 (0.96-22.16)	0.030
INR1	1.3 ± 0.4 (0.86-2.35)	1.829 ± 0.648 (1.12-5.57)	0.000
Cr (mg/dL)[1]	0.9 ± 0.8 (0.15-3.92)	1.1 ± 0.8 (0.35-5.06)	0.042
Alb (g/L)[1]	35.1 ± 4.7 (26-50.5)	33.4 ± 3.4 (28-44.3)	0.016
ALT (U/L)[1]	122.6 ± 96.3 (28-399)	112.9 ± 167.2 (26.5-1400)	0.156
γ-GT (U/L)[1]	515.7 ± 578.2 (17-2374)	119.9 ± 119.6 (11.7-588)	0.000
MELD/PELD score[1]	19.2 ± 6.6 (8-39)	22.0 ± 6.5 (11-38)	0.023
Previous surgery[1]	1.8 ± 1.3 (0-5)	0.1 ± 0.2 (0-1)	0.000

[1]Data are presented as the mean ± SD; ranges are provided in the parentheses; [2]Data are presented as the *n* (%). MELD risk score = [0.957 × log (creatinine; mg/L) + 0.378 × log (bilirubin; mg/dL) + 1.120 × log (INR) + 0.643] × 10. Used for patients aged 12 years and older. Laboratory values less than 1.0 were set to 1.0 to calculate the MELD score. PELD risk score = [0.48 × log (bilirubin; mg/dL) + 1.857 × log (INR) - 0.687 × log (albumin) + infants less than 1 year 0.436 + growth failure 0.667] × 10. Used for patients aged 11 years and younger. Laboratory values less than 1.0 were set to 1.0 to calculate the PELD score. WBC: White blood cell; Hb: Hemoglobin; PLT: Platelets; TBIL: Total bilirubin; INR: International normalised ratio; Cr: Serum creatinine; Alb: Albumin; ALT: Alanine transarninase; r-GT: Glutamyl transpeptidase; MELD: Model for end-stage liver disease; PELD: Paediatric end-stage liver disease.

A significant difference in operation time was observed between the two groups ($P < 0.05$), but intra-operative blood loss was similar in the ESBD and ESLD groups ($P > 0.05$). The overall incidence of complications in the ESBD group was 17 (39.5%), of which 10 (23.3%) were Clavien-Dindo grade Ⅲ and 7 (16.3%) were Clavien-Dindo grade Ⅳ to Ⅴ. The overall incidence of complications in the ESLD group was 27 (31.4%), of which 13 (15.1%) were Clavien-Dindo grade Ⅲ and 14 (16.3%) were Clavien-Dindo grade Ⅳ to Ⅴ. Overall, the incidence of complications in the two groups was similar ($P > 0.05$). The data for complications after LT are presented in Table 3.

Kaplan-Meier analysis was used to estimate the overall patient survival rates with a median patient follow-up time of 43 mo (range: 1-130 mo). The 1-, 3-,

Table 3 Comparison of operation conditions and postoperative hospital stay in the two groups

Index	ESBD group	ESLD group	*P* value
Operative time (min)[1]	527.4 ± 98.8 (300-720)	443.0 ± 101.0 (300-660)	0.000
Intraoperative blood loss (mL)[1]	3062.8 ± 2632.9 (200-12000)	2745.3 ± 1893.1 (500-10000)	0.751
Total complications[2]	17 (39.5)	27 (31.4)	0.358
Clavien-Dindo grade Ⅲ			
Hepatic artery embolisation	2	0	
Re-operation for bile leakage	1	3	
Intraabdominal bleeding	4	7	
Hepatic vein stenosis	0	1	
Bile duct stenosis	0	1	
Portal vein embolisation	1	1	
Intestinal fistula	2	0	
Clavien-Dindo grade Ⅳ-Ⅴ			
Acute kidney failure	3	5	
MODS	4	9	
Intensive care time (d)[1]	12.74 ± 6.6 (6-41)	10.0 ± 7.5 (5-52)	0.000
Hospital stays (d)[1]	32.53 ± 7.5 (20-50)	30.42 ± 6.9 (18-52)	0.079

[1]Data are presented as the mean ± SD, ranges are provided in parentheses; [2]Data are presented as *n* (%). MODS: Multiple organ dysfunction syndrome.

Table 4 Causes of death after liver transplantation for patients in the end-stage biliary disease and end-stage liver disease groups

Cause of death	ESBD group		ESLD group	
	n	Total deaths (%)	*n*	Total deaths (*n*)
Graft failure	3	7.0	4	4.7
Recurrent hepatitis	0	0.0	2	2.3
Biliary complication	2	4.7	2	2.3
Portal vein thrombosis	1	2.3	0	0.0
Multisystem organ failure	4	9.3	10	11.6
Cardiovascular	0	0.0	2	2.4
Myocardial infarction	0	0.0	1	1.2
Arrhythmia	0	0.0	1	1.2
Graft-vs-host disease	0	0.0	1	1.2
Upper gastrointestinal haemorrhage	0	0.0	1	1.2
Central nervous system	1	2.3	2	2.3
Epilepsy	1	2.3	0	0.0
Intracranial haemorrhage	0	0.0	1	1.2
Brain infarction	0	0.0	1	1.2
Lung cancer	1	2.3	0	0.0
Total	9	20.9	20	23.3

Some percentages do not sum to the correct totals because they were rounded up or down. LT: Liver transplantation; ESBD: End-stage biliary disease; ESLD: End-stage liver disease.

and 5-year patient survival rates were 90.7%, 88.4%, and 79.4%, respectively, for the 43 patients with ESBD and 84.9%, 80.92% and 79.0%, respectively, for the matched ESLD group. The log-rank test revealed that the difference between the two groups was not statistically significant (χ^2 = 0.194, *P* = 0.660). Kaplan-Meier estimates of allograft survival revealed 1-, 3-, and 5-year survival rates of 90.7%, 85.2%, and 72.7%, respectively, in the ESBD group and 84.9%, 81.0%, and 77.5%, respectively, in the ESLD group. The log-rank test indicated that the difference between the two groups was not statistically significant (χ^2 = 0.003, *P* = 0.958). The graft losses were primarily due to the death of the patients. The causes of death in both groups are listed in Table 4.

Univariate analysis was performed to determine the effect of individual variables on the survival of the patients in the study group. Demographic parameters such as age, sex and blood type did not affect survival. Pre-transplant medical conditions (*e.g.*, previous abdominal surgery, allograft type, re-transplantation, Hb, PLT, serum ALT level, γ-GT level, Alb level, and INR) had no effect on survival. Univariate analysis revealed that bleeding volume and MELD/PELD score had a significant negative effect on survival after LT. White blood cell (WBC) count exhibited a trend toward poorer survival, but this trend was not statistically significant.

The variables identified by univariate analysis with *P* values < 0.100 (*i.e.*, WBC count, MELD/PELD score, and bleeding volume) were subjected to multivariate analysis. In this model, the MELD/PELD score exhibited

Table 5 Univariate and multivariate analyses of post-transplant survival with a Cox proportional hazard regression model for patients who underwent liver transplantation for end-stage biliary disease

Study variable	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P* value	HR	95%CI	*P* value
MELD/PELD score	1.213	1.081-1.362	0.001	1.132	1.005-1.275	0.041
WBC count	0.761	0.553-1.046	0.093	0.823	0.563-1.203	0.314
Blood loss	0.103	0.020-0.538	0.007	0.171	0.026-1.146	0.069

MELD: Model for end-stage liver disease; PELD: Paediatric end-stage liver disease; WBC: White blood cell.

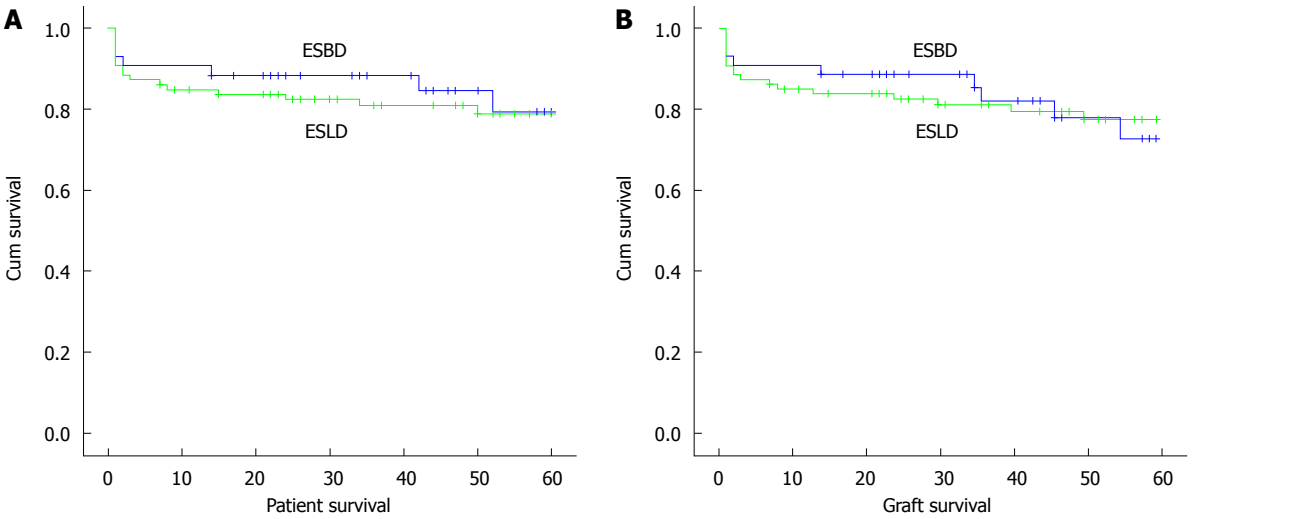

Figure 1 Kaplan-Meier survival curves for patients (A) and allografts (B). Forty-three patients who underwent LT for end-stage biliary disease and eighty-six case-matched end-stage liver disease patients are compared. LT: Liver transplantation; ESBD: End-stage biliary disease; ESLD: End-stage liver disease.

a significant and independent effect on outcomes. These findings are summarised in Table 5; whereas the survival curves for the patients and allografts from the two groups are shown in Figure 1.

DISCUSSION

LT has been studied comprehensively as a treatment for benign ESLD. Evaluation and treatment have gradually been subject to more specifications after establishing the MELD system in organ allocation. Biliary diseases are an important indication for LT. Biliary disease is a common indication for LT in European countries and has accounted for 15% of all LTs over the last decade, including primary sclerosing cholangitis (PSC) (5%), primary biliary cirrhosis (PBC) (4%), secondary biliary cirrhosis (1%), congenital biliary disease (4%), and biliary tract carcinoma (0.5%)[7]. A considerable number of patients with advanced biliary diseases have been observed in China; thus, the role of LT in biliary surgery has gained increasing attention. The present study indicated that ESBD has unique characteristics that differ from those of ESLD; however, standard LT for ESBD has not yet been established, and this concept remains ambiguous. Therefore, this study examined several aspects of ESBD.

Concept of ESBD

Based on the present study, we observed that ESBD differs from ESLD. Although ESBD and ESLD share several clinical characteristics, these disease categories should not be confused with each other. Patients with ESBD occasionally also present with cirrhosis and symptoms of portal hypertension, which are typically ESLD-specific symptoms. In addition, most patients with ESBD do not exhibit complete loss of liver parenchymal function. Although MELD scores accurately predict the 3-mo mortality of patients on the LT waitlist, the scores of patients with conditions such as PSC do not[8]. In this study, the MELD/PELD score of patients with ESBD was significantly lower than that of patients in the control group ($P < 0.05$). Assessment of ESBD using classical evaluation criteria does not fully describe the characteristics and severity of the disease, and thus, a clear concept and position of ESBD would be clinically significant.

The concept of ESBD has not yet been defined. As previously described, we maintain that ESBD primarily refers to benign biliary tract disease. In the end-stage of these type of diseases, irreversible changes appear in the diffused liver and biliary system. Without effective treatment, patients typically die of hepatobiliary failure within a short period of time[5]. The current debate focuses on whether ESBD should

include malignant biliary tumours and the diagnosis standards for ESBD. When the developmental process of the disease and the prognosis are considered, some cholangiocarcinomas caused by benign biliary diseases such as cholelithiasis or PSC are chronic, long-term, and gradually progressive. Moreover, the prognosis of malignant biliary tumours is relatively worse than that of HCC because of the difficulty in early diagnosis and the high degree of malignancy[9]. Thus, cholangiocarcinoma belongs in the end-stage disease category when considered from a prognostic perspective. Moreover, we considered ESBD as a related concept to benign ESLD; the exclusion of malignant biliary disease provides a more effective indication of the clinical characteristics of this disease. Therefore, we define ESBD as a group of benign diseases from primary or secondary causes that leads to irreversibly diffuse lesions in the bile duct tree combined with lipid metabolic disorders, presenting with persistent jaundice, recurrent cholangitis, and biliary sludge, cast or stones. This condition eventually causes liver fibrosis and liver failure.

Indications and safety for treatment of ESBD with LT

Benign ESBD, including biliary atresia, cystic fibrosis, PBC, and graft cholangiopathy, is recognised as an appropriate indication for LT[10,11]. ESBD has a lower risk of recurrence than viral-related hepatitis and malignant liver tumours. In the present study, all patients in the ESBD group except three died during the peri-operative period, whereas the remaining patients recovered well after the surgery. The overall 5-year patient survival rate of patients in the ESBD group was 79.4%, which was similar to that of patients in the ESLD group ($P > 0.05$). The classification of PSC as a relapsing form of ESBD remains controversial. The current 1- and 5-year patient survival rates for PSC after LT are 83% to 97.2% and 75% to 95.4%, respectively[12]. Acute cellular rejection and recurrence are considered major risks of LT[13]. Recurrence occurs in 23.5% of patients an average of 4.6 years after LT[14]. Due to the limited sample size of patients with PSC, this finding could not be confirmed in our series.

With advances in LT techniques, surgical safety for patients with ESBD has further improved. However, LT in cases of ESBD is more challenging for surgeons than that for ESLD. We observed that patients with ESBD were more likely to have undergone abdominal surgery prior to LT than patients in the ESLD group ($P < 0.05$). In addition to portal hypertension and varicose veins, the surgical risk is mainly due to severe abdominal adhesions caused by repeated abdominal surgeries and biliary tract infections. These factors all increase the possibility of causing haemorrhage and damage to the surrounding organs during the removal of the liver. To reduce intra-operative bleeding, we used an innovative method known as "dry blood hepatectomy" to optimize the procedure for liver resection. First, we dissociated and blocked the blood flow of the first hepatic portal (including the portal vein), and then we dissociated and excised the diseased liver, simultaneously reducing bleeding, shortening the operation time, and improving safety[15]. Although the difficulty of surgery in patients in the ESBD group was significantly increased, no significant difference was observed between the two groups with respect to the amount of blood loss and post-operative complications ($P > 0.05$).

Optimal timing of LT for the treatment of ESBD

LT has been the final choice for patients with ESBD because traditional surgery has limited effectiveness. The optimal selection of the operation time for patients with ESBD is very important because of the severe shortage of liver donors, the high risks associated with LT, and the high cost of the operation. In contrast to ESBD, a standard operation time has been established for patients with ESLD. The MELD scoring system can dynamically monitor changes in patients with ESLD, and the optimal time for LT can be quantitatively evaluated. The MELD scoring system was adopted in the United States on February 28, 2002, as a liver allocation tool for patients with chronic liver disease who are candidates for LT. Patients with high MELD scores are prioritised for LT[16]. However, whether patients with ESBD should be an exception to the use of the MELD scoring system remains controversial. Several studies have considered conferring additional scores to patients with biliary disorders to fit the characteristics of recurrent infective cholangitis and intractable pruritus[17,18]. However, Goldberg *et al*[19] speculated that the prioritisation of patients with cholangitis for LT is not necessary because morbidity during this period is similar in patients with and without cholangitis. In the present study, we observed that the main characteristics of ESBD are recurrent cholangitis, intractable pruritus, and frequent hospital admissions. Because resolving pruritus is very difficult[20], the occurrence of intractable pruritus may be an indication for LT[21]. Moreover, the curative effect of repeated surgical treatment is very limited for patients with ESBD. However, the MELD score mainly comprises parameters that indicate the synthetic and detoxification functions of the liver, of which only bilirubin is associated with biliary diseases. Moreover, bilirubin is a low-weighted coefficient in the formula that determines the MELD score[22]. Thus, the MELD score does not reflect the main characteristics of biliary diseases. Although the MELD score is more accurate for the prediction of wait-list mortality than post-transplant survival, it is a risk factor for death after LT[23,24]. In our study, multivariate analysis indicated that the MELD/PELD score was the only independent risk factor for poor outcomes. Moreover, the MELD/PELD score of patients in the ESBD group was significantly lower than that of patients in the

control group. When MELD criteria are used, patients with ESBD will be less likely to be prioritised for LT. Our study also indicated a higher risk associated with surgery in patients with ESBD, primarily during the peri-operative period. In the ESBD group, three patients died during the early post-operative period, two experienced liver failure prior to surgery and had a MELD score > 20, whereas the remaining patient developed a recurrent biliary infection that resulted in MODS. This result demonstrates that the risks associated with LT increase when ESBD develops into decompensated biliary cirrhosis or hepatic failure. Safeguards of the MELD scoring system must be developed to avoid futile transplants in recipients with high MELD scores[25]. Therefore, the MELD scoring system is inappropriate for the evaluation of patients with ESBD. An allocation system based on a "sickest patient first" policy is evidently unfair for patients with ESBD and could also contribute to a reduction in pre-LT mortality, worsen post-LT results, and an increase in organ waste. The MELD-based graft allocation system has failed to improve the efficacy of LT[26] and should be re-evaluated and modified[27-29]. According to data from the Organ Procurement and Transplantation Network, as of 2011, 12.2% of LT recipients with PSC received exception points. Since the publication of consensus recommendations that state that exception points be granted to patients with PSC and bacterial cholangitis, no systematic evaluation of their outcome[19] and standardization of exception points for patients have been established[8,30]. Thus, further studies should be performed to establish a model that precisely demonstrates the degree of severity and the clinical stages of ESBD. We proposed that LT should be performed in patients with ESBD to achieve optimal therapeutic effectiveness in the following cases: recurrence of cholangitis or intractable pruritus, when medical and surgical treatment cannot alleviate the condition, and when symptoms of presence of decompensated biliary cirrhosis are present.

In conclusion, this study analyzed the clinical characteristics of ESBD and demonstrated that ESBD is a group of diseases that are independent of ESLD. LT provides satisfactory long-term patient and graft survival rates in patients with liver-biliary failure caused by irreversible biliary disease. In this study, the MELD/PELD score was the only independent risk factor for poor outcome but this score does not adequately measure the clinical characteristics of ESBD. However, our observations have several limitations. The data obtained were retrospective in nature, and we were reliant on the accuracy of the documentation in the medical records for our data. Furthermore, data from a large, prospective, multi-centre trial are needed to confirm our findings at a national and international level. The evaluation of ESBD and the clinical standards of staging will be particularly beneficial for scientific decision-making and the improvement of therapeutic effectiveness.

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COMMENTS

Background

End-stage biliary disease (ESBD), a novel concept that we developed, is one of the main indications for liver transplantation (LT) in China. However, previous research on ESBD has been classified into the category of end-stage liver disease (ESLD). Moreover, the use of an organ allocation system based on the model for end-stage liver disease (MELD) criteria remains controversial for ESBD.

Research frontiers

Studies have noted that the MELD-based graft allocation system has failed to improve the efficacy of LT, and disputes about the modification of the MELD scoring system have continued to arise. Difference among diseases must be considered when allocation systems are used.

Innovations and breakthroughs

In this work, the authors demonstrated that ESBD comprises a subset of diseases that significantly differs from ESLD caused by hepatitis and cirrhosis. However, previous research on ESBD has been classified into the category of ESLD. The MELD scoring system does not adequately measure the clinical characteristics and staging of ESBD before LT. Patients with ESBD are less likely to be prioritized for LT, and therefore an allocation system based on MELD scores is unfair and should be re-evaluated for patients with ESBD. In addition, the concept of ESBD and indications for LT are established in this paper.

Applications

The MELD scoring system does not adequately measure the clinical characteristics and stages of ESBD before LT. The authors propose that LT should be performed in patients with ESBD to achieve optimal therapeutic effectiveness in the following cases: recurrence of cholangitis or intractable pruritus, when medical and surgical treatment cannot alleviate the condition, and when symptoms of decompensated biliary cirrhosis are present. This study provides evidence for the establishment of a model that, in the future, will precisely demonstrate the degree of severity and the clinical stages of ESBD.

Terminology

ESBD, a novel concept that we developed to distinguish these diseases from ESLD caused by hepatitis and cirrhosis, is a group of benign diseases from primary or secondary causes. These diseases lead to irreversible, diffuse lesions in the bile duct tree combined with persistent jaundice, recurrent cholangitis, and biliary sludge, cast or stones. This condition eventually causes liver fibrosis and failure of liver function.

Peer-review

In this article, the authors demonstrate that ESBD is a subset of diseases that significantly differs from ESLD caused by hepatitis and cirrhosis; however, previous research on ESBD has been classified into the category of ESLD. The concept of ESBD is defined, and the indication for LT is established. This study provides evidence supporting the modification of the MELD system.

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ORIGINAL ARTICLE

Observational Study

Sequential blood purification therapy for critical patients with hyperlipidemic severe acute pancreatitis

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Abstract

AIM: To evaluate the efficacy of sequential blood purification therapy in the treatment of critical patients with hyperlipidemic severe acute pancreatitis.

METHODS: Thirty-one intensive care unit (ICU) patients with hyperlipidemic severe acute pancreatitis treated at the Second Affiliated Hospital of Harbin Medical University were divided into either a study group (n = 15; July 1, 2012 to June 30, 2014) or a control group (n = 16; July 1, 2010 to June 30, 2012) based on the implementation of sequential blood purification therapy. The control group received continuous venous-venous hemofiltration (CVVH) on the basis of conventional treatments, and the therapeutic dose of CVVH was 30 mL/kg per hour. The study group received sequential plasma exchange and CVVH on the basis of conventional treatments. The anticoagulation regimen of CVVH is the regional citrate anticoagulation. Mortality rate on day 28, rates of systemic and local complications, duration of ICU, and time to target serum lipid level, as well as physiologic and laboratory indices were compared between the two groups.

RESULTS: The mortality rate on day 28 was significantly lower in the study group than in the control group (13.33% *vs* 37.50%; $P < 0.05$). The duration of ICU stay was significantly shorter in the study group than in the control group (7.4 ± 1.35 d *vs* 9.19 ± 2.99 d, $P < 0.05$). The time to target serum lipid level was significantly shorter in the study group than in the control group (3.47 ± 0.52 d *vs* 7.90 ± 1.14 d, $P < 0.01$). There were no significant differences in the rates of systemic complications and local complications between the two groups (60% *vs* 50% and 80% *vs* 81%, respectively). In the comparisons of physiologic and laboratory indices, serum albumin and C-reactive protein were significantly better in the study group than in the control group after treatment (37.8 ± 4.6 g/L *vs* 38.9 ± 5.7 g/L, and 20.5 ± 6.4 mg/L *vs* 28.5 ± 7.1 mg/L, respectively, both $P < 0.05$). With the exception of plateletcrit, no other indices showed significant differences between the two groups.

CONCLUSION: Sequential blood purification therapy is effective in the treatment of ICU patients with hyperlipidemic severe acute pancreatitis and can improve patient prognosis.

Key words: Continuous venous-venous hemofiltration; Hyperlipidemic severe acute pancreatitis; Sequential blood purification; Plasma exchange

Core tip: Plasma exchange and continuous venous-venous hemofiltration have certain clinical effects in the treatment of hyperlipidemia acute severe pancreatitis, but there is currently no standardized combination therapy. Based on the 2012 Atlanta International Pancreatitis Consensus, we designed a sequential mode of combined application of plasma exchange and continuous venous-venous hemofiltration for the treatment of hyperlipidemia severe acute pancreatitis. This sequential blood purification therapy was found to be effective in the treatment of intensive care unit patients with hyperlipidemic severe acute pancreatitis and improved patient prognosis, and should therefore become the standardized treatment process.

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INTRODUCTION

Acute pancreatitis (AP) is divided into three categories according to the 2012 Atlanta classification: mild, moderate, and severe[1]. In contrast to the low mortality of mild AP, severe AP (SAP) is associated not only with a high mortality rate, but also with a high rate of complications[2]. So far, many treatment strategies for AP have been developed. In addition to common surgical treatment, peritoneal lavage, organ support therapy, and endoscopic retrograde cholangiopancreatography, blood purification therapy is also a promising treatment. Despite these therapies, the treatment of SAP is still a great challenge, and its mortality rate can still reach 15%-25%[3].

SAP is characterized by persistent organ failure, and the risk of death is especially higher in the first several days of organ failure (36%-50%)[4-6]. Therefore, there is an urgent need to further improve the survival rate of patients with SAP. Currently, the utility of a paradigm involving a multidisciplinary team typically including general surgeons, gastroenterologists, radiologists, and intensive care unit (ICU) physicians is advocated in the clinical treatment of SAP[7]. Organ support technology, especially continuous renal replacement therapy(CRRT), has a key role in the critical care of SAP.

The most common causes of AP include gallstone diseases, excessive alcohol consumption, and pregnancy. Hyperlipidemic AP, which occurs when the triglyceride level is > 1000 mg/dL, accounts for only 1%-4% of cases[8,9]. At present, it is believed that hyperlipidemic AP is related to pancreatic tissue (pancreatic ducts and acini cells) injury and microcirculation disturbance caused by free fatty acids that are produced by pancreatic lipase-catalyzed decomposition of triglycerides. With the progression of the disease, the enzymes, fatty acids, and inflammatory mediators in pancreatic tissue enter into the systemic circulation and participate in the development of multiple organ dysfunction, thus leading to the failure of one or multiple organs[2,10]. There is evidence that multiple organ failure subsequent to systemic inflammatory response syndrome caused by pancreatic inflammation is the main cause of death in patients with pancreatitis.

In the past few years, the use of blood purification technology in the treatment of ICU patients with AP has progressively increased. Continuous venous-venous hemofiltration (CVVH) is one of the most commonly used blood purification procedures in ICU patients, and it can selectively remove inflammatory factors in the body and effectively eliminate the inflammatory cytokine storm. For acute kidney injury patients, CVVH can remove toxins in the body and reduce water retention. In patients with hyperlipidemic AP, the application of plasma exchange (PE) allows for rapid and efficient removal of serum lipids, and reduces triglyceride levels and the production of free fatty acids, thus weakening pancreatic self-digestion by trypsin. In addition, PE can improve pancreatic tissue microcirculation and relieve a high blood coagulation state, ultimately achieving the goal of treatment of AP. In theory, PE is very beneficial for the treatment of hyperlipidemic SAP[11-13].

So far, there have been many small sample-sized clinical studies confirming that PE and CVVH are conducive to improving mortality in patients with either SAP or hyperlipidemic SAP. Moreover, a recent study demonstrated that combined use of PE and CVVH has more advantages in patients with SAP[14-16]. However, despite a large number of existing clinical studies, there have been no standardized criteria for the combination of PE and CVVH for SAP, and this has led to conflicting conclusions. In addition, because the patients were selected randomly in many previous studies, they could not effectively evaluate the clinical efficacy of the combination therapy of PE and CVVH. The present study was designed to further evaluate the clinical efficacy of PE combined with CVVH in the treatment of ICU patients with hyperlipidemic SAP.

MATERIALS AND METHODS

Patient characteristics

This study was divided into two stages based on

the implementation of sequential blood purification therapy. The patients (n = 16) treated from July 1, 2010 to June 30, 2012 underwent conventional treatments and CVVH and were included in the control group, and those (n = 15) treated from July 1, 2012 to June 30, 2014 received conventional treatments with sequential blood purification therapy and comprised the study group.

Inclusion criteria were: (1) diagnosis of pancreatitis (meeting at least two of the following three criteria): abdominal pain typical of pancreatitis; serum amylase and/or lipase levels ⩾ three times the upper limit of normal; evidence of pancreatitis upon abdominal imaging; (2) diagnosis of severe pancreatitis: Marshall score ⩾ 2; and (3) diagnosis of hyperlipidemic pancreatitis: serum triglycerides > 1000 mg/dL. Exclusion criteria were: (1) pancreatic cancer; (2) gallstones; (3) > five-year history of heavy drinking (> 50 g/d); (4) younger than 18 years or older than 60 years; and (5) not receiving PE/CVVH within 5 h after admission to ICU. The withdrawal criterion was that the patient himself/herself or the authorized person requested withdrawl from the study. Indications for discontinuation of therapy were: (1) disappearance of specific abdominal symptoms; (2) Marshall score < 2; and (3) serum triglycerides < 500 mg/dL[17].

Research scheme

The study group received sequential blood purification therapy, which involved the initial PE with freshly frozen plasma (⩾ 3000 mL/d) until serum triglycerides < 500 mg/dL and subsequent CVVH until the disappearance of specific abdominal symptoms and Marshall score < 2. The control group received conventional treatments and CVVH, *i.e.*, lipid-lowering drugs (simvastatin with fenofibrate, 20 + 200 mg/d[17]) plus CVVH, until the disappearance of specific abdominal symptoms, Marshall score < 2, and serum triglycerides < 500 mg/dL.

CRRT settings

PE was performed using a FLEX system with the TPE 2000 set *via* a polysulfone filter, and the velocity was set at 30 mL/min. CVVH was performed using a FLEX system with the M100 set *via* an AN69 filter, and the parameters were as follows: therapeutic dose, 30 mL/kg per hour; blood flow velocity, 150-180 mL/min; dilution mode, pre-dilution 100%; frequency of filter replacement, 8-12 h (depending on transfilter pressure); permissible transfilter pressure, 0-300 mmHg; anticoagulation regimen, regional citrate anticoagulation (4% sodium citrate and 100 mmol/L calcium chloride); detection range for free Ca^{2+} ion before filter, 0.25-0.35 mmol/L; and detection range for free Ca^{2+} ion after filter, 1.12-1.20 mmol/L[18,19].

Conventional treatments

Conventional treatments applied to the study and control groups included fasting, fluid resuscitation, oxygen therapy, gastrointestinal decompression, somatostatin, and organ support therapy.

Clinical parameters

The primary outcome measure was mortality on day 28[14], and secondary outcome measures[15] were rate of systemic complications, rate of local complications, duration of ICU stay, and time to target serum triglyceride level (< 500 mg/dL). Systemic complications included: (1) pulmonary insufficiency (PaO_2 < 8 kPa); (2) renal insufficiency (Cr > 2 mg/dL); (3) shock (systolic blood pressure < 12 kPa, systolic blood pressure was decreased > 40 mmHg compared with the baseline); and (4) upper gastrointestinal bleeding > 500 mL/24 h. Local complications included pylorus dysfunction, peripancreatic effusion, pancreatic pseudocyst, spleen vein and portal vein thrombosis, colon necrosis, necrotic collection, and walled-off necrosis. In addition, physiologic and laboratory indices were also compared after treatment between the two groups. The physiologic indices included body temperature (°C), heart rate (beats/min), blood pressure (mm Hg), RR interval (beats/min), mean arterial pressure (mmHg), and PaO_2/FiO2. Laboratory indices included WBC (10^9/L), plateletcrit (10^9/L), albumin (g/L), alanine transaminase (U/L), total bilirubin (mmol/L), blood urea nitrogen (mmol/L), serum creatinine (mmol/L), Ca^{2+} (mmol/L), bicarbonate (mmol/L), serum amylase (U/L), urine amylase (U/L), C-reactive protein (mg/L), and procalcitonin (ng/mL).

Statistical analysis

Measurement data are expressed as mean ± SD, and count data are expressed as number of cases (or percentage). Survival analysis was performed using the Kaplan-Meier method. Mortality on day 28, secondary indices (systemic complications, rate of local complications, duration of ICU stay, and time to target serum triglyceride level), as well as physiologic and laboratory indices after treatment were compared between the two groups using the t test. Statistical analyses were performed using SAS 9.1.3 statistical software (SAS Institute Inc., Cary, NC, United States), and P < 0.05 were considered statistically significant.

RESULTS

Table 1 shows the baseline characteristics of patients in the study and control groups. There were no significant differences in the baseline characteristics between the two groups.

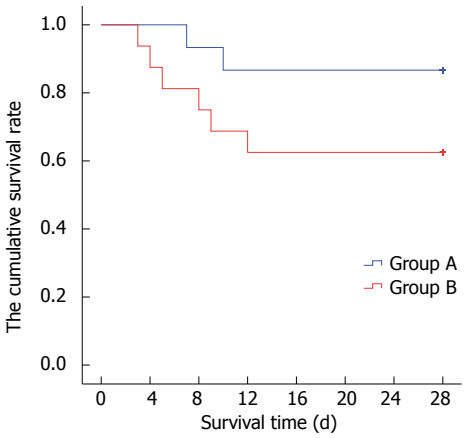
Kaplan-Meier survival analysis showed that the mortality rate on day 28 was 13.33% and 37.50% in the study group and control group, respectively. As time went on, the mortality rate was significantly lower in the study group than in the control group (P < 0.05) (Figure 1).

For secondary outcome measures, the duration of ICU stay and the time to target serum lipid level

Table 1 Baseline characteristics of patients in the two groups

Characteristic	Study group (n = 15)	Control group (n = 16)	P value
Age (yr)	42.6 ± 9.9	40.9 ± 12.6	0.6806
BMI (kg/m^2)	27.4 ± 4.1	28.5 ± 3.7	0.4387
Sex (male/female), n	10/5	11/5	1.0000
Bacterial culture positive, n	3	4	1.0000
Marshall score	2.6 ± 1.7	2.5 ± 1.4	0.8590
APACHE II score	21.3 ± 2.9	22.5 ± 2.1	0.1952
Upper gastrointestinal bleeding, n	7	8	1.0000
ARDS, n	12	13	1.0000
Heart failure/pulmonary edema, n	3	2	0.6539
DIC, n	1	2	1.0000
Surgical debridement, n	1	1	1.0000
Use of vasopressors, n	15	16	1.0000
Mechanical ventilation, n	10	10	1.0000

ARDS: Acute respiratory distress syndrome; BMI: Body mass index; DIC: Disseminated intravascular coagulation.



Group	Mean	SE	95%CI	
			Lower	Upper
A	25.400	1.717	22.034	28.766
B	20.063	2.606	14.954	25.171
Overall	22.645	1.652	19.407	25.883

Figure 1 Kaplan-Meier survival curve. Study group = Group A; Control group = Group B.

were significantly shorter in the study group than in the control group (both $P < 0.05$) (Table 2). The rate of systemic complications was 60% for the study group and 50% for the control group. The rate of local complications was 80% for the study group and 81% for the control group. There were no significant differences in the rates of systemic complications and local complications between the two groups (Table 2).

The comparisons of physiologic and laboratory indices between the two groups are shown in Tables 3 and 4. After treatment, serum albumin and C-reactive protein were significantly better in the study group than in the control group (both $P < 0.05$). With exception of procalcitonin, none of the other indices were significantly different between the two groups.

Table 2 Comparison of secondary outcome measures

Variable	Study group	Control group	P value
Duration of ICU stay (d)	7.40 ± 1.35	9.19 ± 2.99	0.0420
Time to target TG (d)	3.47 ± 0.52	7.90 ± 1.14	< 0.0001
Systemic complications, n (%)	9 (60.00)	8 (50.00)	0.7224
Local complications, n (%)	12 (80.00)	13 (81.25)	1.0000

ICU: Intensive care unit; TG: Triglyceride level.

Table 3 Comparison of physiologic variables

Variable	Study group (n = 15)	Control group (n = 16)
Body temperature (℃)	37.5 ± 0.6	37.3 ± 0.4
Heart rate (beats/min)	86 ± 14	90 ± 19
RR interval (beats/min)	16 ± 4	18 ± 5
Mean arterial pressure (mmHg)	70.2 ± 9.3	67.9 ± 6.0
PaO_2/FiO_2	179.1 ± 41.9	167.7 ± 38.9

Table 4 Comparison of laboratory variables

Variable	Study group (n = 15)	Control group (n = 16)
WBC (10^9/L)	11.5 ± 2.3	13.1 ± 2.9
PLT (10^9/L)	196.5 ± 40.5	199.6 ± 58.7
ALB (g/L)	37.8 ± 4.6[a]	38.9 ± 5.7
ALT (U/L)	54.3 ± 20.4	59.7 ± 23.1
TBIL (mmol/L)	20.1 ± 3.9	25.3 ± 4.2
BUN (mmol/L)	7.8 ± 2.6	9.7 ± 2.8
Scr (mmol/L)	149.8 ± 30.2	139.3 ± 37.5
Ca^{2+} (mmol/L)	2.1 ± 0.5	2.0 ± 0.3
Serum amylase (U/L)	74.4 ± 28.3	82.1 ± 20.7
Urine amylase (U/L)	399.7 ± 59.7	387.1 ± 51.4
CRP (mg/L)	20.5 ± 6.4[a]	28.5 ± 7.1
PCT (ng/mL)	1.33 ± 0.42[a]	1.71 ± 0.61

[a]$P < 0.05$ *vs* control group. ALB: Albumin; ALT: Alanine transaminase; BUN: Urea nitrogen; CRP: C-reactive protein; PCT: Procalcitonin; PLT: Platelet; Scr: Serum creatinine; TBIL: Total bilirubin; WBC: White blood cell.

DISCUSSION

Hyperlipidemic SAP is clinically characterized by severe symptoms, many complications, easy recurrence, and poor prognosis. It is common in obese, young, or middle-aged men, most of which have bad living habits such as a high-fat diet. Evidence has shown that control of serum triglyceride < 500 mg/dL can prevent further progression of pancreatitis[17]. It is well known that hyperlipidemia causes AP mainly through the complex interplay among high serum triglyceride level, increased free fatty acids, reduced activity of trypsin, and activated inflammatory factors to eventually lead to pancreatic tissue inflammation. A high level of serum triglyceride destroys the protectional function of the pancreas, causes abnormal pancreatic enzyme activation and pancreatic self-digestion, and results in the release of a large amount of various proinflammatory factors, thereby causing a cascade effect[10]. Therefore, lowering the serum

triglyceride level is the primary goal for early treatment of hyperlipidemic SAP.

To lower high serum triglyceride levels in patients with pancreatitis, oral lipid-lowering drugs (typically fibrates) are usually used in clinical settings. Meanwhile, fat-free parenteral nutrition preparations are often administered. When patients with hyperlipidemic SAP become seriously ill and are transferred to the ICU, physicians may utilize the PE technology to rapidly lower serum triglyceride levels so as to prevent the progression of pancreatic inflammation. When hyperlipidemia is effectively relieved, the cause of disease progression and aggravation is effectively eliminated. Previous clinical studies on the use of PE in the treatment of hyperlipidemic SAP have demonstrated that PE has a good curative effect (especially for lowering serum lipid) and is safe[20,21]. The present study compared the time to target serum triglyceride level (< 500 mg/dL) between PE and use of oral lipid-lowering drugs and found that the former has certain advantages.

When serum triglyceride levels in pancreatitis patients are effectively controlled, the clinical treatment goal shifts to regulating the body's inflammatory response and volume status. CVVH achieves the purpose of treatment by regulating fluid balance and removing inflammatory mediators and toxins[22]. PE combined with CVVH for the treatment of hyperlipidemic SAP can improve the clinical effects of PE alone in terms of rapidly decreasing the body's inflammatory factors and, at the same time, adjusting and optimizing the patient's circulatory status[23,24]. For this reason, the present study sequentially utilized PE to lower lipid levels and then CVVH to effectively reduce the systematic inflammatory response syndrome and optimize the circulatory status. In addition, application of CVVH can clear allergic reactions related to antigens and antibodies generated during PE treatment due to the transfusion of allogeneic plasma, and further optimize the therapeutic effect, thus ensuring the safety of the treatment. Fortunately, no allergic transfusion reactions occurred in our patients, thus reducing the impact on the implementation of PE. In view of this, although the rate of systemic complications (60%) in the study group was higher than that in the control group (50%), the duration of ICU stay was significantly shorter in the study group than in the control group.

Although this study is not a randomized controlled trial, its design is different from that of many previous studies with arbitrarily selected patients, in which the duration of blood purification treatment as well as the combination of blood purification therapies are arbitrary. In the present study, a target oriented sequential therapy protocol was used. This allowed us to monitor the relatively complete clinical course of patients with hyperlipidemic SAP, thus ensuring the credibility of subsequent evaluation of disease outcome and prognosis. However, given that hyperlipidemic SAP is not relatively common[25,26], recruitment of many more patients is somewhat difficult. Future larger sample-sized, randomized controlled, multicenter clinical studies are expected. In addition, despite many unpredictable variations in clinical trials and that there were no significant differences in the secondary outcome measures between the two groups, the sequential treatment group was associated with a better survival rate and some significantly improved laboratory indices, such as C-reactive protein, suggesting that the application of sequential blood purification treatment in the management of ICU patients with hyperlipidemic SAP is feasible, effective, and safe.

Hyperlipidemia is either one of the causes or a consequence of AP[27-30], and even may be both in some cases. However, there is still controversy over this point of view. The pathogenesis of hyperlipidemia-induced AP is still under study, and signaling pathways that have an exact association with hyperlipidemic SAP are being explored. Future treatment of pancreatitis may be based on targeted therapies that can block hyperlipidemia-induced pancreatic injury. Currently, there have been no standardized criteria for the selection of blood purification procedures and parameters for ICU patients with different stages of pancreatitis. These will be our future important research topics and directions. It is currently well recognized that the treatment of hyperlipidemic SAP relies greatly on early, rapid lowering of blood lipid levels in combination with routine therapies for AP. For early lipid-lowering effects, plasma exchange has better efficacy than the use of lipid-lowering drugs. The subsequent application of blood filtration technology and conventional treatments for AP is the current practice for the treatment of ICU patients with AP.

COMMENTS

Background

Hyperlipidemia is a common cause of acute pancreatitis, and leads to pancreatic tissue inflammation. The pancreatic tissue inflammation can lead to secondary systemic inflammatory response syndrome and multiple organ failure. Multiple organ failure is the main cause of death in patients with severe acute pancreatitis (SAP). Many studies showed that plasma exchange (PE) combined with continuous venous-venous hemofiltration (CVVH) in the treatment of SAP had certain clinical benefits. However, many clinical studies did not have the standardized treatment. Therefore, we cannot assess the validity of their study.

Research frontiers

In the past few years, the use of blood purification treatment of pancreatitis in the intensive care unit (ICU) has progressively increased. In the treatment of hyperlipidemic pancreatitis, the current research focus is how to reduce the mortality and improve the prognosis of patients with the combined application of PE and CVVH.

Innovations and breakthroughs

Based on the 2012 Atlanta International Pancreatitis Consensus, the authors designed a sequential application of PE and CVVH for the treatment of hyperlipidemic SAP. Compared with the past research, the authors designed a relatively reasonable treatment process for improving hyperlipidemic SAP patients' prognosis.

Applications

This sequential blood purification therapy is effective for the treatment of ICU patients with hyperlipidemic SAP and can improve their prognosis.

Terminology

SAP is refers to acute pancreatitis with more than 48 h persistent multiple organ failure (Marshall Score = 2). Hyperlipidemic acute pancreatitis refers to acute pancreatitis with triglyceride levels > 1000 mg/dL.

Peer-review

This observational study is a very interesting manuscript evaluating the efficacy of sequential blood purification therapy for ICU patients with hyperlipidemic SAP and its impact on prognosis.

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ORIGINAL ARTICLE

Randomized Controlled Trial

Modified sequential therapy *vs* quadruple therapy as initial therapy in patients with *Helicobacter* infection

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Author contributions: Wei JQ and Cong YY designed the study; Cong YY and Liao XM performed the research; Liao XM, Nong GH, Chen MZ, Huang XP, Cong YY, Huang YY, Wu BH and Wei JQ contributed technical support; Wei JQ critically revised the manuscript; and Liao XM analyzed the data and wrote the paper.
Ethics approval: This study was reviewed and approved by the 5th Affiliated Hospital of Sun Yat-sen University Institutional Review Board.
Clinical trial registration: This study is registered at http://app1.sfda.gov.cn/datasearch/face3/base.jsp. The registration identification number is 2007S00884.
Informed consent: All study participants, or legal guardians where applicable, provided informed written consent prior to study enrollment.
Conflict-of-interest: Jin-Qi Wei has received fees for serving as an Associate Professor for the 5th Affiliated Hospital of Sun Yat-sen University. Jin-Qi Wei has received research funding from the 5th Affiliated Hospital of Sun Yat-sen University. Jin-Qi Wei is an employee of the 5th Affiliated Hospital of Sun Yat-sen University. Jin-Qi does not own shares in the 5th Affiliated Hospital of Sun Yat-sen University.
Data sharing: No additional data are available.

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Abstract

AIM: To evaluate the efficacy and safety of modified sequential therapy and to compare modified sequential therapy with standard quadruple therapy for *Helicobacter pylori* (*H. pylori*) eradication.

METHODS: In total, 200 consecutive patients who were diagnosed with *H. pylori*-infected chronic gastritis by electronic endoscopy and rapid urease testing from December 2012 to October 2013 were enrolled in this study. The patients had not previously received *H. pylori* eradication treatment, and were randomized into two groups. The patients in Group A (n = 101) were treated with ilaprazole + bismuth potassium citrate + amoxicillin and clavulanate potassium + levofloxacin, and the patients in Group B (n = 99) were administered a modified sequential therapy composed of ilaprazole at 5 mg *bid* and amoxicillin and clavulanate potassium at 914 mg for the first five days followed by ilaprazole at 5 mg *bid*, furazolidone at 100 mg *bid* and levofloxacin at 500 mg *qid* for the next five days. Four to six weeks after the end of treatment, a 14C-urea breath test was performed for all the subjects to confirm the eradication of *H. pylori*. The intention-to-treat and per-protocol eradication rates were determined.

RESULTS: A total of 190 of the 200 patients completed the study. All 200 patients were included in the intention-to-treat analysis, whereas 190 patients were included in the per-protocol analysis. In the intention-to-treat analysis, the rates of *H. pylori* eradication in Groups A and B were 85.15% (86/101) and 81.82% (81/99), respectively. In the per-protocol analysis, the *H. pylori* eradication rates in Groups A and B were 88.66% (86/97) and 87.09% (81/93), respectively. No significant difference was observed (χ^2 = 0.109, P = 0.741) in the eradication rate between Groups A and B. The rates of adverse effects observed in the groups were similar at 6.19% (6/97) for Group A and 7.53% (7/93) for Group B (P > 0.05). No mortality or major morbidities were observed in any of the patients. Symptomatic improvements in the presentation of stomachache, acid regurgitation, and burning sensation were not significantly different between the two groups.

CONCLUSION: Ilaprazole-based 10-d standard quadruple therapy does not offer an incremental benefit over modified sequential therapy for the treatment of *H. pylori* infection, as both treatment regimens appear to be effective, safe, and well-tolerated as initial treatment options.

Key words: *Helicobacter pylori*; Chronic gastritis; Sequential therapy; Quadruple therapy; Initial therapy; Ilaprazole

Core tip: As the prevalence of antibiotic-resistant *Helicobacter pylori* (*H. pylori*) has increased in recent years, the eradication rate of *H. pylori* has simultaneously declined each year. The aim of this randomized controlled clinical trial was to better characterize the safety and efficacy of a modified sequential therapy regimen for the initial treatment of *H. pylori* and to compare this treatment regimen with a 10-d standard quadruple treatment regimen for the eradication of *H. pylori*.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) infection is widespread in humans. An epidemiological study has indicated that the prevalence of *H. pylori* infection in China remains high, reaching 40%-60% in adults[1]. *H. pylori* is involved in chronic gastritis, non-ulcerative dyspepsia, peptic ulcer disease, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphomas. Additionally, *H. pylori* is related to unexplained iron deficiency anemia, chronic idiopathic thrombocytopenic purpura, Alzheimer's disease, colorectal adenomas and colon cancer and is possibly related to atherosclerosis, diabetes, hypertension, obesity and other diseases[2]. The eradication of *H. pylori* facilitates the control of *H. pylori*-related diseases. However, as the prevalence of antibiotic-resistant *H. pylori* has increased in recent years, the eradication rate of *H. pylori* has declined yearly. In 2012, the Chinese Consensus Report showed that the resistance rates to metronidazole, clarithromycin, and levofloxacin were 60%-70%, 20%-38%, and 30%-38%, respectively; however, the resistance rates to amoxicillin, furazolidone and tetracycline remained low (1%-5%). According to a 2013 antibiotic resistance study in Guangdong province in China, the resistance rates to metronidazole, furazolidone, amoxicillin, clarithromycin and levofloxacin were 88.4%, 61.1%, 47.4%, 42.1% and 21.1%, respectively[3]. In 2014, a review noted that the lack of therapeutic compliance and the incidence of side effects might lead to the development of antibiotic resistance. Resistance to metronidazole has reached approximately 40% in developed countries and exceeds 90% in developing countries. The resistance to clarithromycin has been increasing, reaching more than 20% in southern Europe[4]. The Fourth Chinese National Consensus Report[1] showed that the eradication rate of the standard triple therapy [proton pump inhibitor (PPI) + clarithromycin + amoxicillin or PPI + clarithromycin + metronidazole] is lower or far lower than 80%. Increasing the duration of the standard triple therapy from 7 to 10 or 14 d could increase the eradication rate by 5%. To improve the *H. pylori* eradication rate, several regimens of *H. pylori* eradication therapy have been recommended internationally, including sequential therapy (5 d of PPI + amoxicillin followed by 5 d of PPI + clarithromycin + metronidazole, for a total of 10 d), concomitant therapy (PPI + clarithromycin + amoxicillin + metronidazole taken simultaneously) and levofloxacin triple therapy (PPI + levofloxacin + amoxicillin). There has been no controlled study comparing the efficacy of the PPI + amoxicillin + fluoroquinolone regimen with and without the addition of bismuth; however, the use of PPI + amoxicillin + fluoroquinolone + bismuth quadruple therapy as a rescue therapy was shown to be safe and effective in several studies[1].

In this randomized controlled trial, we selected 200 patients with *H. pylori*-positive chronic gastritis who had never received *H. pylori* eradication treatment. These patients were treated for 10 days with ilaprazole + bismuth potassium citrate + amoxicillin and clavulanate potassium + levofloxacin or ilaprazole + amoxicillin and clavulanate potassium + levofloxacin + furazolidone. The aim of the study was to better characterize the safety and efficacy of the modified

sequential therapy regimen for the initial treatment of *H. pylori* and compare it with the standard quadruple treatment for *H. pylori* eradication.

MATERIALS AND METHODS

This was a prospective study. The protocol was approved by the Ethical Investigation Committee of our institution, and informed consent was obtained from all the patients after a full informative session. All patients were managed by a single gastroenterologist, and their details were recorded.

The inclusion criteria were as follows: 200 consecutive patients from December 2012 to October 2013 who visited our hospital clinic for upper abdominal pain, heartburn, acid reflux and other gastrointestinal symptoms, aged 18-65 years old, male or female, and *H. pylori*-positive with chronic gastritis confirmed by electronic endoscopy and a rapid urease test.

The exclusion criteria were as follows: (1) pregnant or breast-feeding women; (2) merged ulcers and ulcer complications; (3) cancer patients; (4) previous upper gastrointestinal surgery; (5) therapy with PPIs, H2 receptor antagonists, bismuth, or antimicrobial drugs 2 wk before treatment; (6) a history of previous *H. pylori* eradication therapy; (7) significant organ dysfunction (hepatic, cardiorespiratory, renal diseases, neoplastic diseases, or coagulopathy); (8) allergy to any of the drugs used in the study or similar drugs; and (9) other cases of interference studies.

A total of 200 patients participated in the study and were randomized into 2 groups. The patients in Group A were treated with standard quadruple therapy (n = 101) consisting of AIBL (amoxicillin and clavulanate potassium at 914 mg *bid*, ilaprazole at 5 mg *bid*, bismuth potassium citrate at 220 mg *bid*, and levofloxacin at 500 mg *qid* for 10 d). The patients in Group B (n = 99) were administered a modified sequential therapy composed of ilaprazole at 5 mg *bid*, amoxicillin and clavulanate potassium at 914 mg *bid*, for the first 5 d followed by ilaprazole at 5 mg *bid*, furazolidone at 100 mg *bid* and levofloxacin at 500 mg *qid*, for the next five days. The proton pump inhibitor and bismuth potassium citrate were administered 30 min before meals, whereas the antibiotics were administered after meals. These drugs were prescribed to the patient one time, and a specific gastroenterologist contacted the patients by telephone to ask them to take the prescribed medication and to inquire whether adverse drug reactions occurred at a fixed time daily. The patients were advised of the possibility of experiencing nausea, taste disturbance, diarrhea, vomiting, dizziness and headaches during the treatment period. The patients were asked to return at the end of eradication therapy to assess the side effects and compliance with the therapy. The incidence of adverse effects was evaluated by a specific questionnaire. Eradication was defined as a negative result on the ^{14}C-urea breath test which was performed 4-6 wk after the end of the course of treatment. None of the patients used antibacterial drugs, PPIs, H_2 receptor antagonists, or bismuth after the treatment until a review was conducted regarding the ^{14}C-urea breath test after *H. pylori* infection.

A technician who was blinded to the assigned protocol performed all of the ^{14}C-urea breath tests.

Statistical analysis

The eradication rates in the intention-to-treat (ITT) and per-protocol (PP) analyses were calculated (Figure 1). The patients who took at least one dose of drugs were included in the ITT analysis, whereas the patients who completed the entire therapy period and completed the follow-up were considered in the PP analysis. The data were analyzed using SPSS 13.0 software. The χ^2 test was used for a comparison between the groups, and values of $P < 0.05$ were considered significant.

RESULTS

A total of 200 patients were included in the ITT analysis, whereas 190 patients were considered in the PP analysis. A total of 97 patients were included in Group A, and 55.67% and 44.33% of these patients were male and female, respectively. The mean age ± SD of the patients in Group A was 40.91 ± 12.10 years. A total of 93 patients were included in group B; 51.61% were male, and 48.39% were female. The mean age ± SD of the Group B patients was 42.88 ± 11.59 years. Of the Group A patients, 23 smoked and 32 used alcohol; in Group B, 25 patients smoked and 30 patients used alcohol. No significant differences between the two groups in terms of drinking, smoking, gender or age were detected ($P > 0.05$). According to the ITT analysis, *H. pylori* eradication was achieved in 86 of the 101 patients in the standard quadruple treatment group (Group A) and in 81 of the 99 patients in the modified sequential treatment group (Group B). The ITT eradication rates of the standard quadruple therapy and sequential therapy were 85.15% and 81.82%, respectively (P = 0.741). The PP eradication rates of the standard quadruple therapy and sequential therapy were 88.66% and 87.09%, respectively (P = 0.741). The reported side effects included diarrhea (three patients in total), nausea (one patient in total), dizziness or headache (four patients in total), insomnia (three patients in total), nausea (five patients in total), vomiting (one patient in total), taste disturbance (six patients in total), menstrual period extension (two patients in total); some patients experienced multiple side effects. However, the extent of the mild adverse reactions could be tolerated and disappeared after treatment. Only 10 patients showed poor compliance (four patients in group A and six patients in group B), and 4 patients (two patients in group A and two patients in group B) were lost to follow-up because of job transfers. The rates of adverse effects for the two groups were similar at 6.19% (6/97) for Group A and

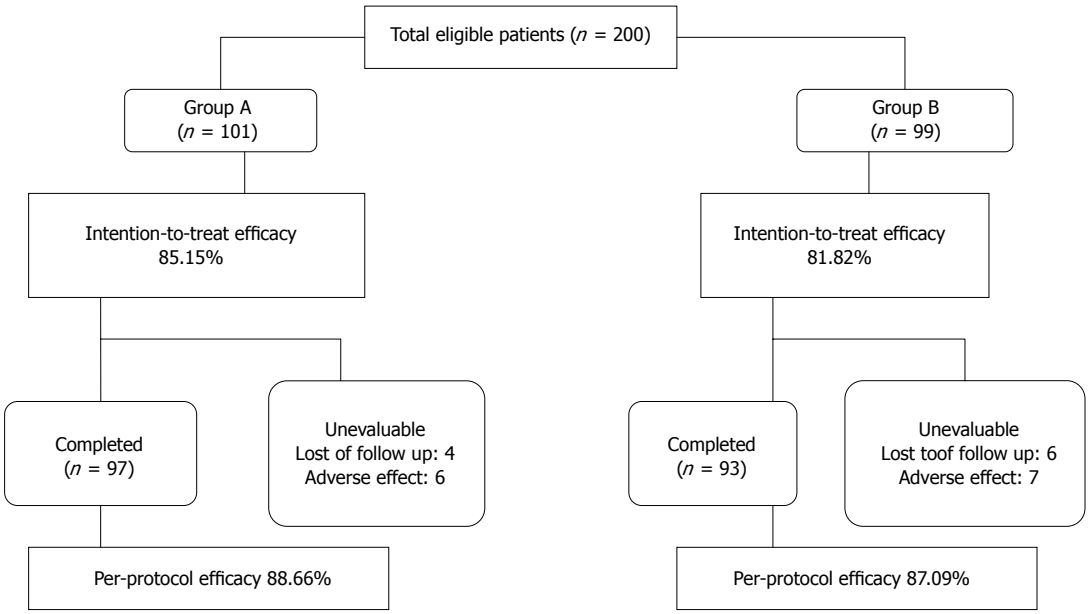

Figure 1 Flow chart to illustrate the study design used in our trial.

Table 1 Adverse events resulting from antibiotic therapy in the study population *n* (%)

Side effects	13 (6.8)
Nausea	5 (2.6)
Taste disturbance	6 (3.2)
Vomiting	1 (0.5)
Diarrhea	3 (1.6)
Dizziness, headache	4 (2.1)
Insomnia	3 (1.8)
Menstrual period extension	2 (1.1)

7.53% (7/93) for Group B ($P > 0.05$). No mortality or major morbidity was recorded in the study (Table 1).

DISCUSSION

Over the past decade, eradication programs regarding *H. pylori*-related diseases have been based on standard triple therapy worldwide. However, the eradication rate of the standard triple therapy (PPI + clarithromycin + amoxicillin or PPI + clarithromycin + metronidazole) is lower or far lower than 80% with the increase in drug-resistant *H. pylori*. Increasing the duration of standard triple therapy from 7 to 10 or 14 d could increase the eradication rate by 5%[1]. The cure rates of *H. pylori* infection are influenced by several factors such as antibiotic susceptibility, insufficient inhibition of acid secretion [*e.g.*, the cytochrome P450 2C19 (CYP2C19) genotype, the PPI dose, and the PPI treatment schedule], bacterial genotypes that reduce virulence (*e.g.*, cagA-negative strains and the vacA s2 genotype), the environment (*e.g.*, smoking), and protocol compliance[5,6]. Reports indicated that the effectiveness of eradication can be influenced by the genetic type of *H. pylori*, better effects of eradicative treatment can be expected if one is infected with the strains of smaller virulence, and cure rates seem to be higher for patients with cagA+/vacA s1 *H pylori* strains[7,8]. A previous randomized open trial[9] showed that smoking significantly decreased the cure rate of *H. pylori* infection, while another study suggested that smoking and drinking habits when analyzed jointly are more useful for predicting the outcome of *H. pylori* eradication than when analyzed separately[10]. Because of the decreased eradication rate, the search for more effective treatment programs or the use of new alternative drugs for *H. pylori* eradication therapy has become imperative. Recently[1,11], *H. pylori* treatment with bismuth-containing quadruple therapy or sequential therapy was recommended as the first-line treatment. A review article[12] showed that quadruple therapy should be considered the first-line treatment in areas of high clarithromycin resistance. A 10-d sequential therapy as a novel therapy shows an impressive eradication rate greater than 90%. The rationale for sequential therapy includes the following: (1) amoxicillin would decrease the bacterial load and the risk of the selection of a clarithromycin-resistant mutant strain; and (2) amoxicillin might disrupt the efflux pump, preventing clarithromycin resistance. Choi *et al*[13] performed a meta-analysis (8 Italian studies) that showed a trend in preferring sequential therapy to triple therapy. Others have suggested that there is insufficient data to recommend sequential therapy as an alternative first-line therapy for *H. pylori* therapy in Asia[14].

This trial was conducted to establish simple and short-term regimens with effective and nontoxic agents for an applicable initial therapy for *H. pylori* eradication in China. Our study showed that 10-d standard quadruple ilaprazole and modified sequential therapy were satisfactory and safe, and they appear

to be well tolerated for initial therapy. In this trial, we used a new PPI, ilaprazole [the compound designated as IY-81149,2 {[(4-methoxy-3-methyl)-2-pyridinyl]-ethylsulfinyl}-5-(1H-pyrrol-1-lyl) 1H-benzimidazole], which belongs to a class of substituted benzimidazole molecules that are chemically related to omeprazole and lansoprazole. The mechanism of action for the suppression of gastric acid secretion is almost identical in ilaprazole and omeprazole. For both drugs, the protonated substituted benzimidazoles suppress gastric acid secretion through the inhibition of H+/K+-ATPase at the secretory surfaces of the gastric parietal cells[15]. Pre-clinical studies and both national and international phase I and Ⅱ clinical trials showed that ilaprazole is a strong, stable, long-lasting inhibitor of gastric acid secretion. A multicenter, randomized, double-blinded, positive-controlled clinical trial which was conducted at 20 hospitals in China concluded that ilaprazole was not affected by CYP2C19 polymorphisms[16]. An additional article reported that ilaprazole provided a higher suppression of gastric acid secretion in a dose-dependent manner, a longer half-life, higher bacteriostasis, and a safety profile similar to that of omeprazole[16]. In agreement with an open randomized crossover study[17] and a previous review[18], which indicated that the metabolism of ilaprazole was not related to CYP2C19 and showed that ilaprazole at 5 mg resulted in an effect comparable to 20 mg of omeprazole, 10 mg and 20 mg of ilaprazole provided a significantly greater and prolonged suppression of gastric acid. The resistance of *H. pylori* to antimicrobial drugs is an important reason for the low eradication rate[19,20]. Amoxicillin and clavulanic acid are two antibiotics that are frequently used in the treatment of *H. pylori*. Amoxicillin is a semi-synthetic β-lactam antibiotic with high selectivity and low toxicity, whereas clavulanic acid is a β-lactamase inhibitor that blocks the activity of the β-lactamase produced by bacteria. Clavulanic acid can reduce bacterial resistance and enhance the antibacterial effect of amoxicillin when used in combination with amoxicillin and other β-lactam antibiotics[21]. Although *H. pylori* does not produce β-lactamase, others[21] have shown that an amoxicillin and clavulanate potassium-containing eradication regimen is safe and effective; therefore, we selected amoxicillin and clavulanate potassium for our study. In this study, we included amoxicillin, levofloxacin and furazolidone in the treatment regimens according to a 2013 antibiotic resistance study in Guangdong province in China which showed that the resistance rates against metronidazole and clarithromycin were 88.4% and 42.1%, respectively, which were relatively lower than the resistance rates for amoxicillin and clavulanate potassium, levofloxacin and furazolidone[3]; the difference in resistance rate between Guangdong province and the entire country might have been related to the specific geography, ethnicity, economic level, drug habits and time span in Guangdong province. A previous randomized controlled trial[22] indicated that bismuth salts had a synergistic effect on antibiotics by destroying bacteria in the manner of an antiseptic. A meta-analysis[23] concluded that bismuth for the treatment of *H. pylori* is safe and well-tolerated, the only adverse event occurring significantly more commonly was dark stools. Recently, a study[24] including one hundred and forty-two *H. pylori*-positive patients in Turkey showed that the 14-d modified sequential treatment, including bismuth, achieved a significantly high eradication rate in patients with *H. pylori* infection, with satisfactory patient compliance and minor side effects. Therefore, we added this agent to the initial therapy regimen.

In this clinical study, 200 consecutive patients with *H. pylori*-positive chronic gastritis who had never received *H. pylori* eradication treatment were randomized into two groups and administered ilaprazole + bismuth + amoxicillin and clavulanate potassium and levofloxacin in a 10-d standard quadruple treatment or amoxicillin and clavulanate potassium + levofloxacin and furazolidone for 10 d in a modified sequential program. The results showed that the ITT eradication rates with the standard quadruple therapy and modified sequential therapy were 85.15% and 81.82%, respectively (P = 0.741). The PP eradication rates with the standard quadruple therapy and modified sequential therapy were 88.66% and 87.09%, respectively (P = 0.741). The *H. pylori* eradication rates in both groups were significantly higher than those in patients in this region who received *H. pylori* eradication treatment containing ilaprazole or esomeprazole + amoxicillin and clavulanate potassium and furazolidone in a 7-d standard triple therapy or amoxicillin and clavulanate potassium + clarithromycin and furazolidone in a 10-d sequential therapy[25]. The eradication rates for both of our study groups were also higher than the eradication rates reported in a national multicenter study evaluating bismuth-containing ilaprazole + amoxicillin and clarithromycin in a 7-d quadruple therapy[26]. These findings suggest that the experimental therapy used in the present study is reasonable regarding the antimicrobial resistance in this region and that antimicrobial drugs and prolonged treatment would improve *H. pylori* eradication by ilaprazole in combination with bismuth quadruple and sequential programs. The rates of side effects in both groups were similar (6.19% *vs* 7.53%, P > 0.05), which indicated that ilaprazole in the 10-d standard quadruple and modified sequential regimen had a better safety profile in the treatment of *H. pylori*-positive patients with chronic gastritis and offered a clinical basis for *H. pylori*-positive treatment programs in the region. Regarding the sequential therapy, our results are similar to a previous randomized, double-blinded, comparative clinical trial in China which reported that the eradication rate with sequential therapy (20 mg of omeprazole *bid* and 1000 mg of amoxicillin

for 5 d followed by 20 mg of omeprazole, 500 mg of metronidazole, and 500 mg of clarithromycin for an additional 5 d) was 88.89%[27]. In the same year, 2012, a randomized study in Japan reported that the eradication rate of non-bismuth quadruple therapy (lansoprazole at 30 mg, amoxicillin at 750 mg, clarithromycin at 200 mg and metronidazole at 250 mg, twice daily for 7 d) was 94.9% and 98.3%, respectively, by ITT analysis and PP analysis[28]. Recently, a Korean article reported that 7-d and 14-d quadruple therapy with PPI, tripotassium dicitrato bismuthate, tetracycline, and metronidazole showed eradication rates of 66.4% and 71.1%, respectively, by an ITT analysis and 76.5% and 83.8%, respectively, by PP analysis[29].

This study has two limitations. The eradication rate with these two regimens did not achieve the desirable eradication rate of 90%[30]. Selecting the modified sequential therapy as an initial treatment, which requires three antibiotic drugs, has the possibility of increasing the adverse reactions of the drugs and reducing the availability of antibiotic drugs when therapeutic failure occurs.

In conclusion, our study suggests that for *H. pylori* patients in the Guangdong province, China, ilaprazole-based 10-d standard quadruple therapy does not offer an incremental benefit over modified sequential therapy, as both regimens appear to be effective, safe, and well-tolerated as initial treatment options. Additional studies comparing the treatment dose and duration are needed to further evaluate these two regimens as initial therapy protocols in our population.

COMMENTS

Background

As the prevalence of antibiotic-resistant *Helicobacter pylori* (*H. pylori*) has increased in recent years, the eradication rate of *H. pylori* has simultaneously declined annually. To improve the rate of *H. pylori* eradication in patients, several regimens of *H. pylori* eradication therapy have been recommended internationally, including sequential therapy [5 d of proton pump inhibitor (PPI) + amoxicillin followed by 5 d of PPI + clarithromycin + metronidazole for a total of 10 d], concomitant therapy (PPI + clarithromycin + amoxicillin + metronidazole taken simultaneously), and levofloxacin triple therapy (PPI + levofloxacin + amoxicillin).

Research frontiers

Controlled studies have not been conducted comparing the relative efficacies of a PPI + amoxicillin + fluoroquinolone regimen with and without the addition of bismuth. However, the use of PPI + amoxicillin + fluoroquinolone + bismuth quadruple therapy as a rescue therapy has been shown to be safe and effective in several studies.

Innovations and breakthroughs

Firstly, because no controlled studies have been conducted comparing the efficacy of a PPI + amoxicillin + fluoroquinolone regimen with and without the addition of bismuth, the authors evaluated the efficacy and safety of a new sequential therapy regimen and compared this regimen with the standard quadruple treatment regimen for *H. pylori* eradication. Secondly, the authors chose amoxicillin, levofloxacin, and furazolidone for these regimens based on an antibiotic resistance study conducted in patients from the Guangdong province in China. Finally, the authors chose two four-drug regimens as initial therapies despite the fact that standard triple therapy is considered standard of care in many countries. Furthermore, both of the regimens tested were well-tolerated as initial therapies.

Applications

The results of this study suggest that ilaprazole-based 10-d standard quadruple therapy does not offer an incremental benefit over modified sequential therapy for the treatment of *H. pylori* patients, as both regimens are effective, safe, and well-tolerated as initial treatment options.

Terminology

H. pylori infection is widespread in humans. *H. pylori* is involved in chronic gastritis, non-ulcerative dyspepsia, peptic ulcer disease, gastric adenocarcinoma and gastric mucosa-associated lymphoid tissue (MALT) lymphomas. Additionally, *H. pylori* is related to idiopathic iron deficiency anemia, chronic idiopathic thrombocytopenic purpura, Alzheimer's disease, colorectal adenomas and colon cancer, and may be related to atherosclerosis, diabetes, hypertension, and obesity among other diseases. The eradication of *H. pylori* could ameliorate many of these *H. pylori*-related diseases.

Peer-review

This is a well-designed, performed and written clinical trial study to compare the efficacy and safety of a modified sequential therapy with the standard quadruple treatment for *H. pylori* eradication in 200 consecutive patients who were diagnosed with *H. pylori*-infected chronic gastritis in China were the eradication rate of *H. pylori* has yearly declined.

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SYSTEMATIC REVIEWS

Integrative analysis of aberrant Wnt signaling in hepatitis B virus-related hepatocellular carcinoma

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Abstract

AIM: To comprehensively understand the underlying molecular events accounting for aberrant Wnt signaling activation in hepatocellular carcinoma (HCC).

METHODS: This study was retrospective. The HCC tissue specimens used in this research were obtained from patients who underwent liver surgery. The Catalogue of Somatic Mutations in Cancer (COSMIC) database was searched for the mutation statuses of *CTNNB1*, *TP53*, and protein degradation regulator genes of *CTNNB1*. Dual-luciferase reporter assay was performed with TOP/FOP reporters to detect whether *TP53* gain-of-function (GOF) mutations could enhance the transcriptional activity of Wnt signaling. Methylation sensitive restriction enzyme-quantitative PCR was used to explore the methylation status of CpG islands located in the promoters of *APC*, *SFRP1*, and *SFRP5* in HCCs with different risk factors. Finally, nested-reverse transcription PCR was performed to examine the integration of *HBx* in front of *LINE1* element and the existence of *HBx-LINE1* chimeric transcript in Hepatitis B virus-related HCC. All results in this article were analyzed with the software SPSS version 19.0 for Windows, and different groups were compared by χ^2 test as appropriate.

RESULTS: Based on the data from COSMIC database, compared with other solid tumors, mutation frequency of *CTNNB1* was significantly higher in HCC ($P < 0.01$). The rate of *CTNNB1* mutation was significantly less frequent in Hepatitis B virus-related HCC than in other etiologies ($P < 0.01$). Dual-luciferase reporter system and TOP/FOP reporter assays confirmed that *TP53* GOF mutants were able to enhance the transcriptional ability of Wnt signaling. An exclusive relationship between the status of *TP53* and *CTNNB1* mutations was observed. However, according to the COSMIC database, *TP53* GOF mutation is rare in HCC, which indicates that *TP53* GOF mutation is not a reason for the aberrant activation of Wnt signaling in HCC. *APC* and *AXIN1* were mutated in HCC. By using methylation sensitive restriction enzyme-quantitative PCR, hypermethylation of *APC* was detected in HCC with different risk factors, whereas *SFRP1* and *SFRP5* were not hypermethylated in any of the HCC etiologies, which indicates that

the mutation of *APC* and *AXIN1*, together with the methylation of *APC* could take part in the overactivation of Wnt signaling. Nested-reverse transcription PCR failed to detect the integration of *HBx* before the *LINE1* element, or the existence of an *HBx-LINE1* chimeric transcript, suggesting that integration could not play a role in the aberrant activation of Wnt signaling in HCC.

CONCLUSION: In HCC, genetic/epigenetic aberration of *CTNNB1* and its protein degradation regulators are the major cause of Wnt signaling overactivation.

Key words: β-catenin; CTNNB1; Hepatitis B virus; Hepatocellular carcinoma; TP53; Wnt signaling

Core tip: Abnormal activation of Wnt/β-catenin signaling can be detected in approximately 50%-70% of hepatocellular carcinoma (HCC). It is necessary to take the analysis about the cause of Wnt/β-catenin signaling pathway aberration with the etiologic differences into consideration. In this review, the suggested genetic/epigenetic aberrations and their involvement in the abnormal Wnt/β-catenin overactivation in HCC were comprehensively analyzed, with focus on the cause of hepatitis B virus-related HCC. We suggest that genetic/epigenetic aberration of *CTNNB1* and its protein degradation regulators are the major cause of Wnt signaling overactivation. *TP53* gain-of-function mutation is seldom involved, and *HBx-LINE1* chimeric transcripts created by viral integration may not be present.

Ding SL, Yang ZW, Wang J, Zhang XL, Chen XM, Lu FM. Integrative analysis of aberrant Wnt signaling in hepatitis B virus-related hepatocellular carcinoma. *World J Gastroenterol* 2015; 21(20): 6317-6328 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6317.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6317

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most deathly human malignancy[1]. The leading causative factors of HCC include chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, exposure to aflatoxin-contaminated food and alcohol consumption. Chronic HBV carriers have a 5-15-fold increased risk of HCC compared with the general population[2]. China alone accounts for more than half of the world's annually diagnosed new HCC cases, predominately due to high prevalence of HBV infection and consequent cirrhosis[3].

The development of HCC is a multistage process, during which numerous genetic/epigenetic abnormalities are involved. The Wnt/β-catenin signaling pathway, which plays key roles in development and adult tissue homeostasis, is highly conserved throughout evolution. Abnormal activation of this pathway could induce different diseases, especially tumors[4]. When Wnt signaling is activated, canonical Wnt signals are transduced through Frizzled family receptors and LRP5/LRP6 co-receptors located on the cell membrane, initiating the β-catenin signaling cascade[5]. β-catenin is the core component of the Wnt/β-catenin pathway and is sequestered in the cytoplasm by the "destruction complex", which includes Axin, glycogen synthase kinase 3, and adenomatous polyposis coli (APC). This multi-protein destruction complex could target the proto-oncogene β-catenin for ubiquitin-mediated proteolysis[6,7]. Activation of the wnt signaling could prevent glycogen synthase kinase 3β (GSK-3β)-mediated β-catenin degradation, leading to accumulation and nuclear translocation of β-catenin[8]. The nuclear accumulated β-catenin could then combine with T-cell factor/lymphoid enhancer factor, and thereby promote the transcription of downstream target genes, including *FGF20*, *DKK1*, *WISP1*, *MYC*, *CCND1*, and so on. It has been shown that 50%-70% of HCC tissues have abnormal β-catenin protein accumulation[9,10]. Furthermore, β-catenin expression, especially in poorly differentiated tumors, is an indicator of poor prognosis, as HCC patients with β-catenin positive grade Ⅲ tumors have a significantly poorer prognosis[11]. Therefore, β-catenin could play important roles in the development and prognosis of HCC.

Many mechanisms have been shown to be involved in the aberrant activation of Wnt signaling. First, mutation of the β-catenin coding gene *CTNNB1* causes aberrant activation of Wnt signaling in many tumors, including sporadic colorectal cancer[12], anaplastic thyroid carcinoma[13], gastric cancer[14], and HCC[15]. Second, aberration of several constitution molecules such as APC, AXIN, secreted Frizzled related protein (SFRP) 1 and SFRP5 in Wnt signaling could also affect the activation of Wnt signaling. In addition, *TP53* gain-of-function (GOF) mutations were reported to activate Wnt signaling[16]. However, whether all these mechanisms play important roles in HCC, especially in the HBV-related HCC, have not been fully understood. Furthermore, a recent study reported that *HBx* could integrate into human genome and form an *HBx-LINE1* chimeric transcript, and this transcript could activate Wnt signaling as an long noncoding RNA[17]. Through literature review and experimental detection, the possible presence of *HBx-LINE1* in primary live tumor was also addressed. In this study, by integrative analysis of these potential factors, we summarize the known molecular mechanisms of aberrant activation of Wnt signaling in HCC.

The aim of the study was to understand the underlying molecular events accounting for aberrant Wnt signaling activation in HCC, particularly in the HCC with background of chronic HBV infection.

Table 1 *CTNNB1* mutation case resources in each article

PubMed ID	Total cases	HBV infection cases	HCV infection cases	HBV and HCV coinfection cases	Non-viral infection cases
9635572	14/75	NA/NA	NA/NA	NA/NA	NA/NA
9671767	8/31	NA/NA	NA/NA	NA/NA	NA/NA
10487827	14/32	2/5	2/13	0/0	10/13
10595907	13/22	0/0	13/22	0/0	0/0
10665646	9/38	NA/NA	NA/NA	NA/NA	NA/NA
10700176	13/100	NA/NA	NA/NA	NA/NA	NA/NA
10980116	57/421	27/265	23/95	3/44	4/17
11282485	6/32	NA/NA	NA/NA	NA/NA	NA/NA
11375957	26/137	3/33	12/31	0/9	11/41
11429783	0/22	0/0	0/0	0/0	0/22
11443619	7/60	5/48	0/2	0/0	2/10
11477549	1/14	0/1	1/12	0/0	0/1
11570580	15/34	NA/NA	NA/NA	NA/NA	NA/NA
12101426	14/73	NA/NA	NA/NA	NA/NA	NA/NA
12375019	10/57	0/0	10/57	0/0	0/0
12439747	184/1123	NA/NA	NA/NA	NA/NA	NA/NA
12845670	24/100	5/26	16/51	0/0	3/23
14999698	3/61	0/29	0/7	0/1	0/0
15067328	10/89	NA/NA	NA/NA	NA/NA	NA/NA
15151624	1/61	NA/NA	NA/NA	NA/NA	NA/NA
15288479	2/16	0/0	0/4	0/0	2/6
15305374	44/781	NA/512	NA/151	NA/52	NA/66
15814635	37/265	NA/161	NA/53	NA/26	NA/25
17187432	34/120	NA/NA	NA/NA	NA/NA	NA/NA
17393110	7/29	NA/NA	NA/NA	NA/NA	NA/NA
17510384	13/81	2/13	8/44	1/2	2/18
17531558	4/42	0/7	4/23	0/3	0/9
18171349	1/36	0/21	0/4	0/0	1/11
18282277	11/52	NA/NA	NA/NA	NA/NA	NA/NA
18358501	4/54	3/43	0/1	0/2	1/8
18467159	47/223	NA/NA	NA/NA	NA/NA	NA/NA
18701503	28/81	0/0	28/81	0/0	0/0
19101982	10/32	0/0	3/6	0/0	7/26
20347502	1/1	0/0	1/1	0/0	0/0
20923573	7/15	1/4	4/10	0/0	2/2
20963515	2/20	2/20	0/0	0/0	0/0
21457159	19/44	3/11	10/23	1/2	5/8
21822264	28/139	5/50	13/43	1/2	9/44
22561517	42/149	3/32	7/24	1/4	31/89
25021421	53/176	13/78	NA/NA	NA/NA	NA/NA

Data are presented as the number of mutation cases/category total. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NA: Not available.

MATERIALS AND METHODS

Database resources

Catalogue of Somatic Mutations in Cancer (COSMIC) database (http://cancer.sanger.ac.uk/) was searched for summarizing the mutation statuses of *CTNNB1*, *APC*, *AXIN1*, *AXIN2*, and *TP53*. *TP53* GOF mutants were defined including *S127Y*, *P151S*, *R156P*, *Y163N*, *Y163C*, *V173L*, *R175H*, *C176Y*, *H179R*, *L194R*, *Y205C*, *H214R*, *Y220C*, *Y234C*, *M237I*, *S241F*, *G245C*, *G245S*, *G245V*, *G245D*, *R248W*, *R248G*, *R248Q*, *R273C*, *R273L*, *R273H*, *R273P*, *C275Y*, *D281G*, and *R282W,* as suggested[16]. For investigating the link between gene mutation and etiology, we also consulted the articles referred by the COSMIC database, and determined the etiology of each case. The mutation data of *CTNNB1*, *AXIN1*, and *TP53* is listed in Tables 1-9.

HCC tissue samples

HCC tissue samples were obtained from patients who underwent routine curative surgery at Henan Oncology Hospital in Zhengzhou, Henan Province of China. This study was retrospective, and all tissues were obtained during surgeries. All patients were HBV-positive, which was indicated with serum HBsAg or HBV-DNA presence. For detecting the methylation status of *APC*, *SFRP1*, and *SFRP5*, seven pairs of tissue specimens from HCC patients with serum anti-HCV-positive and ten pairs of tissue specimens from HCC patients without HBV/HCV viral infection were collected. Six tumor-free tissues from patients with hepatic hemangioma were used as controls. All tissues were snap frozen in liquate nitrogen until use. The study was approved by the Ethics Committee in the university, and the informed consents were obtained

Table 2 *AXIN1* mutation case resources in each article

PubMed ID	Total cases	HBV infection cases	HCV infection cases	HBV and HCV coinfection cases	Non-viral infection cases
10700176	9/100	NA/NA	NA/NA	NA/NA	NA/NA
11375957	12/112	8/32	0/31	2/9	2/40
12101426	4/56	0/9	0/5	0/2	4/40
15067328	25/89	NA/NA	NA/NA	NA/NA	NA/NA
18171349	9/36	5/21	3/4	0/0	1/11
21499249	1/1	0/0	1/1	0/0	0/0
22561517	19/149	5/32	5/24	1/4	8/89
25021421	25/176	13/78	NA/NA	NA/NA	NA/NA

Data are presented as the number of mutation cases/category total. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NA: Not available.

from all patients and donors before the start of the study.

DNA methylation detection

DNA was extracted from tissues by digestion of frozen samples with 1% proteinase K, followed by standard phenol/chloroform and ethanol precipitation. The DNA methylation status was detected by DNA methylation-sensitive restriction endonuclease digestion, followed by subsequent quantitative (q)PCR assay as described previously[18]. In brief, 2 μg DNA was treated with *Hha*I methylation-sensitive enzyme at 37 ℃ for 16 h. *Hha*I can digest the GCGC sequence if the cytosine is not methylated. Then, qPCR was performed to amplify the target template with primers between which *Hha*I cutting sites were located. The reaction was performed in a 96-well plate on Roche Lightcyler 480 Ⅱ Real-Time PCR System (Roche, Basel, Switzerland). Methylation intensity was quantified between 0% and 100% by calculating Ct values of tissues treated either with or without *Hha*I digestion. Primers used for *APC*, *SFRP1*, and *SFRP5* methylation detection are: *APC*, F-CGGACCAGGGCGCTCCCCATTCC and R-TGACACCCTGGCGGGCTGCACCAA; *SFRP1*, F-TCGCCCCGCCGGGAGCTGATTG and R-GGCTGGAGTGCGCGGGGCTCCT; *SFRP5* F-CCAGTGCAGCGCCCCCAGCAGCA and R-CGCGGCGCGCACCTGGAGAG.

Reverse transcription-PCR assay

Total RNA was extracted with TRI-Reagent (Invitrogen of Thermo Fisher Scientific, Waltham, MA, United States) according to the manufacturer's protocol, and reverse transcription was then performed using Reverse Transcription Kit (Thermo Fish Scientific). Nested-PCR was performed to detect *HBx-LINE1* transcript (primers: first round: F-TCCCCGTCTGTGCCTTCTC and R-TAGTGCTGCAATAAACATGGGA; second round: F-ACGCGGTCTCCCCGTCTGT and R-GCTGGATCATATGGAAGCTCTGG). β-actin was used as the calibrator gene (primers: F-CTACAGCTTCACCACCACGG and R-TCAGGCAGCTCGTAGCTCTTC).

Plasmid construction

PcDNA3.1-TP53 expression vector was kept in our lab, which covers the coding sequence of *TP53* and contains a myc tag. Using this vector as a template, three different GOF mutation sites were precisely mutated. The primers used: R273C, F-AACAGCTTTGAGGTGTGTTTGTGCCTGTCCTGGG and R-GACAGGCACAAACACACACCTCAAAGCTGTTCCGTC; R273H, F-AACAGCTTTGAGGTGCATGTTTGTGCCTGTCCTGGG and R-GACAGGCACAAACATGCACCTCAAAGCTGTTCCGTC; Y220C, F-GTGTGGTGGTGCCCTGTGAGCCGCCTGAGGTTGGCT and R-ACCTCAGGCGGCTCACAGGGCACCACCACACTATGT.

Luciferase reporter assays

The TOP/FOP reporter plasmids were co-transfected with Renilla luciferase vector into HEK 293T, SMMC 7721, and Huh-7 cell lines using Lipofectamine 2000 (Invitrogen). Twenty-four hours after transfection, cells were washed twice with PBS and lysed in passive lysis buffer. PGL3-basic vector was used as a negative control. Luciferase activity was analyzed using a luminometer and a dual luciferase assay kit according to the manufacturer (Promega Corp., Madison, WI, United States). Luciferase counts were normalized using Tk-Renilla-luciferase (Promega).

Statistical analysis

All analyses were performed with the software SPSS version 19.0 (IBM, Armonk, NY, United States). Different groups were compared by χ^2 tests as appropriate. All statistical tests were two-sided, and $P < 0.01$ was considered as statistically significant.

RESULTS

CTNNB1 mutation is a major causative factor of aberrant Wnt signaling activation in HCC

CTNNB1 mutation, especially mutation at the phosphorylation sites in N-terminal domain, could affect β-catenin protein stability and its combining capability with APC and AXIN. Firstly, we summarized the mutation frequency of *CTNNB1* in different tumors, including HCC, stomach, lung, ovary, colon tumor and esophageal squamous cell carcinoma, based on the information collected from COSMIC database. As shown in Figure 1A, the *CTNNB1* mutation rate

Table 3 *TP53* mutation case resources in each article

PubMed ID	Total cases	HBV infection cases	HCV infection cases	HBV and HCV coinfection cases	Non-viral infection cases
1311638	0/21/36	0/21/36	0/0/0	0/0/0	0/0/0
1327523	3/20/61	3/12/37	0/4/17	0/3/4	0/1/3
1655254	2/9/43	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
1672732	0/5/10	0/5/8	0/0/NA	0/0/NA	0/0/NA
1849234	0/8/16	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
7903205	2/20/35	2/15/28	0/NA/NA	0/NA/NA	0/NA/NA
8093350	1/17/53	0/3/7	NA/NA/NA	NA/NA/NA	NA/NA/NA
8093978	0/3/20	0/NA/17	0/NA/NA	0/NA/NA	0/NA/NA
8100480	1/3/20	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
8108145	2/6/15	2/6/15	0/0/0	0/0/0	0/0/0
8261444	5/12/16	1/2/3	4/8/10	0/1/2	0/1/1
8290606	0/19/80	0/8/28	0/NA/NA	0/NA/NA	0/NA/NA
8302580	1/6/22	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
8380058	0/2/15	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
8382111	0/12/38	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
8384081	0/4/45	0/4/28	0/0/11	0/0/2	0/0/4
8389246	0/11/34	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
8390289	0/10/15	0/8/13	0/NA/NA	0/NA/NA	0/NA/NA
8390407	0/1/18	0/0/3	0/0/5	0/0/1	0/1/9
8393166	1/1/7	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
8407553	3/19/63	2/10/16	1/4/15	0/0/0	0/5/32
8565124	0/2/12	0/0/3	0/0/2	0/0/1	0/2/6
8655704	3/10/38	NA/NA/19	NA/NA/10	NA/NA/5	NA/NA/4
8655958	2/2/20	1/1/4	NA/NA/NA	NA/NA/NA	NA/NA/NA
8672994	0/1/18	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
8895490	0/9/12	0/0/0	0/1/3	0/2/3	0/3/6
9012469	0/8/16	0/1/8	0/NA/NA	0/NA/NA	0/NA/NA
9270015	2/26/105	2/24/78	0/NA/NA	0/NA/NA	0/NA/NA
9463584	0/2/8	0/NA/NA	0/NA/NA	0/NA/NA	0/NA/NA
9699537	2/6/97	NA/NA/16	NA/NA/NA	NA/NA/NA	NA/NA/NA
9781942	1/2/15	0/0/0	1/2/15	0/0/0	0/0/0
10389750	0/17/31	0/11/17	0/NA/NA	0/NA/NA	0/NA/NA
10389978	0/12/24	0/10/19	0/NA/NA	0/NA/NA	0/NA/NA
10564952	2/9/11	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
10662591	0/5/27	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
10699891	4/11/18	0/0/0	0/0/0	0/0/0	4/11/18
10743047	0/35/40	0/33/38	0/NA/NA	0/NA/NA	0/NA/NA
11051249	2/21/83	0/14/50	1/6/16	0/0/0	1/1/17
11191353	0/14/55	0/NA/45	0/NA/NA	0/NA/NA	0/NA/NA
11282486	2/6/22	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
11375957	1/36/137	1/15/33	0/10/31	0/3/9	0/8/41
11704835	8/30/71	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
12483005	3/7/34	2/5/27	0/1/3	0/0/0	1/1/4
12640682	0/2/18	0/1/5	0/1/8	0/0/4	0/0/1
12759240	2/16/33	2/16/33	0/0/0	0/0/0	0/0/0
12845670	0/14/100	0/4/26	0/7/51	0/0/0	0/3/23
14499690	0/18/68	0/NA/52	0/NA/2	0/0/0	0/NA/14
14675778	0/5/22	0/0/0	0/5/22	0/0/0	0/0/0
14687797	3/9/80	2/7/NA	0/1/NA	0/0/NA	1/1/NA
15017592	4/22/50	NA/NA/13	NA/NA/33	NA/NA/3	NA/NA/1
15126338	3/27/48	NA/NA/NA	NA/NA/NA	NA/NA/NA	NA/NA/NA
15943041	8/8/20	8/8/20	0/0/0	0/0/0	0/0/0
16078640	2/7/41	NA/NA/1	NA/NA/26	NA/NA/NA	NA/NA/NA
16570275	1/11/40	1/4/22	0/2/5	0/2/5	0/3/8
16685387	0/28/55	0/NA/49	0/NA/NA	0/NA/NA	0/NA/NA
16697535	0/7/23	0/7/23	0/0/0	0/0/0	0/0/0
17066440	0/5/51	0/0/14	0/4/30	0/0/0	0/1/7
17266182	1/28/50	1/24/41	0/NA/NA	0/NA/NA	0/NA/NA
17350822	4/16/83	NA/2/13	NA/13/64	NA/0/2	NA/1/4
17510384	0/25/81	0/5/13	0/15/44	0/2/2	0/3/18
17531558	2/7/42	1/1/7	1/4/24	0/1/2	0/1/9
21499249	0/1/1	0/0/0	0/1/1	0/0/0	0/0/0
21760996	0/9/26	0/6/13	0/2/4	0/0/0	0/0/4
21822264	4/39/139	2/20/50	1/10/43	0/2/2	1/7/44
22561517	0/26/149	0/9/32	0/7/24	0/2/4	0/8/89
22634756	3/14/27	3/7/11	0/7/14	0/0/0	0/0/2
22922871	1/1/10	1/1/10	0/0/0	0/0/0	0/0/0

Data are presented as the number of gain-of-function mutation cases/number of mutations/category total. HBV: Hepatitis B virus; HCV: Hepatitis C virus; NA: Not available.

Table 4 Mutation case numbers and rates of *CTNNB1* in different tissues

Cancer type	Mutation cases (*n*)	Total cases (*n*)	Mutation rate (%)	*P* value[1]
HCC	671	3720	18.04	
Colon Adenocarcinoma	126	1318	9.56	< 0.001
Ovary carcinoma	102	1599	6.38	< 0.001
Stomach Adenocarcinoma	54	1137	4.75	< 0.001
Lung Adenocarcinoma	38	1219	3.12	< 0.001
ESCC	5	347	1.44	< 0.001

[1]The *P* value *vs* HCC. ESCC: Esophageal squamous cell carcinoma; HCC: Hepatocellular carcinoma.

Table 5 Mutation case numbers and rates of CTNNB1 in hepatocellular carcinoma etiologies

Viral background	Mutation cases (*n*)	Total cases (*n*)	Mutation rate (%)	*P* value[1]
HBV	74	686	10.79	
HCV	155	554	27.98	< 0.001
HBV and HCV	7	69	10.14	0.999
Non-viral	90	348	25.86	< 0.001

[1]The *P* value *vs* HBV. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

in HCC was 18.04%, which was significantly higher than the mutation rates in other tumors (Table 4), implicating that *CTNNB1* mutation could be one of the major reasons for aberrant activation of Wnt signaling commonly seen in HCC. Next, the HCC patients were classified into different groups according to the background of viral infection, and then we compared the rates of *CTNNB1* mutation among different HCC groups. As shown in Figure 1B, the *CTNNB1* mutation rate in chronic HBV-related HCC was 10.79%, which was similar to that in HBV/HCV coinfection-related HCC, but significantly lower than those with HCV-related HCC or non-viral HCC (Table 5). Nevertheless, the *CTNNB1* mutation rate in chronic HBV-related HCC was still higher than that in several other human tumors such as esophageal squamous cell carcinoma, lung cancer, and gastric cancer.

TP53 GOF mutants may not contribute to aberrant Wnt signaling activation in HCC

Tumor suppressor gene *TP53* is the most frequently mutated gene in cancer. Among these p53 mutants, some mutations not only lose the tumor suppressive functions, but also gain novel oncogenic activities, including promotion of tumor cell proliferation, survival, metabolic changes, angiogenesis, and metastasis, which were defined as p53 GOF activities[19]. It has been reported that β-catenin expression and the Wnt signaling pathway are highly activated in tumors harboring GOF p53 mutants[16]. To investigate whether *TP53* GOF mutant could activate the Wnt signaling

Table 6 Mutation case numbers and rates of *AXIN1* in different tissues

Cancer type	Mutation cases (*n*)	Total cases (*n*)	Mutation rate (%)
Hepatocellular carcinoma	75	872	8.60
Colon adenocarcinoma	20	513	3.90
Ovary carcinoma	3	749	0.40
Stomach adenocarcinoma	18	377	4.77
Lung adenocarcinoma	6	697	0.86
Esophageal squamous cell carcinoma	3	130	2.31

Table 7 Mutation case numbers and rates of *AXIN1* in hepatocellular carcinoma etiologies

Viral background	Mutation cases (*n*)	Total cases (*n*)	Mutation rate (%)	*P* value[1]
HBV	31	172	18.02	
HCV	9	65	13.85	0.964
HBV and HCV	3	15	20.00	0.999
non-viral	15	180	8.33	0.122

[1]The *P* value *vs* HBV. HBV: Hepatitis B virus; HCV: Hepatitis C virus.

pathway in HCC, we performed a TOP/FOP luciferase assay by overexpressing Y220C, R273C, and R273H *TP53* GOF mutants in HEK 293T, SMMC7721, and Huh-7 cell lines. Compared with wild-type p53, Y220C enhanced the TOP/FOP value in Huh-7 cells, whereas R273H and R273C enhanced the TOP/FOP value in SMMC 7721 cells. As a positive control, all three GOF mutations enhanced TOP/FOP values in HEK 293T cells (Figure 2A). The effect of p53 GOF mutants in those hepatic origin cells was insignificant and inconsistent. Consistent with the results of the *in vitro* analysis, searching of the COSMIC database revealed that, though the total *TP53* mutation rate in HCC was as high as 29.33%, the rate of *TP53* GOF mutation in HCCs was only 4.27%, substantially lower than that observed in other tumors (Figure 2B and Table 8). Furthermore, such a low rate of *TP53* GOF mutation was constantly present among HCCs of different etiologies (Figure 2B). Taken together, although *TP53* GOF mutants activate Wnt signaling in HCC cell lines *in vitro*, they seldom occur in HCC and likely do play a major role in aberrant activation of Wnt signaling commonly present in HCC.

Frequent genetic/epigenetic aberrations in negative regulators involved in Wnt signaling activation in HCC

Since APC and AXINs can form a degradation complex with GSK-3β to prompt the ubiquitination-dependent degradation of β-catenin protein, aberration of either APC or AXINs might affect the activity of the Wnt/β-catenin signaling pathway. Based on the COSMIC database, the mutation rates of *AXIN1*, *AXIN2*, and *APC* in HCC were 8.60%, 0.42%, and 1.33%, respectively (Figure 3A). The high frequent mutation rate of *AXIN1* suggests that it commonly contributes

Table 8 Mutation case numbers and rates of TP53 in different tissues

Cancer type	GOF mutation cases (*n*)	Mutation cases (*n*)	Total cases (*n*)	GOF mutation rate (%)	Non-GOF mutation rate (%)	Total mutation rate (%)	*P* value[1]
HCC	91	825	2813	3.23	26.09	29.33	
Colon adenocarcinoma	331	1867	3596	9.20	42.71	51.92	< 0.001
Ovary carcinoma	392	1604	3329	11.78	36.41	48.18	< 0.001
Stomach adenocarcinoma	270	1160	3459	7.81	25.73	33.54	< 0.001
Lung adenocarcinoma	421	2414	6394	6.58	31.17	37.75	< 0.001
ESCC	224	972	1858	12.06	40.26	52.31	< 0.001

[1]GOF mutation rate *vs* HCC. ESCC: Esophageal squamous cell carcinoma; GOF: Gain-of-function; HCC: Hepatocellular carcinoma.

Table 9 Mutation case numbers and rates of TP53 in hepatocellular carcinoma etiologies

Viral background	GOF mutation cases (*n*)	Mutation cases (*n*)	Total cases (*n*)	GOF mutation rate (%)	Non-GOF mutation rate (%)	Total mutation rate (%)
HBV	35	321	819	4.27	34.92	39.19
HCV	9	101	398	2.26	23.12	25.38
HBV and HCV	0	18	41	0.00	43.90	43.90
non-viral	7	60	346	2.02	15.32	17.34

GOF: Gain-of-function; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

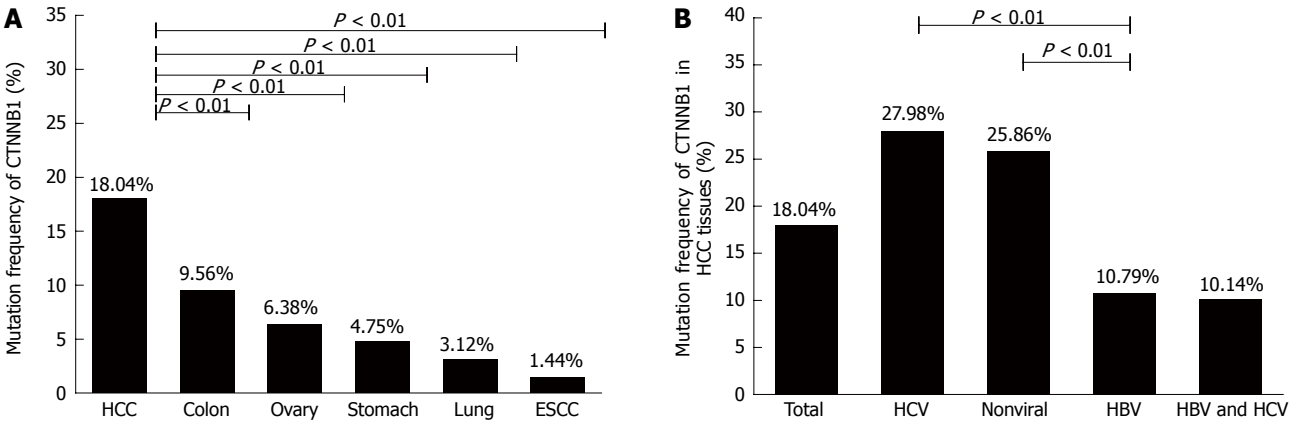

Figure 1 *CTNNB1* mutation rate in tumors based on the information collected from COSMIC database. *CTNNB1* mutation rates in A: Different human tumors; and B: Hepatocellular carcinoma tissues with different risk factors. ESCC: Esophageal squamous cell carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

to the aberrant Wnt signaling activation in HCC.

SFRP1 and *SFRP5* encode SFRP, which is the antagonist of the Wnt signaling pathway. The epigenetic downregulation of SFRP has been shown to be involved in hepatocarcinogenesis[20]. We previously demonstrated that the promoter CpG island of *APC* is hypermethylated in HCC with HBV infection[21]. To further demonstrate the involvement of the negative regulators in Wnt/β-catenin overactivation in HCC, methylation sensitive restriction enzyme-qPCR was performed to detect the methylation statuses of *APC*, *SFRP1*, and *SFRP5* in HCC with different risk factors, including between HCV-related HCC and those without viral infection. The results showed extensive hypermethylation of the *APC* promoter in all HCC of different etiologies (Figure 3B). As shown in Figure 3C, hypermethylation of *SFRP1* was present in 15% (3/20) of HBV-infected tumor tissues, 0% (0/7) of HCV-infected tumor tissues, 20% (2/10) of non-infected tumor tissues, and 0% (0/6) of hepatic hemangioma tissues, whereas hypermethylation of *SFRP5* was present in 5% (1/20) of HBV-infected tumor tissues, 28.6% (2/7) of HCV-infected tumor tissues, 0% (0/10) of non-infected tumor tissues, and 0% (0/6) of hepatic hemangioma tissues. These results suggest that *SFRP1* and *SFRP5* are not primary causes of aberrant Wnt signaling activation in HCCs.

HBx-LINE1 transcripts are not detected in tissues of HBV-related HCC

A recent report described that *HBx* frequently forms a chimeric transcript (*HBx-LINE1*) after integrating into the *LINE1* element in 8p11.21 of the host genome, and the authors further suggested that *HBx-LINE1* could activate the Wnt signaling pathway as a long noncoding RNA[17]. To explore whether *HBx-LINE1*

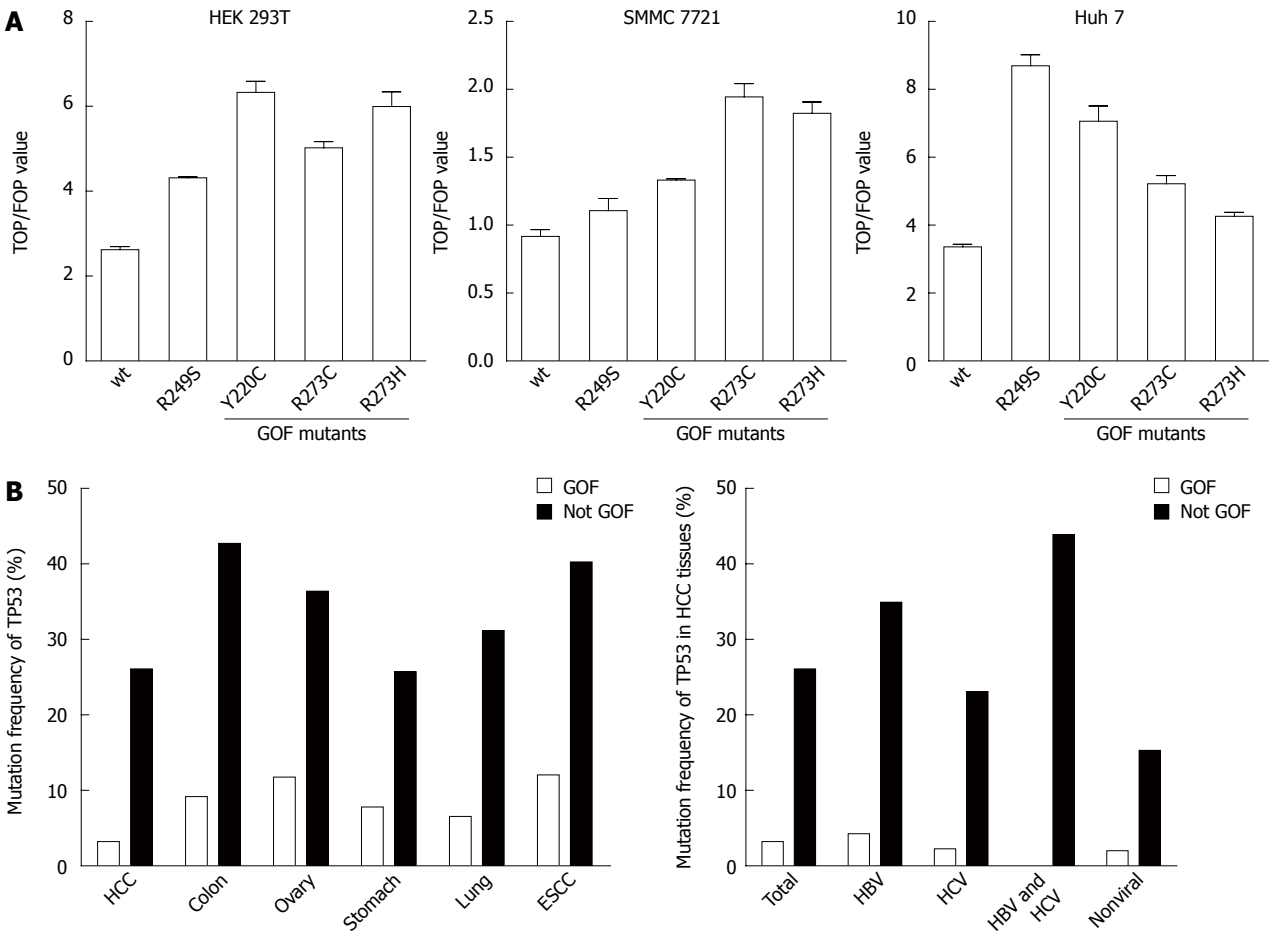

Figure 2 ***TP53*** **GOF mutation does not likely contribute to the aberrant activation of Wnt signaling.** A: *TP53* GOF mutants activate Wnt signaling *in vitro;* B: *TP53* mutation rate in different tumors and different etiologies of HCC. Although *TP53* mutation was commonly detected in HCC, GOF mutation was a rare event in HCC with different etiologies. ESCC: Esophageal squamous cell carcinoma; GOF: Gain-of-function; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

could take part in the aberrant activation of Wnt signaling in HBV-related HCC, the expression of this transcript was measured in up to 30 HCC tissues with a chronic HBV infection background. Unfortunately, even by nested-PCR, no *HBx-LINE1* chimeric transcript was detected. To exclude the possibility that the absence was due to an experimental failure, PCR primers were designed to detect the *HBx-LINE1* viral-host junction sequences at the DNA level. Still, no *HBx-LINE1* integration was detected (data not shown). Moreover, we carefully reviewed the available viral integration information from published data. In a total of 1115 HBV integration sites derived from 299 HBV-HCC patients, only one was found mapped at chromosome 8p11.21. However, further precise analysis excluded the possibility to form an *HBx-LINE1* transcript[22], because the integration site was away from the *LINE1* site. Collectively, *HBx* was not expected to exactly integrate at such an accurate site to form the *HBx-LINE1* chimeric transcript, at least not at the high frequency as described by the report[17]. As a result, *HBx-LINE1*, the suggested viral-host junction transcript, may not commonly present in HBV-infection related HCCs.

DISCUSSION

Abnormal activation of Wnt/β-catenin signaling is detected in 50%-70% of HCC cases, making it the most common signaling pathway aberration in this cancer[10]. However, it is necessary to analyze the cause of Wnt/β-catenin signaling pathway aberration while considering the different etiologic causes of HCC. In the present study, we summarized all the suggested factors relevant to the aberrant activation of the Wnt signaling pathway in HCC with different causative etiologies. Those genetic/epigenetic events include *CTNNB1* gene mutation, *TP53* GOF mutation, the presence of an *HBx-LINE1* chimeric transcript, and aberrations of other genes within this signaling pathway.

CTNNB1, which encodes β-catenin, has been recognized as one of the most frequently mutated genes in primary HCC. Indeed, according to the COSMIC database, the mutation of *CTNNB1* was detected in 671/3720 HCC cases, with detailed

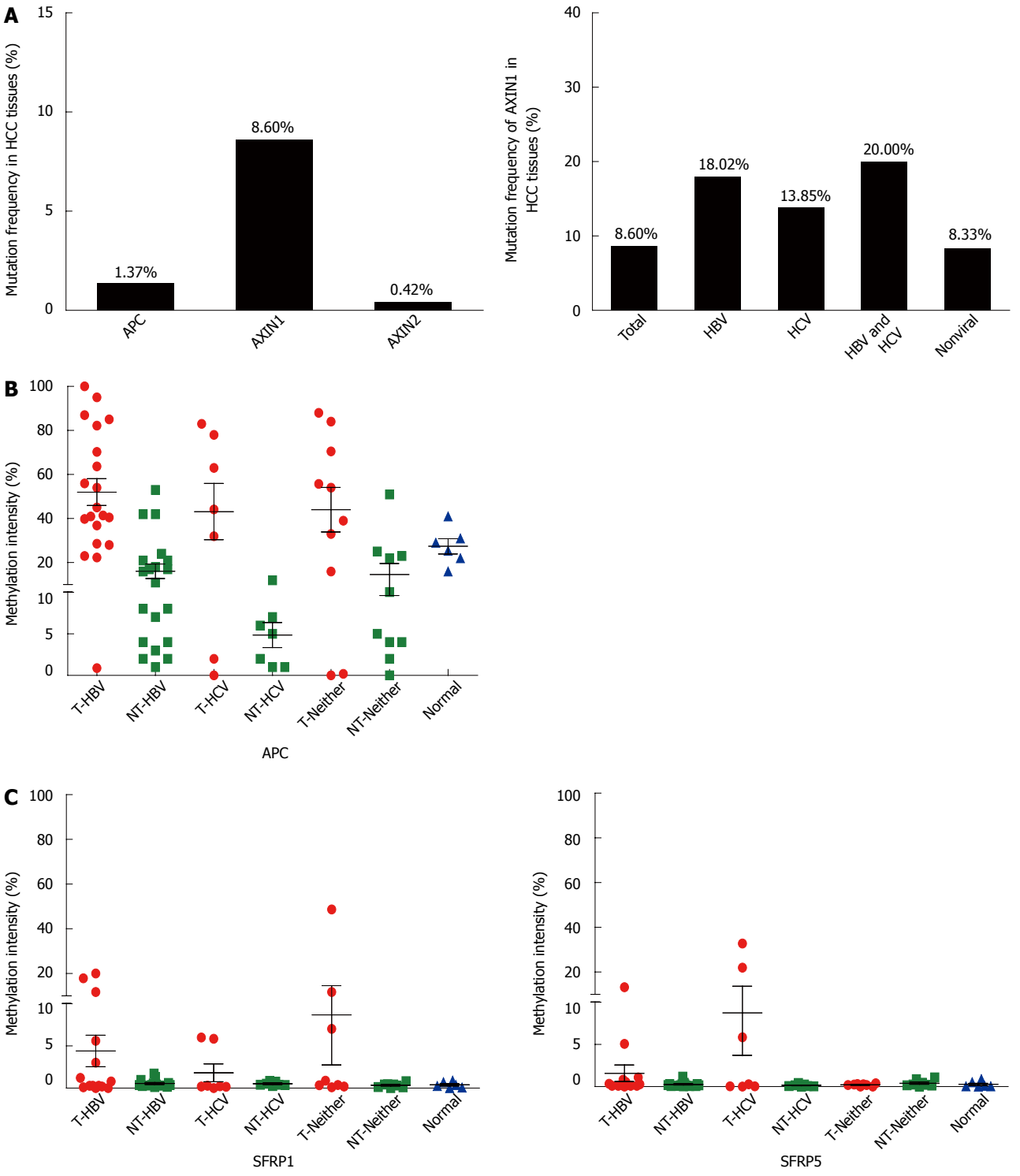

Figure 3 Frequent genetic/epigenetic aberrations of the negative regulators in Wnt signaling in hepatocellular carcinoma. A: *AXIN1* was frequently mutated in HCCs with different etiologies; B: *APC* promoter hypermethylation was frequently found in HCCs with different etiologies; C: Neither *SFRP1* nor *SFRP5* was frequently hypermethylated in HCCs with different risk factors. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma.

mutation information provided for 543 of these cases (http://cancer.sanger.ac.uk/). It is worthwhile to note that among the 543 identified mutations, 477 were point mutations located at N-terminal domain, 309 of which were at β-catenin phosphorylation sites (serines 33, 37, and 45 and threonine 41). This result is consistent with the fact that the stability of β-catenin relies on the phosphorylation of its N-terminal domain.

The large percentage (18.04%; 671/3720) of *CTNNB1* mutations makes it one of the major causative mechanisms contributing to the aberrant activation of Wnt signaling in HCC. However, the mutation rate of *CTNNB1* in chronic HBV-related HCC was only 10.79%, much lower than in HCV-related

cases or of other etiologic backgrounds. The lower rate of *CTNNB1* mutation in HBV-related HCC has been reported previously by other laboratories, in which relatively small patient cohorts were used[1,23]. The other gene with a high frequency of mutation in HCC is *AXIN1*, another component of the "destruction complex". Interestingly, in contrast to *CTNNB1*, the rate of *AXIN1* mutation was higher in HBV-related HCC compared to HCC of other etiologic backgrounds.

The above results suggest that mutation of genes comprising the "destruction complex", together with their target *CTNNB1* mutation, are common events contributing to the altered Wnt signaling pathway activation in HCC. In addition to genetic mutations, epigenetic modulation can also cause aberrant gene expression. We previously reported that in HBV-related HCCs, the CpG islands in the promoter regions of *APC* and *AXIN2* were frequently hypermethylated[20]. Frequent *APC* hypermethylation in HBV- and HCV-related HCC tissues has also been reported by Feng *et al*[24]. In this study, we compared the methylation status in HCC with different etiologies. However, there was no noticeable difference among HCCs with different etiologic backgrounds. Taken together, these results suggest that *APC* promoter hypermethylation is a causative factor for aberrant Wnt signaling activation in HCC tissues. In addition, our data and those from other laboratories suggest that *SFRP* methylation may not be a primary factor for aberrant Wnt signaling activation in HCC[24].

The recent report that *HBx-LINE1* chimeric transcripts can activate Wnt signaling has attracted some recognition[17,25,26]. However, we doubted the possibility of such a high frequency of identical integration (present in 23% of primary HCC tissues, as described by the author[17]). As a matter of fact, the integration of HBV into the host cellular genome is generally a random event, and it is hard to imagine that HBV integrates precisely at chr.8p11.21 in close to one-quarter of HCC tumor tissues. Moreover, in order to make an *HBx-LINE1* junction, the HBV genome must also be broken exactly at the same site of its genome. Our lab had searched up to 1115 HBV host genome-adjacent sequences from several articles, including our data, and none of them locate at the site of the *LINE1* element, which is essential for the formation of the *HBx-LINE1* chimeric transcripts[27-31]. In addition, the effort to detect the presence of *HBx-LINE1* chimeric transcripts by the powerful nested Reverse transcription-PCR, or PCR at the tumor genome level, failed. In addition, the previous report utilized Sanger sequencing to confirm the formation of *HBx-LINE1* transcripts[17]; we noticed that the viral nucleic acid sequence was exactly the same, not even a single nucleotide variant to this high mutation-rate virus. Therefore, we had sufficient cause to doubt that *HBx* can accurately integrate with *LINE1* with such an extremely high frequency.

Besides the above factors, some other mechanisms that can activate Wnt signaling should be further explored, such as the phosphorylation of extracellular signal-regulated kinase and protein kinase B by HBx, which may be the major mechanism for Wnt signaling activation in HBV-related HCC[32,33]. Additionally, it was reported that HBx can competitively combine with APC protein to release GSK-3β[34]. However, further experimental evidences are still needed to confirm this postulation.

Together with other previous reports, we propose that several possible mechanisms account for the aberrant Wnt signaling activation in HCC, including *CTNNB1* mutation, as well as hypermethylation of *APC* and *AXIN1* mutation. In contrast, hypermethylation-mediated silencing of *SFRP1* and *SFRP5* expression was not a common event. Additionally, although *TP53* GOF mutations have the potential to activate Wnt signaling, they rarely occur in HCC, and therefore, it should not be counted as a causative factor of aberrant Wnt signaling overactivation in HCC. Unfortunately, our results do not support the hypothesis that *HBx-LINE1* chimeric transcripts activate Wnt signaling in HCC, as none of the 1115 known HBV viral-host cellular genome junction sequences involved the *LINE1* sequence, and furthermore, the *HBx-LINE1* chimeric transcript was not detected at the mRNA or genomic DNA level.

As our understanding about Wnt signaling pathways continues to grow, the potential clinical value of our knowledge on Wnt signaling and HCC should be further studied.

COMMENTS

Background

The development of hepatocellular carcinoma (HCC) is a multistage process, during which numerous genetic/epigenetic factors could be involved. As one of the most important factors, the Wnt/β-catenin signaling pathway is frequently activated, about 50%-70% of HCC tissues show abnormal β-catenin protein accumulation, which predicts poor prognosis. It has become recognized that the Wnt signaling pathway plays an important role in the development and prognosis of HCC.

Research frontiers

Many mechanisms have been reported to be involved in the aberrant activation of Wnt signaling in HCC. Mutation of *CTNNB1*, which encodes β-catenin, could cause cytoplasmic accumulation of β-catenin. Genetic or epigenetic aberrations of several constitution molecules in the Wnt signaling pathway could also affect its activation. Furthermore, *TP53* gain-of-function mutations have the ability to upregulate the expression of *CTNNB1*. Finally, a recent report suggested a frequent integration of *HBx* into *LINE1* elements of the human genome and formation of *HBx-LINE1* chimeric transcripts, which enhance the transcriptional activity of Wnt signaling.

Innovations and breakthroughs

Previous reports have suggested the presence of different underlying mechanisms for the aberrant activation of Wnt signaling in HCC. However, whether all these mechanisms could really take part in this process is not known. In this article, by integrative analysis of the potential factors, the involvement of the following suggested mechanisms in the aberrant activation of Wnt signaling in HCC were investigated: the mutation rate of *CTNNB*1, *TP53*, *APC*, *AXIN1*, and *AXIN2* by searching in COSMIC database; and the epigenetic aberrations of the constituent molecules in Wnt signaling, such as *APC*, *SFRP1*, and *SFRP5*, by determining their CpG island methylation status using a methylation sensitive restriction enzyme-quantitative PCR technique developed in the laboratory. In addition, in order to judge whether *HBx* integration in *LINE1*

elements could activate Wnt signaling in HBV-related HCC, the proposed *HBx-LINE* integration was also examined at both the genome and RNA levels among HBV-related HCC tissue specimens by nested reverse transcription-PCR and PCR.

Applications

This integrative study provides a panoramic view of the underlying mechanisms relevant to the aberrant activation of Wnt signaling in HCC. The discovery will enhance our understanding of hepatocarcinogenesis.

Terminology

The Wnt/β-catenin pathway is highly conserved throughout evolution, and plays key roles in development in adult tissue homeostasis. β-catenin is the core component which is precisely regulated. β-catenin can be degraded by the destruction complex composed of APC, AXIN, and GSK-3β. Wnt signaling activation leads to nuclear translocation of β-catenin, where it promotes the transcription of several downstream target genes.

Peer-review

The authors present a comprehensive study. The methodology is correct. The conclusions are consistent with the results obtained. This study represents a significant contribution to advance our study on the process of hepatocarcinogenesis.

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META-ANALYSIS

Risk factors for new onset diabetes mellitus after liver transplantation: A meta-analysis

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Abstract

AIM: To determine the risk factors for new-onset diabetes mellitus (NODM) after liver transplantation by conducting a systematic review and meta-analysis.

METHODS: We electronically searched the databases of MEDLINE, EMBASE and the Cochrane Library from January 1980 to December 2013 to identify relevant studies reporting risk factors for NODM after liver transplantation. Two authors independently assessed the trials for inclusion and extracted the data. Discrepancies were resolved in consultation with a third reviewer. All statistical analyses were performed with the RevMan5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). Pooled odds ratios (OR) or weighted mean differences (WMD) with 95% confidence intervals (CIs) were calculated using either a fixed effects or a random effects model, based on the presence (I^2 < 50%) or absence (I^2 > 50%) of significant heterogeneity.

RESULTS: Twenty studies with 4580 patients were included in the meta-analysis, all of which were retrospective. The meta-analysis identified the following significant risk factors: hepatitis C virus (HCV) infection (OR = 2.68; 95%CI: 1.92-3.72); a family history of diabetes (OR = 1.69, 95%CI: 1.09-2.63, P < 0.00001); male gender (OR = 1.53; 95%CI: 1.24-1.90; P < 0.0001); impaired fasting glucose (IFG; OR = 3.27; 95%CI: 1.84-5.81; P < 0.0001); a family history of diabetes (OR = 1.69; 95%CI: 1.09-2.63; P = 0.02); use of tacrolimus (OR = 1.34; 95%CI: 1.03-1.76; P = 0.03) and body mass index (BMI)(WMD = 1.19, 95%CI: 0.69-1.68, P < 0.00001). Other factors, such as hepatitis B virus infection and alcoholism, were not found to be associated with the incidence of NODM.

CONCLUSION: The study showed that HCV infection, IFG, a family history of diabetes, male gender, tacrolimus and BMI are risk factors for NODM after liver transplantation.

Key words: Diabetes mellitus; Meta-analysis; Risk

factor; Liver transplantation; Hepatitis C virus

Core tip: New-onset diabetes mellitus (NODM) is a serious complication of liver transplantation (LT) that negatively affects patient and graft survival. However, the risk factors for NODM after LT have not been well elucidated. It has been reported that many factors are involved in the development of NODM. This meta-analysis demonstrated that hepatitis C virus infection, impaired fasting glucose, a family history of diabetes, male gender, tacrolimus and body mass index are risk factors for NODM after liver transplantation.

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INTRODUCTION

New-onset diabetes mellitus (NODM) is a serious complication of liver transplantation (LT) that negatively affects patient and graft survival. The reported incidence of NODM after LT ranges from 9% to 63.3%[1-6]. Similar incidence rates of and risk factors for NODM have also been reported in renal transplantation[7-9]. NODM contributes to an increased risk of infections, cardiovascular disease and rejection, all of which are leading causes of mortality among LT recipients[6,10-12]. However, the mechanisms underlying NODM after LT are poorly understood.

Age, gender, body mass index (BMI), hepatitis C virus (HCV) infection, immunosuppressive regimens, and a family history of diabetes have been reported as the main risk factors for the development of NODM after LT[1,13-15], although many controversial issues remain. For example, HCV-induced cirrhosis, one of the most studied risk factors, is the leading indication for LT[16] and has been identified as a major risk factor for the development of NODM in solid organ transplant recipients. A recent study based on the OPTN/UNOS database demonstrated that HCV infection is an independent risk factor for NODM in the liver transplant population. NODM was found to occur more frequently in HCV-positive patients (28.3% *vs* 23.7%, HR = 1.155), although some studies did not find any statistical association between HCV infection and post-transplant NODM[3,6,17]. However, comparing the rates of NODM between studies is often complicated by the varying definitions of NODM and differing follow-up periods.

The aim of this meta-analysis was to identify risk factors for the development of NODM after LT.

MATERIALS AND METHODS

Search strategy and data extraction

Two of the authors searched studies published between January 1980 and December 2013 *via* MEDLINE, EMBASE, and the Cochrane Library. The search strategy included the terms "diabetes mellitus", "diabetes", "liver transplantation" and related synonyms. Two authors independently screened the titles and abstracts of the retrieved papers, and full-text copies were obtained of most of the potentially relevant studies. The reference lists of the retrieved publications were also comprehensively reviewed to identify additional potentially relevant studies. Discrepancies were resolved in consultation with a third reviewer. This search was limited to human studies, without any language limitations; both case-controlled studies and observational studies were included.

Criteria for inclusion

The studies included in the meta-analysis had to satisfy the following criteria: (1) randomized controlled trials and prospective or retrospective cohort and case-control studies investigating patients with NODM after LT; (2) adult recipients aged more than 18 years with no history of diabetes mellitus pre-transplantation; (3) follow-up period > 6 mo; and (4) description of an accurate incidence of NODM after LT that could be extracted for the meta-analysis.

Criteria for exclusion

We excluded studies meeting the following criteria: (1) recipient age < 18 years; (2) recipients with diabetes mellitus before transplantation; (3) complete data that were unavailable for the meta-analysis; (4) use of a definition of NODM that did not meet the criteria of the 2003 International Consensus Guidelines; (5) follow-up time less than 6 mo or loss to follow-up rate greater than 10%; and (6) studies enrolling patients who had undergone multiple transplants.

Definition

NODM was defined according to the American Diabetes Association/World Health Organization (ADA/WHO) criteria (see Table 1)[18,19], as described in the 2003 International Consensus Guidelines for the diagnosis of post-transplantation NODM [fasting blood glucose > 126 mg/dL (7.0 mmol/L) on at least two separate occasions, and/or 2-h post-prandial blood sugar > 200 mg/dL (11.1 mmol/L)]. Alternatively, DM was defined as a requirement for glucose-lowering medications (insulin or oral hypoglycemic agents for > 1 mo)[20].

Quality assessment

Study quality was evaluated using the Newcastle-Ottawa scale, which was designed especially for observational case control and cohort studies. The scale includes three separate categories, using counts

Table 1 American Diabetes Association Criteria for diabetes mellitus, impaired fasting glucose, and impaired glucose tolerance

		Terminology
FPG (mg/dL)	< 100	Normal
	100-125	IFG
	> 126	Diabetes mellitus
2-h glucose after 75 g oral glucose load	< 140	Normal
	140-199	IGT
	> 200	Diabetes mellitus

FPG: Fasting plasma glucose; IFG: Impaired fasting glucose; IGT: Impaired glucose tolerance.

of 1-9 as the assessment score. The total score is 9, including 4 for selection part, 2 for comparability part, and 3 for outcome part. A total score ⩾ 7 represents high quality (see Table 2).

Statistical analysis

The meta-analysis was performed using RevMan 5.0, according to the Cochrane Handbook for Systematic Reviews of Interventions, as recommended by the Cochrane Collaboration. Odds ratios (OR) and mean differences (MD) were calculated for each principal outcome for dichotomous and continuous variables, respectively. The 95% confidence intervals (95%CIs) were calculated for all parameters. Heterogeneity among the trials was assessed with the Cochran's *Q* test and I^2 statistics. The meta-analysis was performed with a random-effect or fixed-effect model, based on the presence (I^2 < 50%) or absence (I^2 > 50%) of significant heterogeneity. Potential publication bias was assessed using a funnel plot, if necessary. A sensitivity analysis was also conducted by excluding individual studies in turn to evaluate the influence of a single study on the pooled estimates.

RESULTS

Literature review

We identified 1408 potentially relevant citations with our initial search strategy, 418 of which were excluded due to duplication. A further 941 were excluded after reviewing the titles and abstracts because they were not relevant to our analysis, and 29 more were excluded after reviewing the full articles, mainly because they did not meet the inclusion criteria. Ultimately, 19 studies involving 4580 patients were included in our meta-analysis[1-3,5,6,10,13-15,17,21-29]. The process used for article selection is presented in Figure 1. Quality assessment of the included studies was shown in Table 2, and all studies got a total score ⩾ 6.

Patient characteristics

Some of the principal demographic and clinical characteristics of subjects enrolled in the included clinical trials are shown in Tables 3 and 4. Four of the

Table 2 Newcastle-Ottawa scoring system for cohort studies

Study	Selection score	Comparability score	Outcome score	Total score
Saliba *et al*[1]	4	2	3	9
Parolin *et al*[14]	3	1	3	7
Baid *et al*[10]	3	2	3	8
Schmilovitz *et al*[21]	3	1	3	7
Moon *et al*[6]	4	1	2	7
Kishi *et al*[22]	4	2	3	9
Khalili *et al*[13]	3	2	3	7
Yoshida *et al*[15]	3	2	3	8
Gelley *et al*[23]	3	2	3	8
Dehghan *et al*[17]	4	2	2	8
Harada *et al*[24]	3	2	2	7
Anderson *et al*[25]	3	1	3	7
Ling *et al*[26]	3	2	2	7
Zhao *et al*[27]	3	2	1	6
Sánchez-Pérez *et al*[28]	3	2	3	7
Mirabella *et al*[5]	3	2	3	8
Driscoll *et al*[29]	3	1	3	7
Honda *et al*[3]	3	2	3	8
Carey *et al*[2]	4	1	3	8

studies (20%) were from Europe; eight (40%) from North or South America; and eight (40%) from Asia. The overall incidence of NODM post-LT among the included studies was 30.2% (1385/4580), ranging from 10.2%[28] to 63.3%[6].

Summary estimates of the outcomes

HCV infection: A total of 14 studies including 3362 LT recipients were included in the meta-analysis to explore the relationship between NODM and HCV infection. The incidence of NODM was 25.4% (855/3362) overall, 34.0% (372/1095) among HCV (+) recipients, and 21.3% (483/2267) among HCV (-) patients. HCV infection was associated with a statistically significantly higher incidence of NODM in a random effects model, with a pooled OR of 2.68 (95%CI: 1.92-3.72; Figure 2). This result is consistent with most previous studies. There was high heterogeneity among the studies (P < 0.05, I^2= 65%), and thus a random effects model was used.

Hepatitis B virus infection: Figure 3 shows the association between HBV infection and the risk of NODM after LT based on 6 studies with a total of 681 recipients. The pooled OR (OR = 1.04; 95%CI: 0.54-2.00) indicated no significant association between HBV infection and the risk of NODM after LT. A random effects model was used due to the presence of heterogeneity (χ^2= 11.60; P = 0.04, I^2= 57%).

Gender

Eleven studies were included to analyze the association between gender and NODM after LT (2033 recipients). The results of the meta-analysis are shown in Figure 4. The pooled OR for male *vs* female gender was 1.53 (95%CI: 1.24-1.90), indicating a mild association between male gender and an increased risk of NODM

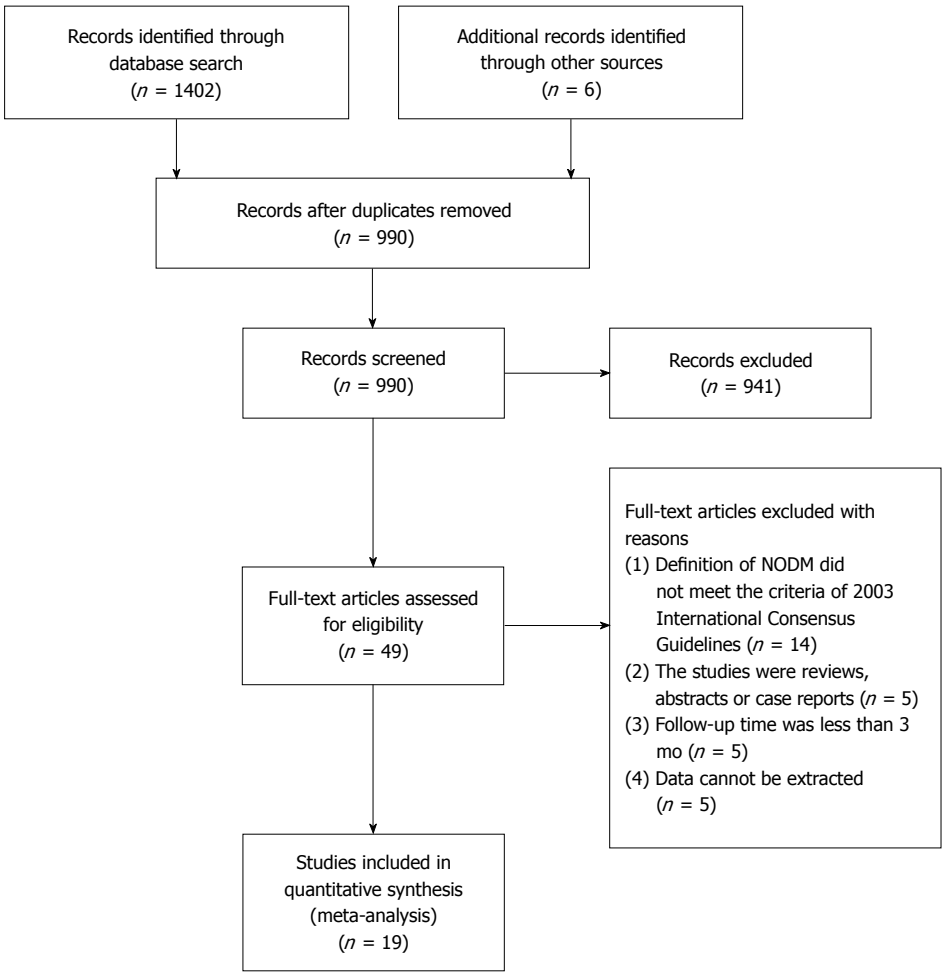

Figure 1 Flow diagram of the study selection.

Table 3 Baseline characteristics of the included studies

Ref.	Year	Country	Total number	NODM	Follow-up	Reference number
Saliba *et al*[1]	2007	France	211	48	6-24 mo	1
Parolin *et al*[14]	2004	Brazil	82	15	⩾ 1 yr	14
Baid *et al*[10]	2001	United States	136	52	> 6 mo	10
Schmilovitz *et al*[21]	2003	Israel	91	27	> 6 mo	21
Moon *et al*[6]	2006	United States	619	392	6-122 mo	6
Kishi *et al*[22]	2006	Japan	205	71	> 6 mo	22
Khalili *et al*[13]	2004	United States	555	209	1.6-6.8 yr	13
Yoshida *et al*[15]	2013	Canada	280	89	> 6 mo	15
Gelley *et al*[23]	2011	Hungary	206	63	> 6 mo	23
Dehghan *et al*[17]	2008	Iran	170	44	6-156 > 6 mo	17
Harada *et al*[24]	2013	Japan	331	128	3.8-11.2 yr	24
Anderson *et al*[25]	2009	United States	45	11	6 mo	25
Ling *et al*[26]	2013	China	125	25	6-61 mo	26
Zhao *et al*[27]	2009	China	66	11	3-38 mo	27
Sánchez-Pérez *et al*[28]	2008	Spain	127	13	> 6 mo	28
Mirabella *et al*[5]	2005	India	830	90	> 10 mo	5
Driscoll *et al*[29]	2006	United States	115	36	12 mo	29
Honda *et al*[3]	2013	Japan	161	22	> 3 mo	3
Carey *et al*[2]	2012	United States	225	39	⩾ 1 yr	2

NODM: New-onset diabetes mellitus.

after LT. As no heterogeneity was found across the studies (P = 0.71, I^2 = 0%), we used a fixed effects model (Figure 4).

Pre-transplant impaired fasting glucose

Three studies investigated the association between pre-transplant impaired fasting glucose (IFG) and

Table 4 Baseline characteristics of the included studies

Ref.	Study year	Study design	Risk factors
Saliba *et al*[1]	2003.10-2004.6	Retrospective	BMI, HCV, IFG, immunosuppression
Parolin *et al*[14]	2004.1-2004.6	Retrospective	Gender, BMI, HCV, a family history of diabetes, alcohol
Baid *et al*[10]	1991.1-1998.10	Retrospective	HCV
Schmilovitz *et al*[21]	1992-2002	Retrospective	Gender, HCV, alcohol, immunosuppression, HBV
Moon *et al*[6]	1996.1-2004.10	Retrospective	Gender, HCV
Kishi *et al*[22]	1996.1-2005.1	Retrospective	HCV
Khalili *et al*[13]	1990-1994	Retrospective	HCV
Yoshida *et al*[15]	1996.1-2006.10	Retrospective	Immunosuppression
Gelley *et al*[23]	1995-2009	Retrospective	HCV
Dehghan *et al*[17]	1994-2006	Retrospective	Gender, HCV, BMI, immunosuppression, HBV
Harada *et al*[24]	1996.1-2011.1	Retrospective	Gender, HCV, alcohol, HBV
Anderson *et al*[25]	2004.1-2005.10	Retrospective	Gender, HCV, BMI, a family history of diabetes, alcohol, HBV
Ling *et al*[26]	2006.11-2009.7	Retrospective	Gender, BMI, HBV
Zhao *et al*[27]	2001-2008.3	Retrospective	Gender, IFG, a family history of diabetes, immunosuppression, HBV
Sánchez-Pérez *et al*[28]	1997.3-2001.10	Retrospective	Immunosuppression
Mirabella *et al*[5]	NR	Retrospective	HCV
Driscoll *et al*[29]	1998.1-2001.8	Retrospective	CMV, gender, BMI, HCV, a family history of diabetes, immunosuppression
Honda *et al*[3]	1998.12-2011.10	Retrospective	CMV, gender, HCV, BMI, a family history of diabetes
Carey *et al*[2]	1999.6-2008.2	Retrospective	Gender, BMI, HCV, IFG, a family history of diabetes, alcohol, immunosuppression

NR: Not reported; HCV: Hepatitis C virus; IFG: Impaired fasting glucose; HBV: Hepatitis B virus; CMV: Cytomegalovirus; BMI: Body mass index.

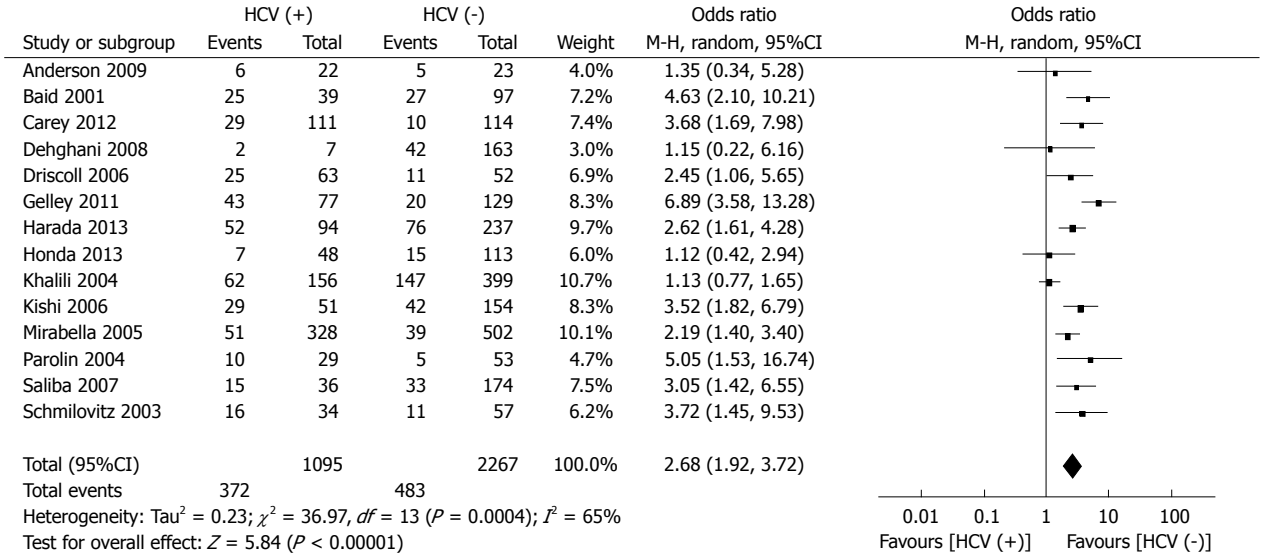

Figure 2 Forest plot of studies finding an association between hepatitis C virus infection and new onset diabetes mellitus. There was significant heterogeneity in the results of the meta-analysis. The pooled OR (OR = 2.68; 95%CI: 1.92-3.72) indicated a significant association between hepatitis C virus infection and the risk of new-onset diabetes mellitus after liver transplantation.

NODM after LT. The meta-analysis revealed that pre-transplant IFG was associated with a significantly higher rate of NODM than normal blood glucose (pooled OR = 3.27; 95%CI 1.84-5.81), with no evidence of heterogeneity (P = 0.68, I^2 = 0%; Figure 5).

Family history

Six studies investigated the association between family history and NODM. The pooled OR was 1.69 (95%CI 1.09-2.63; P = 0.25, I^2= 24%; Figure 6), indicating that there was a significant association between a family history of DM and the risk of NODM after LT.

Immunosuppressive therapy

Tacrolimus-based immunosuppressive therapy has been reported to be an independent risk factor for NODM after LT in many clinical studies, in comparison with cyclosporine. Ten retrospective studies were included in the meta-analysis. The pooled OR (OR = 1.34; 95%CI: 1.03-1.76) showed that tacrolimus was associated with an increased risk of NODM in liver transplant recipients, with moderate heterogeneity

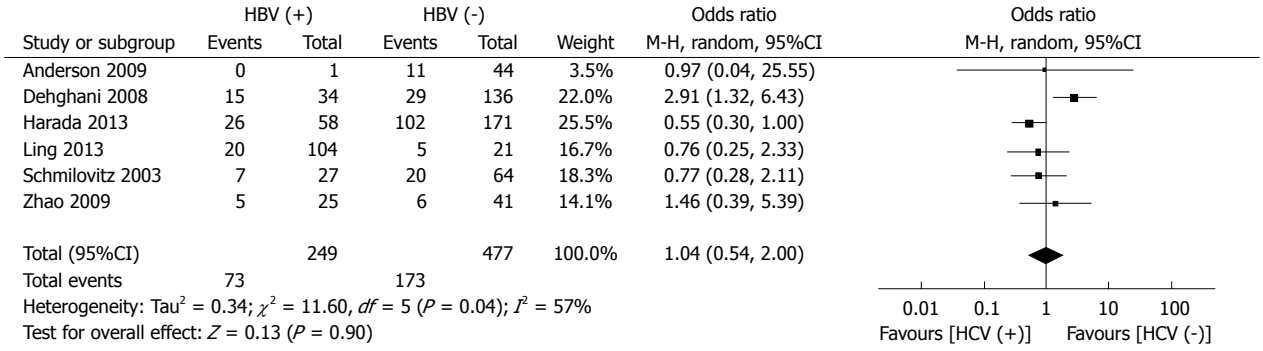

Figure 3 Forest plot of studies finding an association between hepatitis B virus infection and new onset diabetes mellitus. There was significant heterogeneity in the results of the meta-analysis. The pooled OR (OR = 1.04; 95%CI: 0.54-2.00) indicated a significant association between hepatitis B virus infection and the risk of new-onset diabetes mellitus after liver transplantation.

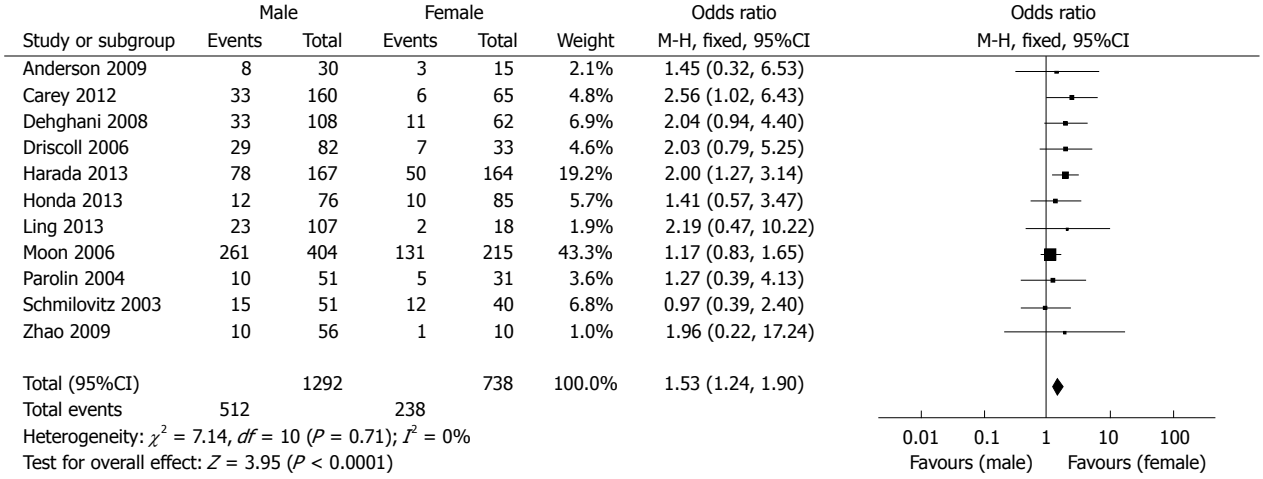

Figure 4 Forest plot of studies finding an association between gender and new onset diabetes mellitus. There was no significant heterogeneity in the results of the meta-analysis. The pooled OR (OR = 1.53; 95%CI: 1.24-1.90) indicated a significant association between male gender and the risk of new-onset diabetes mellitus after liver transplantation.

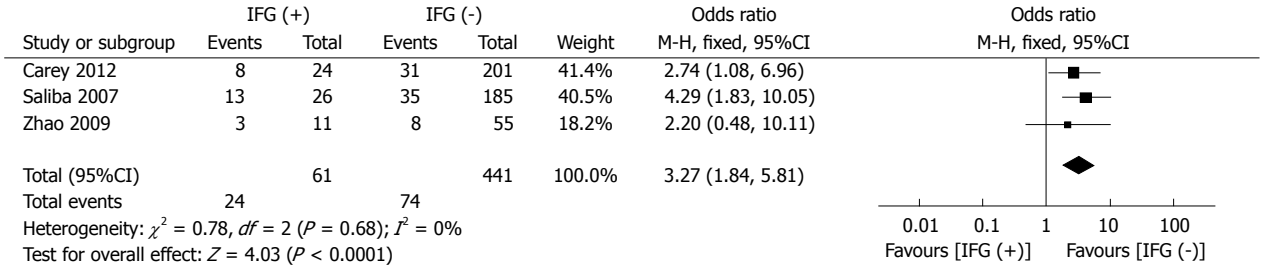

Figure 5 Forest plot of studies finding an association between impaired fasting glucose and new-onset diabetes mellitus. There was no heterogeneity in the results of the meta-analysis. The pooled OR (OR = 3.27; 95%CI: 1.84-5.81) indicated a significant association between pre-transplant impaired fasting glucose and the risk of new-onset diabetes mellitus after liver transplantation.

across the studies (P = 0.15, I^2 = 33%; Figure 7).

Alcoholism and BMI

Five studies provided data on the relationship between alcoholic cirrhosis and NODM after LT, and the pooled OR was 0.71 (95%CI: 0.36-1.37; P = 0.14, I^2 = 43%; Figure 8). Seven studies (1046 participants) reported explicit pre-transplant BMI values and NODM rates for LT recipients. The results of the meta-analysis demonstrate that the pre-transplant BMI of recipients with NODM was significantly higher than that of recipients who did not develop NODM (WMD=1.19, 95%CI: 0.69-1.68; P = 0.15, I^2= 37%; Figure 9).

Subgroup analysis

To investigate any confounding factors that might be related to heterogeneity among studies, we performed a subgroup analysis upon the analyses that

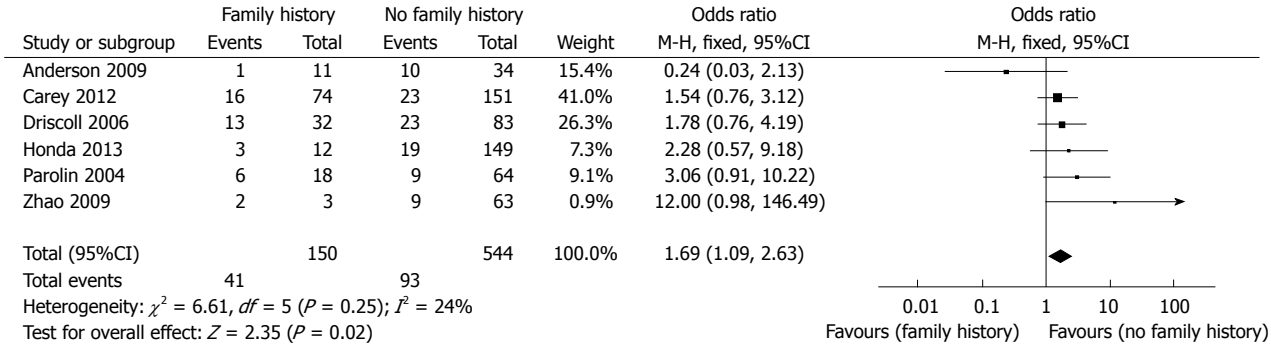

Figure 6 Forest plot of studies finding an association between a family history of diabetes and new-onset diabetes mellitus. There was no significant heterogeneity in the results of the meta-analysis. The pooled OR (OR = 1.69; 95%CI: 1.09-2.63) indicated a significant association between a family history of diabetes and the risk of new-onset diabetes mellitus after liver transplantation.

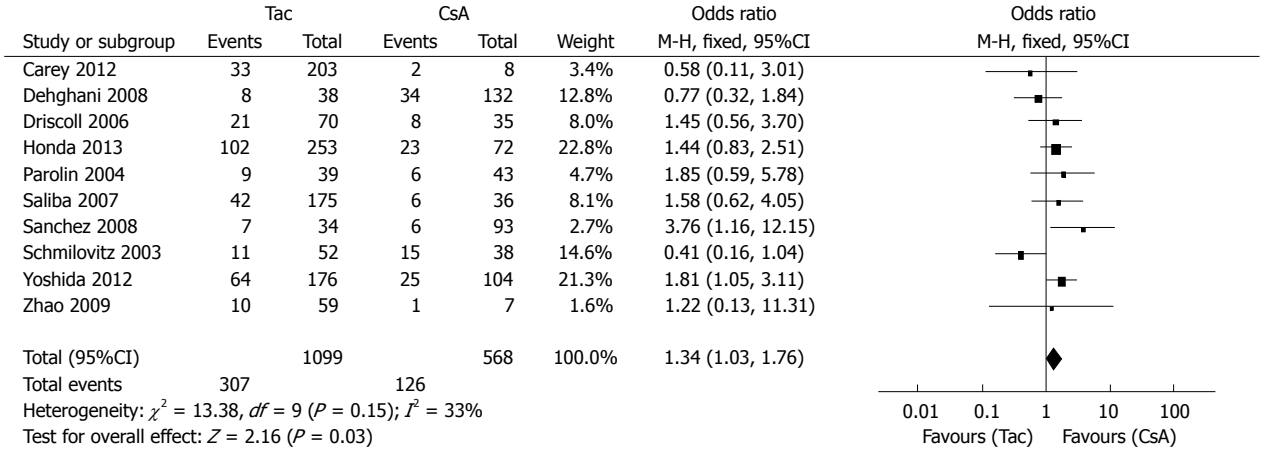

Figure 7 Forest plot of studies finding an association between immunosuppression and new-onset diabetes mellitus. There was no significant heterogeneity in the results of the meta-analysis. The pooled OR (OR = 1.34; 95%CI: 1.03-1.76) indicated a significant association between tacrolimus and the risk of new-onset diabetes mellitus after liver transplantation.

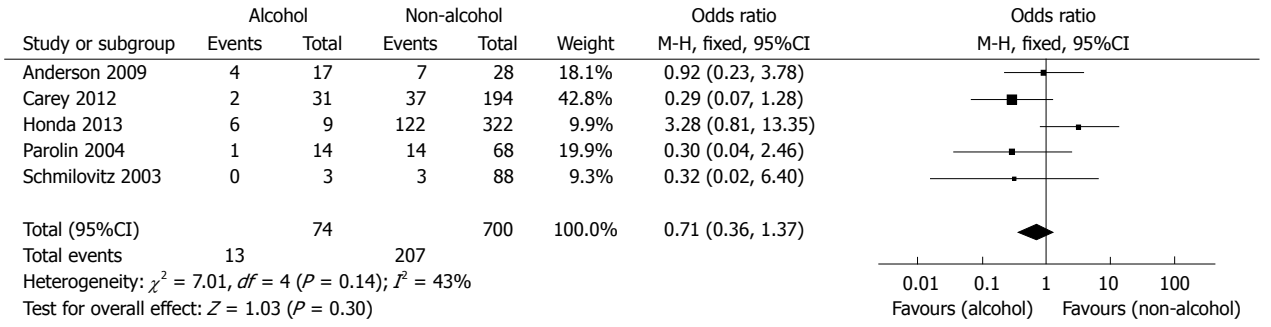

Figure 8 Forest plot of studies finding an association between alcohol-related cirrhosis and new-onset diabetes mellitus. There was no significant heterogeneity in the results of the meta-analysis. The pooled OR (OR = 0.71; 95%CI: 0.36-1.37) indicated no significant association between alcohol-related cirrhosis and the risk of new-onset diabetes mellitus after liver transplantation.

revealed significant heterogeneity (HCV infection and HBV infection), stratifying the studies according to transplant country (United States or other countries) and publication year (before or after 2010; Table 5). In the subgroup analysis for HCV infection, we found that the heterogeneity among the studies increased (P = 0.004, I^2 = 74%) when studies were restricted to the US and decreased (P = 0.06, I^2 = 46%) when studies were restricted to other countries. As for the subgroup analysis of HBV infection, heterogeneity among the studies decreased greatly when the syntheses were stratified by the year of publication. All results from the subgroup analysis were consistent with the results of the overall analysis.

Publication bias assessment and sensitivity analysis

We assessed the publication bias with funnel plots for the studies involving HCV, gender, and

Table 5 Subgroup analysis

Risk factor	Subgroup	Studies, *n*	Effect estimate (95%CI)	*P* value	Heterogeneity
HCV	United States	5	2.29 (1.19-4.41)	< 0.01	$P = 0.004$, $I^2 = 74\%$
	Other	9	2.96 (2.11-4.15)	< 0.01	$P = 0.06$, $I^2 = 46\%$
	Overall	14	2.68 (1.92-3.72)	< 0.01	$P = 0.004$, $I^2 = 65\%$
HBV	Published before 2010	4	1.61 (0.94-2.78)	0.08	$P = 0.23$, $I^2 = 30\%$
	Published after 2010	2	0.59 (0.35-1.00)	0.05	$P = 0.61$, $I^2 = 0\%$
	Overall	6	1.04 (0.54-2.00)	0.9	$P = 0.04$, $I^2 = 57\%$

HCV: Hepatitis C virus; HBV: Hepatitis B virus.

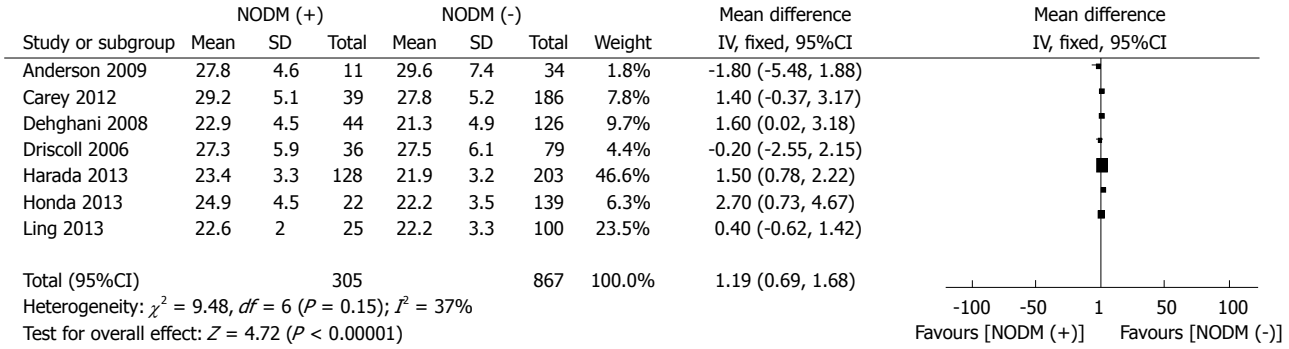

Figure 9 Forest plot of studies finding an association between body mass index and new-onset diabetes mellitus. There was no significant heterogeneity in the results of the meta-analysis. The WMD (WMD = 1.19, 95%CI: 0.69-1.68) indicated a significant association between body mass index and the risk of new-onset diabetes mellitus after liver transplantation.

immunosuppression. The funnel plots for HCV showed slight asymmetry, suggesting possible publication bias. The funnel plots for gender and immunosuppression were both generally symmetrical and suggested a lack of significant publication bias (Figure 10). In the sensitivity analysis, the removal of any study from the analysis did not significantly alter the overall results.

DISCUSSION

NODM is a common complication of LT, with an incidence of 9% to 63.3%, and is associated with impaired long-term liver allograft function and patient survival. The overall incidence of post-LTNODM in the included studies was 30.2% (1385/4580). A number of risk factors for NODM after LT have been reported, including HCV infection, age, race, ethnicity, family history, BMI, acute rejection and type of immunosuppressive agents, but controversy persists regarding risk factors for NODM in LT recipients. The aim of the present study was to determine the risk factors for NODM using meta-analysis.

HCV infection is the leading cause of end-stage liver disease in the United States[4]. Epidemiological studies have shown a significant association between HCV and NODM after solid organ transplantation[7,30], but several studies have also reported a negative relationship between NODM and HCV[22,31]. This controversy may result from the relatively small number of cases and from discrepancies in the studies' follow-up periods and choices of diagnostic criteria for NODM. The present meta-analysis provides retrospective evidence of a 2.68-fold increased risk of NODM among patients with HCV infection compared with HCV-negative recipients. The explicit mechanism between the development of NODM and HCV has yet to be fully elucidated. Chronic HCV infection can impair glucose metabolism in the liver by destroying hepatocytes[32]. Several possible mechanisms for HCV-induced insulin resistance have been proposed. It has been widely reported recently that in addition to causing liver injury, HCV is detrimental to other organs and tissues[33]. A post-mortem study proved that HCV is able to replicate in the pancreas before causing a failure of compensatory hyperinsulinemia by damaging β-cells *via* cytokine-mediated tissue damage[33-36]. The current meta-analysis confirms an association between HCV and NODM post-LT; the potential cause of the increased risk of NODM in HCV-infected LT recipients therefore requires further investigation.

The risk factors for developing NODM have previously been shown to differ between genders[22,24,37-39]. Male gender was identified as an independent risk factor for the presence of post-transplant diabetes in many studies[22]. Saab *et al*[37] reported that males are more likely to have NODM, which is consistent with the results of Stockmann *et al*[38] and Dehghani *et al*[40]. However, other studies have found no relationship between NODM and gender. The current meta-analysis indicated that males were at a significantly greater risk of developing NODM than females, based on a pooled OR of 1.53 (95%CI: 1.24-1.90). This finding is consistent

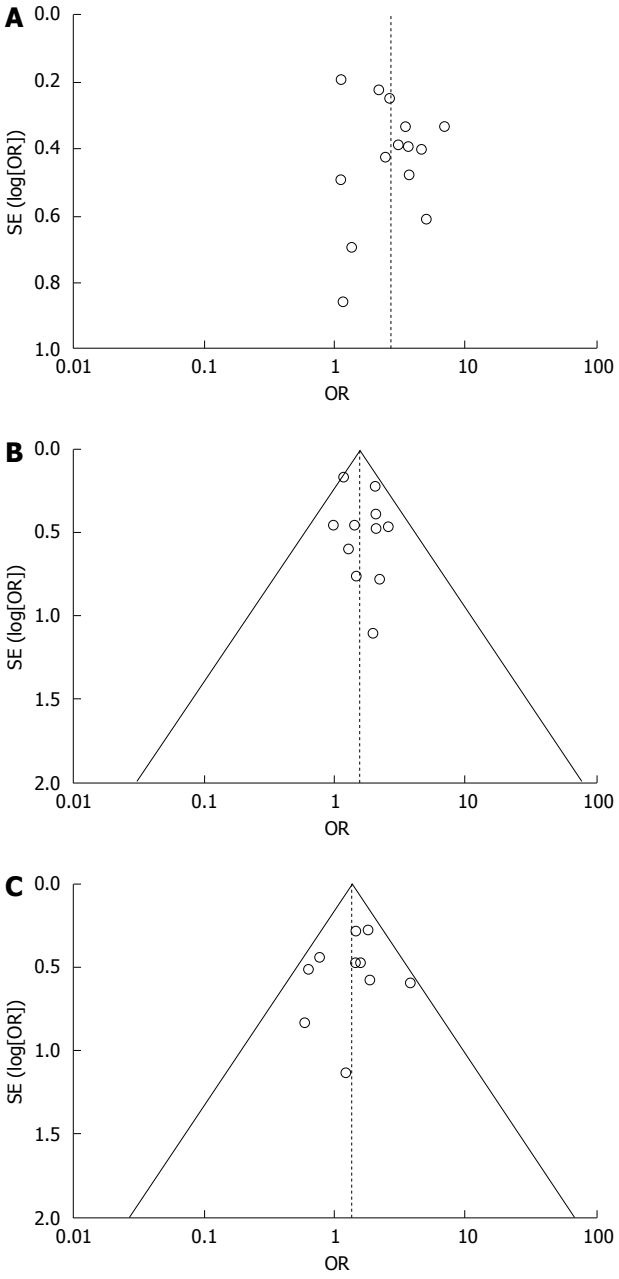

Figure 10 Funnel plots of studies conducted on new-onset diabetes mellitus and the risk factors of hepatitis C virus infection (A), gender (B) and immunosuppression (C).

with a number of studies suggesting that gender is an independent risk factor for NODM. This difference may be a consequence of differences in lifestyles, dietary habits and other social factors between female and male recipients.

The non-modifiable risk factor of a family history of diabetes mellitus was reported to have a positive but non-significant association with NODM after LT in many studies. The results of the pooled OR in the present study suggest that a family history of diabetes mellitus can slightly increase the incidence of NODM after LT, similar to the results found in the general population. IFG prior to transplantation has been shown to be a risk factor for NODM after LT in many studies. Pre-transplant IFG has also been shown to predict NODM in renal transplant recipients[41]. In the general population, fasting plasma glucose is significantly associated with the incidence of type 2 diabetes mellitus[42]. Approximately 70% of individuals with abnormal blood glucose, defined as impaired glucose tolerance or IFG, may ultimately develop diabetes mellitus[43]. In cirrhotic patients, the prevalence of impaired glucose tolerance has been estimated to be approximately 60% to 80%.The current meta-analysis showed a significant relationship between pre-transplant IFG and NODM after LT. Thus, we speculated that pre-transplant IFG has similar effects in transplant recipients and the ordinary population, although further research is required to determine whether the pathogenetic mechanism is the same in the two populations.

The reported incidence of NODM after solid organ transplantation was significantly higher among recipients receiving tacrolimus than cyclosporine; this pattern has been observed in liver, renal, heart and lung transplants[44,45]. Despite its adverse impact on glucose metabolism, tacrolimus significantly reduced the risks of acute rejection, patient death and graft loss after liver transplantation compared with cyclosporin[45]. Sánchez-Pérez *et al*[28] reported that patients treated with tacrolimus were 4 times more likely to develop NODM or IFG post-LT than those treated with cyclosporine, similar to previously reported results in the literature[39,46]. However, discrepancies were still observed among studies[2,17,21,47], perhaps due to the differing definitions of NODM[48]. In the current meta-analysis, the type of immunosuppressant (tacrolimus *vs* cyclosporine A) was found to be an independent risk factor for NODM after LT. The additional risk factors evaluated in this meta-analysis, including HBV infection and alcohol-related cirrhosis before transplantation, were not found to correlate with NODM after LT.

HBV is a leading cause of end-stage liver diseases, such as cirrhosis and hepatocellular carcinoma, in Asia[49]. The relationship between HBV infection and NODM after LT has been investigated in many studies recently, and a study from Iran reported that HBV infection was an independent risk factor for NODM. Still, other studies have found no consistent association. The number of the cases included in these studies is limited, and so more investigations from larger centers are needed to determine the impact of HBV infection on NODM after LT. Alcohol-related cirrhosis was not found to be significantly associated with the risk of NODM post-LT in the current study. One of the potential reasons for this finding is that these factors are significantly modified by transplantation, which could result in a moderate effect on glucose metabolism after LT.

The present meta-analysis has some limitations. First, a major limitation was publication bias. Most of the cases represent Western populations. Studies with statistically significant results are more likely to

be published than those with non-significant results, whereas studies with small sample sizes might be published in a journal from the author's native country; both of these factors might have distorted the results of the meta-analysis. To minimize such bias, we included studies from as many sources as possible. An American study using the Organ Procurement and Transplant Network/United Network for Organ Sharing (OPTN/UNOS) liver transplant database was identified and found to include 15463 recipients between July 2004 and December 2008[4]. Several independent risk factors for NODM were identified by the study, including recipient age, race, BMI, HCV, recipient cirrhosis history, tacrolimus, and diabetic donors. We excluded this study because it did not provide an explicit definition of NODM, sufficient follow-up or sufficiently detailed information for the meta-analysis. Several other studies were also excluded for similar reasons. Next, we tried to contact the authors of these papers. Some of them very kindly replied to us and supplied us with a great deal of useful data; however, these studies did not meet the criteria for inclusion.

Second, heterogeneity was inevitable due to methodological differences among the studies. The calculated I^2 was as high as 76% when assessing the association between HCV infection and NODM, which may be related to differences in the length of follow-up, sample size, race and age. To reduce the effect of high heterogeneity, a random effects model was used when I^2 was greater than 50%.

Third, the current meta-analysis did not distinguish between the subtypes of NODM. NODM can be classified into two subtypes according to the period of persistence: transient-NODM (T-NODM, *i.e.*, NODM that is temporarily present for 1-6 mo after LT) and persistent-NODM(P-NODM, *i.e.*, NODM that is sustained for $\geqslant$ 6 mo after LT)[6]; however, such definitions were applied inconsistently among the studies[13,22]. The two types of NODM were found to be significantly different in terms of risk factors, post-transplant complications, and patient outcomes. Unfortunately, we were unable to pursue any further analysis of the risk factors for T-NODM and P-NODM due to the variable definitions and the limited number of related studies. Fourth, all of the studies included in the meta-analysis were retrospective clinical trials, which are not considered as reliable as prospective studies. No prospective clinical trials were identified. Most of the studies in this meta-analysis did not adjust for potential confounders, including gender, age, BMI, *etc*., and so the potential effect of other confounders on the pooled results could not be excluded. Therefore, further prospective clinical trials are needed to better understand risk factors for NODM.

In conclusion, this meta-analysis found that HCV infection, IFG, a family history of diabetes, male gender, and tacrolimus use are all significantly associated with an increased risk of developing NODM after LT. The mechanism by which these risk factors influence the development of NODM remains unclear. Some of the identified factors are potentially modifiable, including HCV infection and tacrolimus-based immunosuppression. Well-designed prospective clinical trials that are designed to investigate the risk factors for NODM are needed to further confirm our findings.

COMMENTS

Background

New-onset diabetes mellitus (NODM) is a common complication after liver transplantation and is associated with increased rates of rejection, infection, cardiovascular disease, and with decreased survival. To date, information regarding incidence, risk factors, and clinical consequences of NODM in liver transplant recipients has been limited. Most of the published data are from single center studies with relatively small sample sizes, so the authors performed the meta-analysis to analyze the risk factors for NODM in liver transplant recipients.

Research frontiers

Due to the significantly negative impact of NODM on the long term outcome of liver transplantation, the study about NODM has been becoming a new hotspot. In order to identify the risk factors, the meta-analysis included many potential factors which have been frequently reported, including hepatitis C virus (HCV) infection, hepatitis B virus infection, impaired fasting glucose (IFG), a family history of diabetes, male gender, tacrolimus, BMI and alcoholism.

Innovations and breakthroughs

In 2009, a meta-analysis was conducted to analyze the connection between HCV infection and NODM after liver transplantation. However, the number of included studies was limited and many others potential factors were missed to be evaluated. Therefore, firm conclusions could not be drawn. Many high-quality studies with large sample sizes have been published recently. Therefore, it is important to conduct this comprehensive meta-analysis. Except for HCV infection, this study also identified HCV infection, IFG, a family history of diabetes, male gender, tacrolimus and body mass index (BMI) as risk factors for NODM after liver transplantation.

Applications

Some of the identified factors are potentially modifiable, including pre-transplant BMI and tacrolimus-based immunosuppression, and large-scale prospective clinical trials are needed to assess whether modifying these modifiable risk factors will indeed prevent NODM after liver transplantation.

Terminology

NODM after liver transplantation is an incompletely understood phenomenon estimated to occur in 9%-63.3% of recipients who were not diabetic prior to transplant.

Peer-review

The authors have made a systematic review and meta-analysis of risk factors for new onset diabetes mellitus post liver transplantation (LT). The focus of the study is very important as in the Western world almost 1/3 of patients develop diabetes mellitus post LT. Although there is no novel finding in the study, it is nicely performed and written. The results are in congruence with earlier meta-analyses.

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Proton pump inhibitors therapy *vs* H_2 receptor antagonists therapy for upper gastrointestinal bleeding after endoscopy: A meta-analysis

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Abstract

AIM: To compare the therapeutic effects of proton pump inhibitors *vs* H_2 receptor antagonists for upper gastrointestinal bleeding in patients after successful endoscopy.

METHODS: We searched the Cochrane library, MEDLINE, EMBASE and PubMed for randomized controlled trials until July 2014 for this study. The risk of bias was evaluated by the Cochrane Collaboration's tool and all of the studies had acceptable quality. The main outcomes included mortality, re-bleeding, received surgery rate, blood transfusion units and hospital stay time. These outcomes were estimated using odds ratios (OR) and mean difference with 95% confidence interval (CI). RevMan 5.3.3 software and Stata 12.0 software were used for data analyses.

RESULTS: Ten randomized controlled trials involving 1283 patients were included in this review; 678 subjects were in the proton pump inhibitors (PPI) group and the remaining 605 subjects were in the H_2 receptor antagonists (H_2RA) group. The meta-analysis results revealed that after successful endoscopic therapy, compared with H_2RA, PPI therapy had statistically significantly decreased the recurrent bleeding rate (OR = 0.36; 95%CI: 0.25-0.51) and receiving surgery rate (OR = 0.29; 95%CI: 0.09-0.96). There were no statistically significant differences in mortality (OR = 0.46; 95%CI: 0.17-1.23). However, significant heterogeneity was present in both the numbers of patients requiring blood transfusion after treatment [weighted mean difference (WMD), -0.70 unit; 95%CI: -1.64 - 0.25] and the time that patients remained hospitalized [WMD, -0.77 d; 95%CI: -1.87 - 0.34]. The Begg's test (P = 0.283) and Egger's test (P = 0.339) demonstrated that there was no publication bias in our meta-analysis.

CONCLUSION: In patients with upper gastrointestinal bleeding after successful endoscopic therapy, compared with H_2RA, PPI may be a more effective therapy.

Key words: H_2 receptor antagonist; Proton pump inhibitor; Upper gastrointestinal bleeding; Randomized controlled trial; Meta-analysis

Core tip: Recently, the administration of proton pump inhibitors (PPI) or H_2 receptor antagonists (H_2RA) have been used commonly for upper gastrointestinal bleeding patients after successful endoscopic therapy; however, which drug class is more effective, remains controversial. In this meta-analysis, we concluded that in patients with upper gastrointestinal bleeding after successful endoscopic therapy, compared with H_2RA, PPI may be a more effective therapy.

Zhang YS, Li Q, He BS, Liu R, Li ZJ. Proton pump inhibitors therapy *vs* H_2 receptor antagonists therapy for upper gastrointestinal bleeding after endoscopy: A meta-analysis. *World J Gastroenterol* 2015; 21(20): 6341-6351 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6341.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6341

INTRODUCTION

Gastrointestinal (GI) bleeding is a common cause of hospitalization, resulting in approximately 400000 hospital admissions annually, with a mortality rate of 5%-10%[1]. Re-bleeding has been described as the most important factor that affects patient prognosis; therefore, the re-bleeding rate is associated with mortality[2]. Appropriate endoscopic therapy of patients with non-variceal upper gastrointestinal bleeding improves outcomes, including re-bleeding rates, mortality, surgery, blood transfusions and hospitalization time[3,4]. Moreover, *Helicobacter pylori* (*H. pylori*) infection and nonsteroidal anti-inflammatory medication (NSAID) use are believed to be the main causes of non-variceal upper gastrointestinal bleeding (UGIB)[5,6]. Consequently, many factors may affect results of studies that examine UGIB.

Recently, the administration of proton pump inhibitors (PPI) or H_2 receptor antagonists (H_2RA) has been used commonly for upper gastrointestinal bleeding patients after successful endoscopic therapy[7,8]; however, the two drugs possess different pharmacological acid-suppressing activities. PPIs are substituted benzimidazoles that inhibit the parietal cell hydrogen-potassium adenosine-triphosphatase enzyme system in the gastric mucosa, reducing acid output; whereas H_2RAs decrease acid secretion by interfering with the H_2 receptor[9]. PPIs decrease hydrogen ion concentration by 95%-99% in humans at doses of 30-40 mg/d; however, H_2RAs cause less acid inhibition than PPIs[10].

Several valuable randomized controlled trials (RCTs) have compared the therapeutic effect of PPIs and H_2RAs in upper gastrointestinal bleeding patients after successful endoscopic therapy. Additionally, the meta-analysis of Yang *et al*[11] only evaluated the re-bleeding rate in two groups to perform a one-sided comparison of the curative effect of two drugs. Therefore, in this study, we expanded the sample size to analyze the effect of PPI therapy *vs* H_2RAs therapy from these RCTs, with data pertaining to recurrent bleeding rate, mortality, receive surgery rate, blood transfusion units and hospitalization time.

MATERIALS AND METHODS

Data selection

The following keywords, proton pump inhibitors, PPI, H_2 receptor antagonists, H_2RA, endoscopic, bleeding, randomized controlled trial and clinical trial, were used as search terms in the Cochrane library, MEDLINE, EMBASE and PubMed until July 2014.

Inclusion criteria

The inclusion criteria for this study were: (1) all patients in the experimental group were diagnosed with any type of upper gastrointestinal bleeding after successful endoscopic therapy; (2) comparison therapies of proton pump inhibitors or H_2 receptor antagonists in similar baseline level patients; (3) the end-points included recurrent bleeding rate; (4) randomization, controls, and measurable outcomes were reported; and (5) the articles were written in English.

Data extraction

The articles were extracted independently by two investigators and any disagreements were resolved by discussion or by asking the third investigator. The first author's name, publication year, sample size, participants' age, participants' gender, smoking (%), alcohol abuse (%), positive *H. pylori* infection (%), NSAID user (%), drug type, intervention measure and outcome assessment time were extracted. The main outcomes included were: (1) mortality (*n*); (2) re-bleeding (*n*); (3) received surgery (*n*); (4) blood transfused (unit/500 mL); and (5) hospitalization stay time (d).

Study quality assessment

We used the Cochrane Collaboration's tool[12] to evaluate the quality of the articles. The following seven items of risk of bias in the tool were assessed: random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attrition bias), selective reporting (reporting bias) and Other bias, all studies were classified as low risk, high risk and unclear risk. The assessment was performed

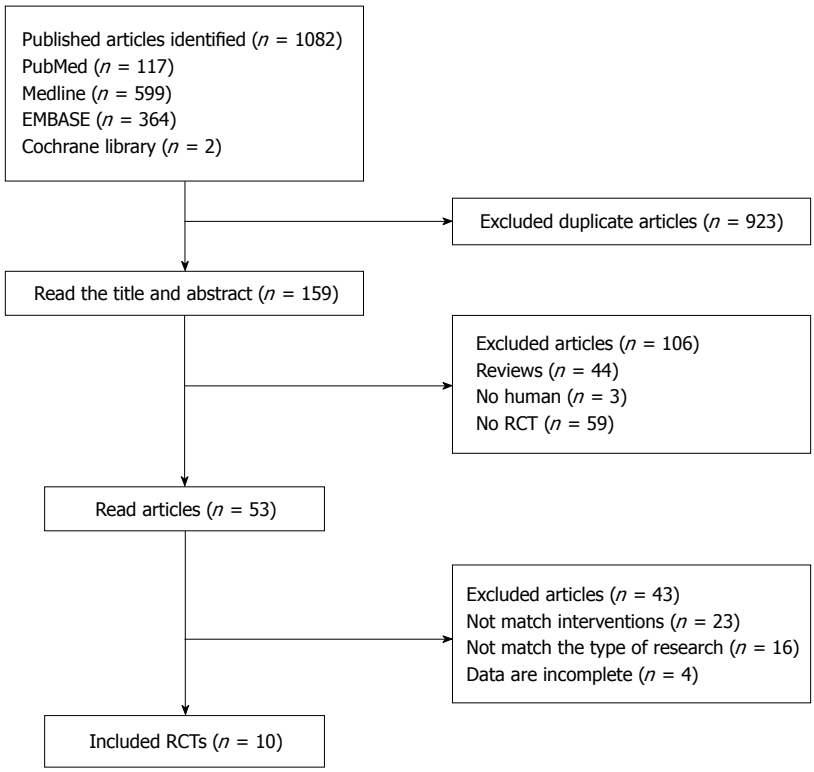

Figure 1 Flow chart of study selection. RCTs: Randomized controlled trials.

independently by two investigators; disagreements were be resolved by discussion or by involving the third investigator.

Statistical analysis

At the end of treatment for the individual trials, the odds ratio (OR) and mean difference (MD) with their 95% confidence interval (CIs) were calculated. The OR and weighted mean difference (WMD) were used as summary estimators. A fixed-effect model weighted by the inverse variance method was used following a homogeneity test. We performed the homogeneity test using a χ^2 test on N-1 degrees of freedom. A P value of 0.05 was regarded as statistically significant, rejecting the assumption of homogeneity ($P < 0.05$), and the random-effect model was then performed using the inverse variance method. Publication biases were evaluated using a Funnel plot, Begg's test and Egger's test; $P \geqslant 0.05$ indicated there was no publication bias.

All statistical analyses were performed using Review Manager 5.3.3 statistical software (Cochrane Collaboration, Oxford, United Kingdom) and Stata 12.0 statistical software (Stata Co. College Station, TX, United States) for the meta-analysis.

RESULTS

Article selection

One-thousand and eighty-two publish articles were identified, and we initially excluded 923 duplicate articles. Then, after reading the title and abstract, 53 articles remained. Finally, by reading the full text of each article, 10 RCTs[13-22] were included in this meta-analysis (Figure 1). The characteristics of all included studies are shown in Table 1. This meta-analysis included 1283 patients, with 678 subjects in the PPI group and the remaining 605 subjects in the H_2RA group. The maximum sample size of the included studies was 200 cases[16]; the minimum was 77 cases[20]. For the study of Lin *et al*[16], which used two randomized controlled programs, the programs were divided into a and b programs as two separate studies and included in the meta analysis.

Use of PPI therapy compared with H_2RA therapy for recurrent bleeding rate and quality assessment

The number of recurrent bleeding subjects reported after treatment was investigated in all included studies[13-22]. According to the different routes of administration, the studies could be divided into three subgroups, intravenous followed by oral, simple intravenous and simple oral. There was not any significant heterogeneity between the included trials ($P = 0.13$, $I^2 = 35\%$) and subgroups ($P = 0.90$, $I^2 = 0\%$); therefore, we used the fixed effects model for the analysis. Seven of the studies[13,16,17,19-22], including 922 subjects, used the first intravenous and then oral administration method; the results of these studies did not statistically significantly reduce the re-bleeding rate (OR = 0.35; 95%CI: 0.24-0.52; Figure 2). However, one trial[15], which included 149 subjects, reported the re-bleeding number after simple intravenous treatment

Table 1 General condition sheet of the included studies

Ref.	Sample size	Age	Gender (M/F)	Smoking (%)	Alcohol abuse (%)	*H. pylori* infection positive (%)	NSAID user (%)	Drug type	Intervention		Outcome assessment time	The main outcomes
									PPI group	H_2RA group		
Hsu *et al*[13]	P:52	P: 63.2 ± 18.	P: 41/11	P: 32.7	P: 13.5	NA	P: 26.9	P: Pantoprazole	40 mg intravenous /12 h 3 d, followed by 40 mg/d orally	50 mg intravenous /8 h, followed by 150 mg /12 h orally	8 wk	[1,2,3,4,5]
	H:50	H: 64.7 ± 13.8	H: 37/13	H: 32.0	H: 8.0		H: 320	H: Ranitidine				
Ye *et al*[14]	P:41	P: 61.2 ± 9.0	P: 28/13	NA	NA	P: 61.0	NA	P: Omeprazole	20 mg/d orally	20 mg/12 h orally	28 d	[1]
	H:41	H: 58.5 ± 9.4	H: 24/17			H: 56.1		H: Famotidine				
Jensen *et al*[15]	P:72	P: 59.6 ± 16.1	P: 51/21	NA	NA	NA	P: 69	P: Pantoprazole	80 mg bolus and 8 mg/h infusion 3 d	50 mg bolus and 6.25 mg/h infusion 3 d	3 d, 7 d, 30 d	[1]
	H:77	H: 55.6 ± 16.8	H: 52/25				H: 71	H: Ranitidine				
Lin *et al*[16]	Pa:67	Pa: 67	Pa: 58/9	NA	NA	NA	Pa: 26.9	P: Omeprazole	a: 40 mg intravenous /12 h 3 d, followed by 20 mg/d orally	400 mg intravenous /12 h 3 d, followed by 400 mg/12 h orally	14 d	[1,2,3,4,5]
	Pb:66	Pb: 71	Pb: 57/9				Pb: 24.2	H: Cimetidine	b: 40 mg intravenous /6 h 3 d, followed by 20 mg/d orally			
	H:67	H: 68	H: 61/6				H: 29.9					
Jeong *et al*[17]	P:85	P: 62.9 ± 9.4	P: 52/33	NA	NA	P: 61.9	NA	P: Pantoprazole	80 mg bolus and 8 mg/h infusion d1, 40 mg intravenous /12 h d2-3, followed by 40 mg/d orally	20 mg intravenous /12 h d2, followed by 20 mg/d orally	24 h, 7 d, 14 d	[1,2,3]
	H:79	H: 63.5 ± 7.8	H: 53/26			H: 64.1		H: Famotidine				
Uedo *et al*[18]	P:64	P: 68.1 ± 8.5	112/33	NA	NA	11.5	11.5	P: Rabeprazole	20 mg/d orally	800 mg/d orally	8 wk	[1]
	H:66	H: 65.7± 7.6						H: Cimetidine				
Imaeda *et al*[19]	P:62	P: 68.4 ± 8.0	P: 47/15	P: 58.1 H: 49.2	NA	P: 61.3	NA	P: Lansoprazole	30 mg intravenous /12 h 2 d, followed by 30 mg/d orally	75 mg intravenous /12 h 2 d, followed by 75 mg/12 h orally	8 wk	[1]
	H:61	H: 67.6 ± 8.5	H: 52/9			H: 62.3		H: Roxatidine				
Sakurada *et al*[20]	P:40	P: 65.8 ± 2.5	P: 35/5	NA	NA	P: 77.5	P: 35.0	P: Omeprazole	20 mg intravenous /12 h 3 d, followed by 20 mg/d orally	20 mg intravenous /12 h 3 d, followed by 20 mg/d orally	6-8 wk	[1,5]
	H:37	H:60.2 ± 1.7	H: 31/6			H: 78.4	H: 29.7	H: Famotidine				
Tomita *et al*[21]	P:77	P: 70.4 ± 8.7	P: 59/18	NA	NA	NA	NA	P: Omeprazole	20 mg/12 h intravenous 3 d, followed by 20 mg/d orally	40 mg bolus/d	8 wk	[1]
	H:79	H: 70.6 ± 9.5	H: 59/20					H: Famotidine				
Lin *et al*[22]	P:50	P: 65 H: 66.5	P: 46/4	P: 34.0	P:12.0	NA	NA	P: Omeprazole	40 mg intravenous and 160 mg infusion 3 d, followed by 20 mg/12 h orally 2 m	300 mg intravenous and 1200 mg infusion 3 d, followed by 400 mg/12 h orally 2 m	3 d, 14 d	[1,2,3,4,5]
	H:50		H:43/7	H: 28.0	H: 12.0			H: Cimetidine				

[1]Recurrent bleeding (*n*); [2]Mortality (*n*); [3]Received surgery (*n*); [4]Blood transfused (unit); [5]Hospital stay (d). P: PPI group; H: H_2RA group; PPI: Proton pump inhibitor; H_2RA: Histamine 2 receptor antagonist; NA: Not applicable.

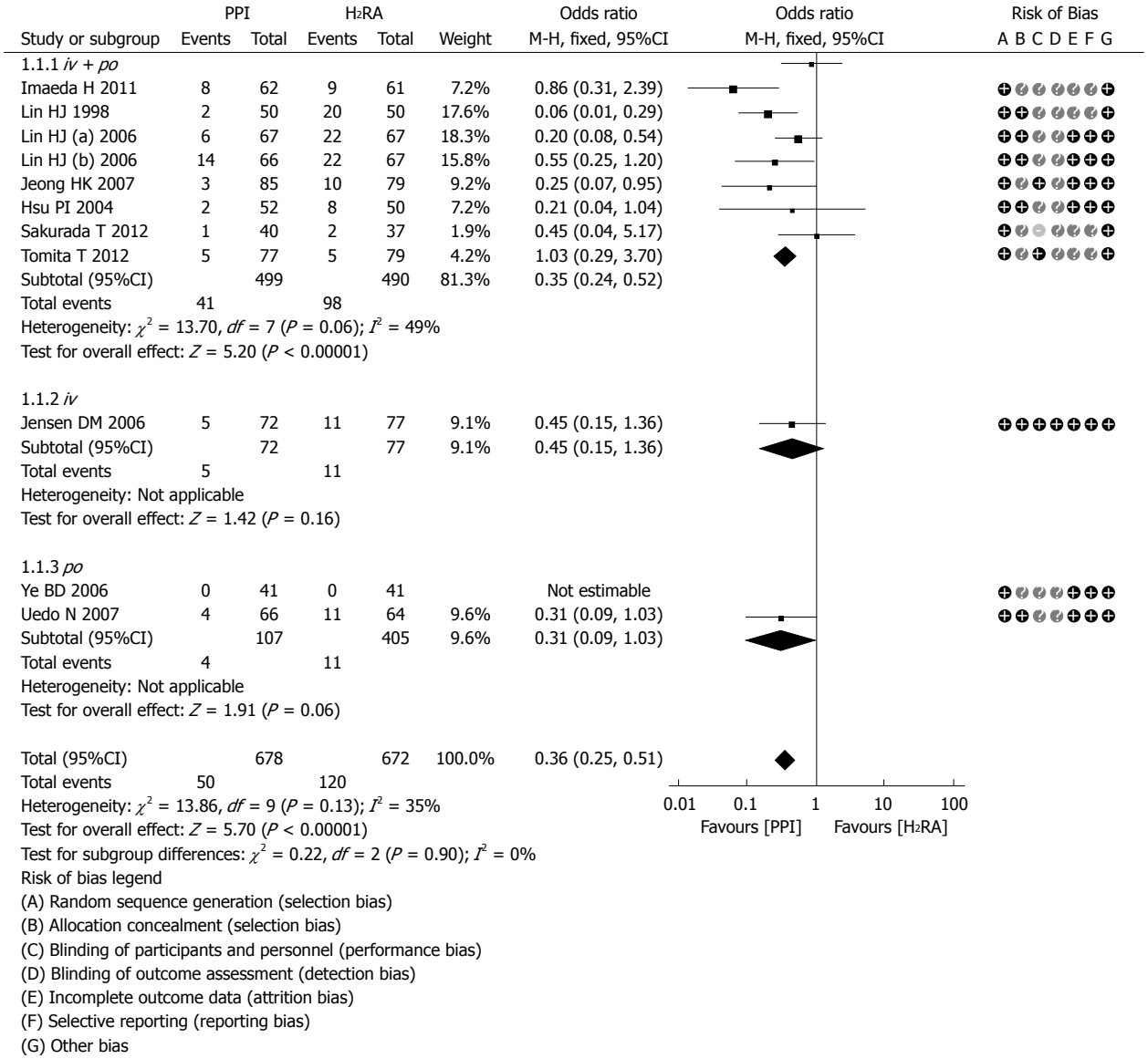

Figure 2 Forest plot and risk of bias summary from the Cochrane risk of bias tool comparing proton pump inhibitors therapy *vs* H_2 receptor antagonists therapy efficacy for the recurrent bleeding rate. PPI: Proton pump inhibitors; H_2R: H_2 receptor antagonists.

and did observe a statistically significant difference (OR = 0.45; 95%CI: 0.15-1.36; Figure 2). Meanwhile, no statistical difference was observed in two trials[14,18] that reported the re-bleeding number after simple oral treatment (OR = 0.31; 95%CI: 0.09-1.03; Figure 2). The meta-analysis results for re-bleeding rate revealed that after successful endoscopic therapy, compared with the H_2RA therapy group, the PPI therapy group had significantly reduced re-bleeding rates (OR = 0.36; 95%CI: 0.25-0.51; Figure 2). Figure 2 shows the quality assessments of these ten articles, as evaluated by the Cochrane Collaboration's tool.

Inconsistencies within the end-point time, nationality and intervention drugs, may have resulted in significant and substantial heterogeneity in our analysis (Table 2). The first subgroup analysis used the time of recurrent bleeding occurrence after successful endoscopic therapy (d). Only one trial[17] reported the re-bleeding number within 24 h and two trials[15,22] reported the re-bleeding number within 3 d. These trials were significantly heterogeneous in reducing the re-bleeding rate (OR = 0.23; 95%CI: 0.07-0.76; Table 2). An additional two trials[15,17] reported the re-bleeding number within 7 d after successful endoscopic therapy and observed a statistically significant reduction in the re-bleeding rate (OR = 0.32; 95%CI: 0.13-0.79; Table 2). Furthermore, heterogeneity was observed in the three trials[16,17,22] that reported the re-bleeding number within 14 d (OR = 0.26; 95%CI: 0.16-0.43; Table 2). Meanwhile, a statistical difference was observed in two trials[14,15] that reported the re-bleeding number within 28-30 days (OR = 0.45; 95%CI: 0.15-1.36; Table 2). Finally, there were five trials[13,18-21] that reported re-bleeding number within 6 wk or more and no statistical difference was observed (OR = 0.53; 95%CI: 0.30-0.94; Table 2). In summary,

Table 2 Subgroup analyses of recurrent bleeding rate

	Number of studies	Number of subjects	OR (95%CI)	Heterogeneity within subgroups	Difference between subgroups
End-point time					
Overall	10	2076	0.33 (0.24-0.45)	No (P = 0.20, I^2 = 23%)	No (P = 0.42, I^2 = 0%)
24 h	1	164	0.08 (0.00-1.46)	NA	
3 d	2	249	0.23 (0.07-0.76)	Yes (P = 0.13, I^2 = 56%)	
7 d	2	313	0.32 (0.13-0.79)	No (P = 0.37, I^2 = 0%)	
14 d	3	464	0.26 (0.16-0.43)	Yes (P = 0.07, I^2 = 58%)	
28-30 d	2	231	0.45 (0.15-1.36)	NA	
6 wk or more	5	588	0.53 (0.30-0.94)	No (P = 0.42, I^2 = 0%)	
Nation					
Overall	10	1283	0.36 (0.25-0.51)	No (P = 0.13, I^2 = 35%)	No (P = 0.42, I^2 = 0%)
Japan	4	486	0.63 (0.33-1.17)	No (P = 0.51, I^2 = 0%)	
China	3	402	0.25 (0.14-0.42)	Yes (P = 0.06, I^2 = 59%)	
South Korea	2	246	0.25 (0.07-0.95)	NA	
United States	1	149	0.45 (0.15-1.36)	NA	
Intervention drug PPI					
Overall	8	1030	0.32 (0.21-0.48)	No (P = 0.13, I^2 = 37%)	No (P = 0.93, I^2 = 0%)
Omeprazole	5	615	0.32 (0.20-0.52)	Yes (P = 0.03, I^2 = 61%)	
Pantoprazole	3	415	0.31 (0.15-0.65)	No (P = 0.69, I^2 = 0%)	
Intervention drug H_2RA					
Overall	8	996	0.33 (0.22-0.48)	No (P = 0.14, I^2 = 36%)	No (P = 0.18, I^2 = 41.6%)
Cimetidine	3	430	0.27 (0.16-0.44)	Yes (P = 0.07, I^2 = 58%)	
Famotidine	3	315	0.85 (0.28-2.61)	No (P = 0.56, I^2 = 0%)	
Ranitidine	2	251	0.34 (0.14-0.85)	No (P = 0.45, I^2 = 0%)	

OR: Odds ratio; CI: Confidence interval; NA: Not applicable.

this subgroup analysis demonstrated that the end-point time after successful endoscopic therapy was not significantly different between studies (OR = 0.33; 95%CI: 0.20-0.45; Table 2) and subgroups (P = 0.42; Table 2).

The second subgroup analysis used the nationality of origin for each study. Four Japanese trials[18-21] had an OR = 0.63 (95%CI: 0.33-1.17; Table 2) and did not demonstrate a statistically significantly reduced re-bleeding rate. Three Chinese trials[13,16,22] showed heterogeneity for reducing the re-bleeding rate (OR = 0.25; 95%CI: 0.14-0.42; Table 2). Two South Korea trials[14,17] and one United States trial[15] had an OR of 0.25 (95%CI: 0.07-0.95; Table 2) and 0.45 (95%CI: 0.15-1.36; Table 2), respectively. In summary, in this subgroup analysis for re-bleeding, the nationality of the study did not significant effect drug use after successful endoscopic therapy between the different studies (OR = 0.36; 95%CI: 0.25-0.51; Table 2) and subgroups (P = 0.42; Table 2).

The third subgroup analysis used groupings by the PPI intervention drug type. Five trials[14,16,19,20,22] used omeprazole as the intervention PPI drug and had significant heterogeneity, with an OR of 0.32 (95%CI: 0.20-0.52; Table 2). These five trials were not statistically significantly different compared with the three pantoprazole studies[13,15,17] (OR = 0.31, 95%CI: 0.15-0.65; Table 2). The fourth subgroup used groupings by the H_2RA intervention drug type. Three trials used cimetidine[16,18,22] as the H_2RA intervention drug and had an OR of 0.27 (95%CI: 0.16-0.44; Table 2) with significant heterogeneity. Three trials used famotidine[14,20,21] as the H_2RA intervention drug (OR = 0.85; 95%CI: 0.28-2.61; Table 2) and two trials[13,15] used ranitidine as the H_2RA intervention drug (OR = 0.34; 95%CI: 0.14-0.85; Table 2). In summary, the H_2RA intervention drug subgroup analysis for the re-bleeding rate revealed that the H_2RA drug type after successful endoscopic therapy drug use had no significant effect in reducing the re-bleeding rate (OR = 0.33; 95%CI: 0.22-0.48; Table 2) or between subgroups (P = 0.18; Table 2).

In general, the subgroup analyses results did not reveal statistically significant differences in patients with upper gastrointestinal bleeding after successful endoscopic therapy (Table 2).

Use of PPI therapy compared with H_2RA therapy for mortality

Five studies[13,15-17,22], involving 715 patients, reported mortality after treatment. According to the different routes of administration, the studies could also be divided into three subgroups, intravenous followed by oral, simple intravenous and simple oral. There was no significant heterogeneity between these trials (P = 0.68, I^2 = 0%) or subgroups (P = 0.22, I^2 = 33.3%); therefore, we used a fixed effects model for the analysis. Four of the studies[13,16,17,22], including 588 subjects, used the first method, intravenous and then oral administration, and the mortality was not statistically significantly reduced (OR = 0.29; 95%CI: 0.08-0.17; Figure 3). Only one trial[15], including 149 subjects, reported the dead number after simple intravenous treatment and no statistically significant

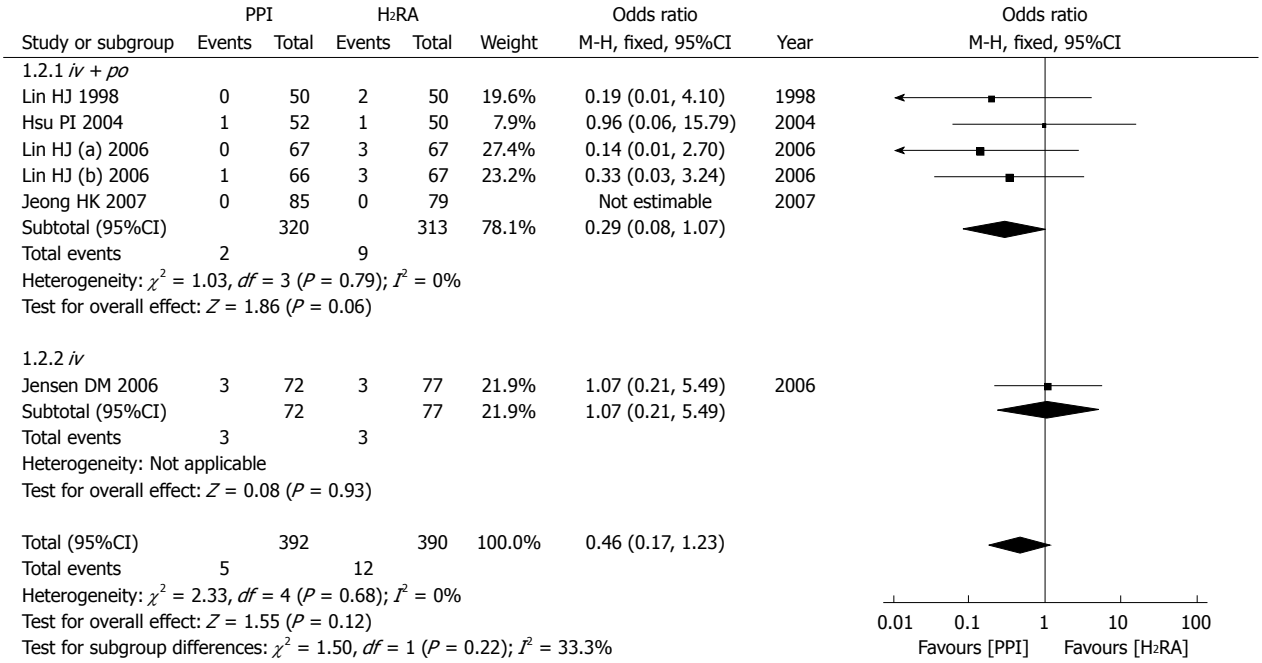

Figure 3 Forest plot comparing the efficacy of proton pump inhibitors therapy *vs* H_2 receptor antagonists therapy for the recurrent bleeding rate mortality. PPI: Proton pump inhibitors; H_2R: H_2 receptor antagonists.

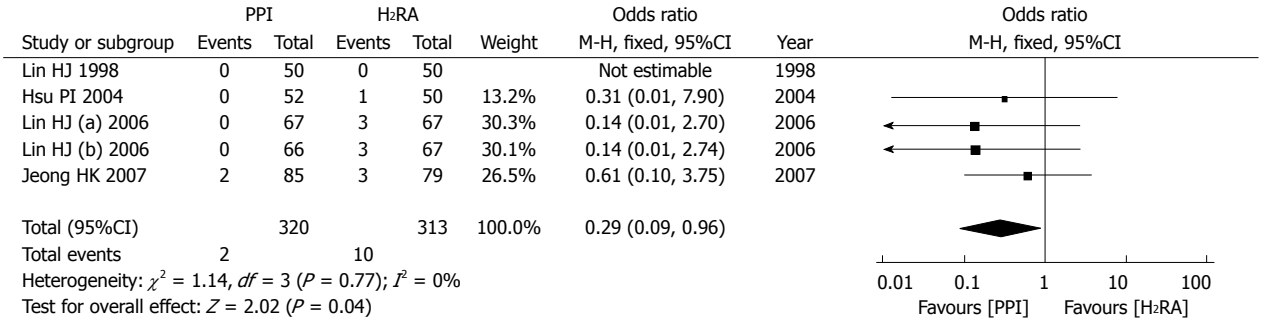

Figure 4 Forest plot comparing the efficacy of proton pump inhibitors therapy *vs* H_2 receptor antagonists therapy for the required surgery rate. PPI: Proton pump inhibitors; H_2R: H_2 receptor antagonists.

difference was observed (OR = 1.07; 95%CI: 0.21-5.49; Figure 3). In summary, this meta-analysis of mortality revealed that after successful endoscopic therapy, there were no significant differences in mortality between the two drugs (OR = 0.46; 95%CI: 0.17-1.23; Figure 3).

Use of PPI therapy compared with H_2RA therapy in the receiving required surgery rate

Four studies[13,16,17,22], involving 566 patients, reported the number of patients who received surgery after treatment. There was no significant heterogeneity between these trials (P = 0.77, I^2 = 0%); therefore, a fixed effects model was used for the analysis. The meta-analysis revealed that after successful endoscopic therapy, compared with H_2RA therapy, PPI therapy significantly decreased the number of patients that received surgery (OR = 0.29; 95%CI: 0.09-0.96; Figure 4).

Use of PPI therapy compared with H_2RA therapy for blood transfusion amounts (units)

Three studies[13,16,22], involving 402 patients, reported the units (mL; 500 mL per unit) for patients who required blood transfusions after treatment. There was significant heterogeneity between these trials (P = 0.0001, I^2 = 89%); therefore, a random effects model was used for the analysis. The meta-analysis revealed that after successful endoscopic therapy, PPI therapy was more effective in decreasing blood transfusion units (WMD: -0.70 unit; 95%CI:-1.64-0.25; Figure 5).

Use of PPI therapy compared with H_2RA therapy for hospitalization time (days)

Four studies[13,16,20,22], involving 479 patients, reported the hospitalization time of patients. There was significant heterogeneity between these trials (P < 0.00001, I^2 = 97%); therefore, a random effects model was used for the analysis. The meta-analysis revealed

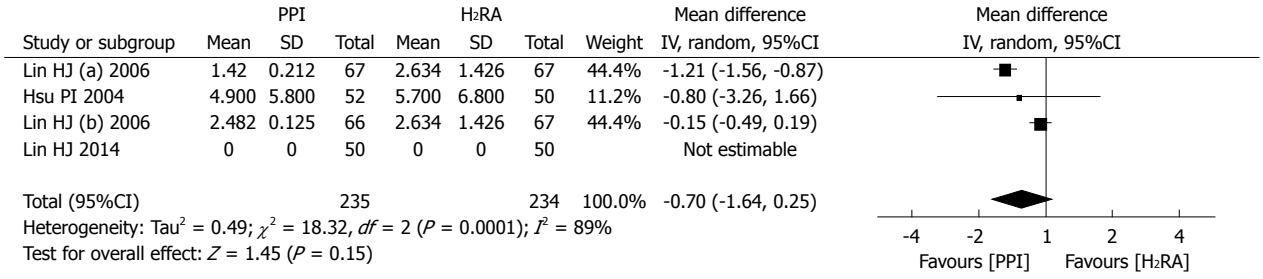

Figure 5 Forest plot comparing the efficacy of proton pump inhibitors therapy *vs* H_2 receptor antagonists therapy for blood transfusion units. PPI: Proton pump inhibitors; H_2R: H_2 receptor antagonists.

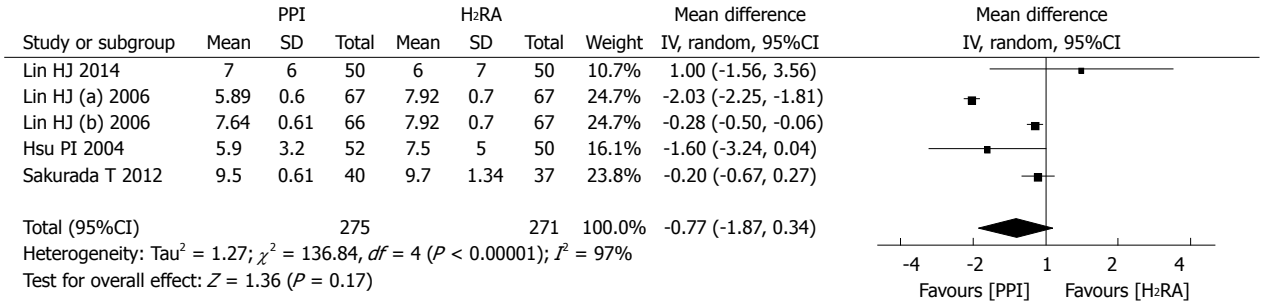

Figure 6 Forest plot comparing the efficacy of proton pump inhibitors therapy *vs* H_2 receptor antagonists therapy for hospitalization time. PPI: Proton pump inhibitors; H_2R: H_2 receptor antagonists.

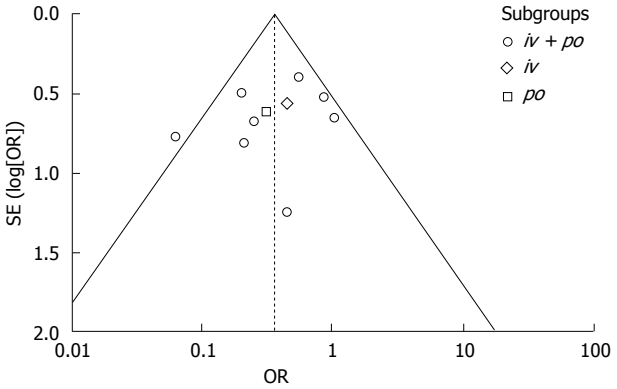

Figure 7 Funnel plot evaluating the publication bias for recurrent bleeding rate.

that after successful endoscopic therapy, PPI therapy was more effective in decreasing hospitalization time (WMD: -0.77d; 95%CI: -1.87-0.34; Figure 6).

Publication bias assessment

Publication bias was evaluated using a Funnel plot (Figure 7). The asymmetrical scatter plot revealed little publication bias in our meta-analysis. In addition, we also performed the Begg's test and Egger's test; both tests demonstrated that there was no publication bias in our meta-analysis (Begg's test: $P = 0.283$; Egger's test: $P = 0.339$).

DISCUSSION

In patients with upper gastrointestinal bleeding after successful endoscopic therapy, compared with H_2RA therapy, PPI therapy was more effective for reducing the re-bleeding rate and received surgery rate, but there were no significant differences in mortality between the two drugs. There was little heterogeneity between the three different methods of administration (*iv* + *po*; *iv*; *po*). The results also revealed that intravenous and oral PPI had similar results, which has also been proposed by several published studies[23,24], and the PPI therapeutic results were more effective than H_2RA therapy. In this meta-analysis, the intravenous followed by oral administration method had better therapeutic value than either simple intravenous therapy or simple oral therapy, which may prompt a standard drug administration method.

For the subgroup analyses of the re-bleeding time, the risk of recurrent bleeding was highest during the first 3 d after therapy and most re-bleeding events occurred in the first 24 h[6]. In this meta-analysis, we concluded that PPI can significantly reduce the re-bleeding rate during the first 3 d after successful endoscopic therapy compared with H_2RA. Additionally, the same result was observed during 7 d and 14 d post endoscopy, which indicated that PPI can decrease the short-term re-bleeding rate. Furthermore, the same result was observed in the group of studies that evaluated 6 wk or more post endoscopy, which illustrated that PPIs may also decrease the long-team re-bleeding rate. However, in the 28-20 d group, the two types of drugs produced similar results, with an OR < 1 (OR = 0.45, Table 2), which indicated that PPI therapy tends to be better than H_2RA therapy.

There was no significant difference between the different populations included in the studies in our meta-analysis (P = 0.16, I^2 = 41.2%, Table 2); therefore, we concluded that in patients with upper gastrointestinal bleeding after successful endoscopic therapy, PPI has better therapeutic value worldwide. However, a meta-analysis by Leontiadis *et al*[25] revealed that PPI therapy for ulcer bleeding was more efficacious in Asia than elsewhere, which conflicts with the results in our study. However, in our meta-analysis, nine out of ten studies were conducted in Asian countries, which may result in geographical limitations.

In the subgroup analysis of PPI type, there was no significant difference between the omeprazole group and pantoprazole group (P = 0.93, I^2 = 0%, Table 2); *i.e.*, the different PPI types had little influence on outcome. However, in the analysis of the H_2RA group, although there was no heterogeneity difference between the groups (P = 0.18, I^2 = 41.6%, Table 2), cimetidine and ranitidine had superior curative effects compared with famotidine (Table 2).

The standard dose of PPI used after successful endoscopic therapy is hard to determine, the included studies showed large differences in the dose administered; however, the meta-analysis of Wu *et al*[26] demonstrated that low-dose intravenous PPI can achieve the same efficacy as high-dose PPI following endoscopic hemostasis. To clarify these conclusions, additional research must be conducted.

In the ten included studies, there were no serious adverse reactions. An evaluation of the acute and chronic adverse reactions of PPIs and H_2RAs is necessary because the treatment period ranged from 3 d to a few weeks. The results suggested that these two types of drugs have short-term adverse reactions that are mild or not obvious. PPIs are a well-tolerated pharmaceutical class, with adverse effects occurring at a rate of 1%-3%, and with no significant differences between PPI types[27]. The adverse effects most commonly observed with PPI use are nausea, rash, headaches, constipation, flatulence, diarrhea, abdominal pain and dizziness[28]. However, in patients with a history of ulcer bleeding, long-term oral PPI is necessary and the safety of long-term PPI use is controversial. Insogna *et al*[29] revealed that long-term PPI use may influence mineral metabolism, specifically calcium absorption, which increased the risk of bone fracture. Ito *et al*[30] demonstrated that long-term PPI use may influence calcium absorption, as well as influence the absorption of vitamin B_{12}, iron and magnesium, which can have important clinical implications. However, tolerance has been reported to prolonged H_2RA therapy, as discussed in several studies. Rackoff *et al*[31] argued that prolonged hypergastrinemia is induced by long-term or high dose H_2RA therapy.

There were several limitations in this study. First, the quality of the included randomized controlled trials was variable, but most of them were of acceptable quality. A number of high-quality, well-designed RCTs are needed for further research. Future studies should describe the grouping method in detail and disclose the number of patients lost to follow-up and exit. Second, heterogeneity between the studies may have skewed the meta-analysis results; the results may be from the baseline and after pharmaceutical therapy. For example, in our meta-analysis, the baseline values of the smoking rate, alcohol abuse rate, positive *H. pylori* infection rate and NSAID use rate in the included studies were different and these factors may have affected the analysis results. Additionally, the agents used in the included studies were different; most studies used pantoprazole or omeprazole for the PPI group, whereas cimetidine, famotidine and ranitidine were mostly used in the H_2RA group. Some factors were influenced by the area, hospital, *etc.*, particularly the mean hospitalization days. Therefore, subject enrollment data should include age, sex, risk and drug types, as well as, follow-up time, endpoint and the number of patients who accepted PPI therapy or H_2RA therapy. Third, the potential for publication bias is always a concern. The number of included studies and differences in sample size may have affected the publication bias. Fourth, it is difficult to publish the results when the results do not identify any significant differences, *i.e.*, PPI therapy has similar efficacy to H_2RA therapy. This phenomenon may have led to bias.

Further research, specifically large-scale double-blind randomization trials, are required to provide more credible data for PPI or H_2RA treatment using different administration methods in upper gastrointestinal bleeding patients.

COMMENTS

Background

In patients with upper gastrointestinal bleeding, recurrent bleeding is the most important adverse effect, resulting in morbidity and mortality. Both proton pump inhibitors and H_2 receptor antagonists are commonly administered to upper gastrointestinal bleeding patients after successful endoscopic therapy.

Research frontiers

A series of randomized controlled trials were conducted to compare proton pump inhibitor (PPI) therapy and H_2 receptor antagonists (H_2RA) therapy after successful endoscopy to determine the appropriate first-line treatment drug for upper gastrointestinal bleeding.

Innovations and breakthroughs

This is the first meta-analysis to evaluate the efficacy and safety of PPI therapy and H_2RA therapy after successful endoscopy in patients with upper gastrointestinal bleeding, using several important endpoints, such as re-bleeding rate, mortality and receiving surgery rate.

Applications

The results suggested that PPI therapy is superior to H_2RA therapy in upper gastrointestinal bleeding patients after successful endoscopic therapy. This result may provide valuable information to clinicians.

Terminology

PPI is a type of H^+/K^+ ATPase inhibitor. Its acid-suppressing activity is potent, highly specific and has a long duration. H_2RA can selectively block wall H_2 receptors on the cell membrane, reducing gastric acid secretion.

Peer-review

This is a well-performed meta-analysis that aimed to determine whether PPI therapy is more efficacious and safe than H_2RA therapy in upper gastrointestinal bleeding patients after successful endoscopic therapy that provides definitive

results.

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META-ANALYSIS

Meta-analysis of the effectiveness and safety of vedolizumab for ulcerative colitis

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Abstract

AIM: To conduct a meta-analysis examining the effectiveness and safety of vedolizumab for the treatment of ulcerative colitis (UC).

METHODS: A search was conducted of MEDLINE, Cochrane, EMBASE, and Google Scholar on July 31, 2013. Inclusion criteria were: (1) Randomized controlled trial (RCT); (2) Patients treated for UC; and (3) Intervention was vedolizumab. The following information/data were extracted from studies that met the inclusion criteria: the name of the first author, year of publication, study design, patient demographic information, response rate, remission rate, and adverse events. The primary outcome was clinical response rate, and the secondary outcomes were clinical remission rate and serious adverse events. Odds ratio (OR) with 95%CI were calculated for each outcome.

RESULTS: Of 224 studies initially identified, three RCTs examining the use of vedolizumab meeting the inclusion criteria were included in the meta-analysis. All studies examined the use of vedolizumab at dosages ranging from 0.5 to 10 mg/kg body weight (one study used a standard dose of 300 mg). The follow-up periods were approximately 6 wk. The total number of patients in the intervention groups was 901, and in the control groups was 221. The mean age of the patients was approximately 41 years, and approximately half were males. The follow-up periods ranged from 43 d to 6 wk. The clinical response and remission rates were significantly higher for patients who received vedolizumab as compared to control patients (clinical response: OR = 2.69; 95%CI: 1.94-3.74, $P < 0.001$ and remission rate: OR = 2.72; 95%CI: 1.76-4.19, $P < 0.001$). Serious adverse events were not higher in patients that received vedolizumab.

CONCLUSION: This analysis supports the use of vedolizumab for the treatment of UC.

Key words: Inflammatory bowel disease; Ulcerative colitis; Vedolizumab; MLN-002; Meta-analysis

Core tip: Studies have suggested that vedolizumab may be effective in reducing intestinal inflammation in patients with ulcerative colitis (UC). This meta-analysis including three randomized controlled trials showed that treatment with vedolizumab results in significantly higher clinical response and remission rates than placebo in patients with UC. Importantly, serious adverse events were not more common in vedolizumab-treated patients than control patients. This analysis supports the use of vedolizumab for the treatment of UC.

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INTRODUCTION

Inflammatory bowel disease (IBD), an inflammatory condition of the colon and small intestine, is an autoimmune disease in which the body's own immune system attacks elements of the digestive system[1,2]. The main forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC), and the primary difference between the two is the location and nature of the inflammatory changes[1,2]. CD may affect any part of the gastrointestinal tract from mouth to anus, and can result in a wide variety of symptoms including abdominal pain, diarrhea, vomiting, and weight loss[1,2]. UC is more common than CD with a reported incidence of nine to 20 cases per 100000 person years, and a prevalence of approximately 150 to 300 cases per 100000 persons[1]. The incidence of both CD and UC has been increasing in China in the past two decades[3].

In UC, characteristic ulcers or open sores are restricted to the colonic mucosa, and the main symptom of active disease is typically the gradual onset of constant diarrhea mixed with blood[1]. UC can occur at any age, but onset is typically between 15 and 30 years of age[1]. Diagnostic studies include tests of blood and stool, colonoscopy or sigmoidoscopy, and imaging studies. Medical treatments for UC include anti-inflammatory agents such as 5-aminosalycilate compounds, systemic corticosteroids, topical corticosteroids, and immunomodulators, but all have certain side effects and are not effective in all cases[1,2]. Despite the success of anti-tumor necrosis factor therapies in the treatment of UC, a considerable proportion of patients are refractory to treatment[4,5]. In severe cases refractory to medical treatment colectomy is necessary.

Integrins are transmembrane receptors that mediate the attachment between a cell and its surroundings, such as other cells or the extracellular matrix[6,7]. Integrins interact with other proteins such as cadherins, immunoglobulin superfamily cell adhesion molecules, selectins, and syndecans to mediate cell-cell and cell-matrix interaction and communication[6,7]. Alpha4beta7 ($\alpha4\beta7$) integrin is found on circulating T lymphocytes, and is involved in the recruitment of leukocytes to the gastrointestinal tract[8]. Integrin antagonists are a new class of agents that inhibit leukocyte adhesion and aim to selectively inhibit the inflammatory pathway[4]. Vedolizumab (MNL-02) is a recombinant humanized IgG1 monoclonal antibody that inhibits adhesion and migration of leukocytes into the gastrointestinal tract by binding the $\alpha4\beta7$ integrin[9]. Early animal[10] and human studies[11] suggested that MLN-02 may be effective in reducing intestinal inflammation in patients with UC.

The purpose of this meta-analysis is to examine the efficacy and safety vedolizumab for the treatment of UC.

MATERIALS AND METHODS

Methods

The procedures performed in this meta-analysis are in accordance with recent guidelines for the reporting of meta-analyses (PRISMA guidelines). Meta-analysis does not involve human subjects and does not require Institutional Review Board review.

Data sources and searches

We conducted a systematic search of electronic databases and the bibliographies of all eligible studies to identify all relevant studies. A search was conducted of MEDLINE, Cochrane, EMBASE, and Google Scholar on July 31, 2013 using combinations of the search terms vedolizumab/MLN0002/MLN-02, inflammatory bowel disease, and ulcerative colitis.

Study selection

Studies were selected for inclusion in this analysis based on the following criteria: (1) Randomized controlled trial (RCT); (2) Patients treated for UC (if the study enrolled patients with IBD, only those with UC were included); and (3) The intervention was vedolizumab. Non-English publications were excluded.

Data extraction and quality assessment

Studies were identified using the search strategy by two independent reviewers. When there was uncertainty regarding eligibility, a third reviewer was consulted. References of identified studies were hand searched for other relevant studies. The following information/data were extracted from studies that met the inclusion criteria: the name of the first author, year of publication, study design, patient demographic information, response rate, remission rate, and adverse events (AEs).

The methodological quality of each study was assessed using the risk-of-bias assessment tool

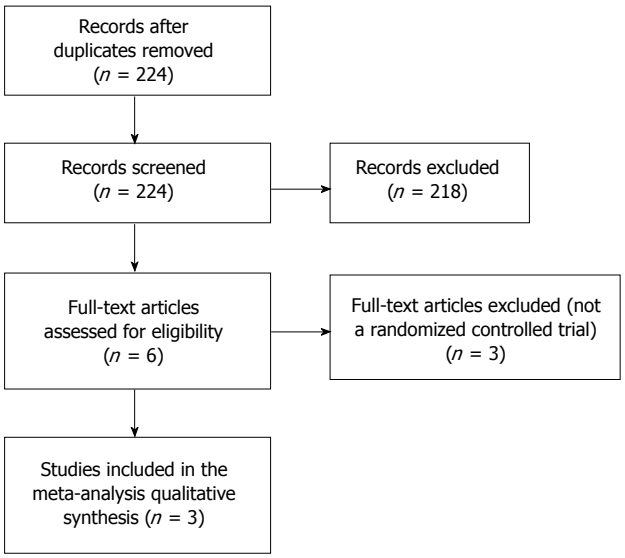

Figure 1 Flow diagram of study selection.

outlined in the Cochrane Handbook for Systematic Reviews of Interventions (version 5.1.0)[12]. Two reviewers subjectively reviewed all studies and assigned a value of "low risk," "high risk," or "unclear" to the following: (1) random sequence generation; (2) allocation concealment; (3) blinding (patients, personnel, and assessor); (4) adequate assessment of each outcome; (5) selective outcome reporting avoided; and (6) if the analysis included an intention-to-treat analysis.

Statistical analysis

The primary outcome was clinical response rate, and the secondary outcomes were clinical remission rate and serious adverse events. For each outcome, the odds ratio (OR) with 95%CI was calculated. Heterogeneity among the studies was assessed by the Cochran's *Q* test and the I^2 statistic. For Cochran's *Q*, a value of $P < 0.10$ was considered to indicate statistically significant heterogeneity. If either the *Q* statistics ($P < 0.1$) or I^2 statistic (> 50%) indicated the existence of significant heterogeneity between studies, a random-effects model of analysis (DerSimonian-Laird method) was used. Otherwise, a fixed-effect model of analysis (Mantel-Haenszel method) was used. Pooled ORs for the three outcomes were calculated; a two-sided *P* value < 0.05 was considered to indicate statistical significance. Sensitivity analysis was performed for the three outcomes based on the leave-one-out approach. As more than five studies are required to detect funnel plot asymmetry[13], publication bias was not assessed if less than five studies were identified with data for a particular outcome measure. All statistical analyses were performed using the statistical software Comprehensive Meta-Analysis, version 2.0 (Biostat, Englewood, NJ, United States).

RESULTS

Literature search

A flow diagram of study selection is shown in Figure 1. A total of 224 potentially relevant studies were identified in the literature search, and after screening 218 studies were excluded. Thus, 6 full-text articles were reviewed of which three was excluded because they were not RCT design. Finally, a total of three RCTs were included in the meta-analysis[14-16].

Description of studies

The characteristics of the three studies included in the meta-analysis are summarized in Table 1. All studies examined the use of vedolizumab at dosages ranging from 0.5 to 10 mg/kg body weight (one study used a standard dose of 300 mg). The total number of patients in the intervention groups was 901, and in the control groups was 221. The mean age of the patients was approximately 41 years, and approximately half were males. The follow-up periods were approximately 6 wk.

Quality assessment

The "risk of bias" summary is presented in Figure 2A, and an overall assessment of risk of bias is presented in Figure 2B. The random sequence and allocation concealment were appropriate in all three studies. The patients and personnel were blinded in two studies; however, none of the studies provided information on the blinding of outcome assessors. All studies were at a low risk of attrition bias and reporting bias. In addition, intention-to-treat analysis was used in all three studies.

Meta-analysis

Clinical response rate: The clinical response rates of the intervention groups ranged from 47.1% to 59.3% and of the control groups ranged from 25.5% to 33.3% (Table 2). There was no evidence of significant heterogeneity when data from the studies were pooled ($Q = 0.113$, $df = 2$, $P = 0.945$, $I^2 = 0\%$); therefore, a fixed-effect model was used for analysis of the clinical response rate (pooled of all intervention groups) (Figure 3A). The overall analysis revealed the clinical response rate was significantly higher for patients who received vedolizumab as compared to control patients (OR = 2.69, 95%CI: 1.94-3.74, $P < 0.001$).

Subgroup analysis for the pooled clinical response rate was performed according to the dosage of intervention drug. The studies of Feagan *et al*[14] and Parikh *et al*[16] were included in the analysis of clinical response rate of patients who received 2 mg of drug per kilogram of body weight and the studies of Feagan *et al*[15] and Parikh *et al*[16] were included in the analysis of clinical response rate of patients who received 6 mg of drug per kilogram of body weight.

Table 1 Characteristics of studies included in the meta-analysis

Ref.	Study type	Follow-up periods	Group	Drug	Drug dosage	Number of cases	Age, yr	Male
Feagan*et al*[14], 2005	RCT	6 wk	Intervention 1	MLN-02	0.5 mg/kg	58	41.6 ± 14.7	56.9%
			Intervention 2		2 mg/kg	60	43.8 ± 14.6	50.0%
			Control	Placebo	NA	63	38.9 ± 13.4	55.6%
Feagan *et al*[15], 2013	Randomized allocation	6 wk	Intervention	Vedolizumab	300 mg	746	40.1 ± 13.2	58.0%
			Control	Placebo	NA	149	41.1 ± 1.25	61.7%
Parikh *et al*[16], 2012	RCT	43 d	Intervention 1	Vedolizumab	2 mg/kg	12	39 (30-49)[1]	33.3%
			Intervention 2		6 mg/kg	14	47 (19-61)[1]	50.0%
			Intervention 3		10 mg/kg	11	41 (26-69)[1]	45.5%
			Control	Placebo	NA	9	33 (21-51)[1]	33.3%

[1]Median (range). RCT: Randomized controlled trial; NA: No data available.

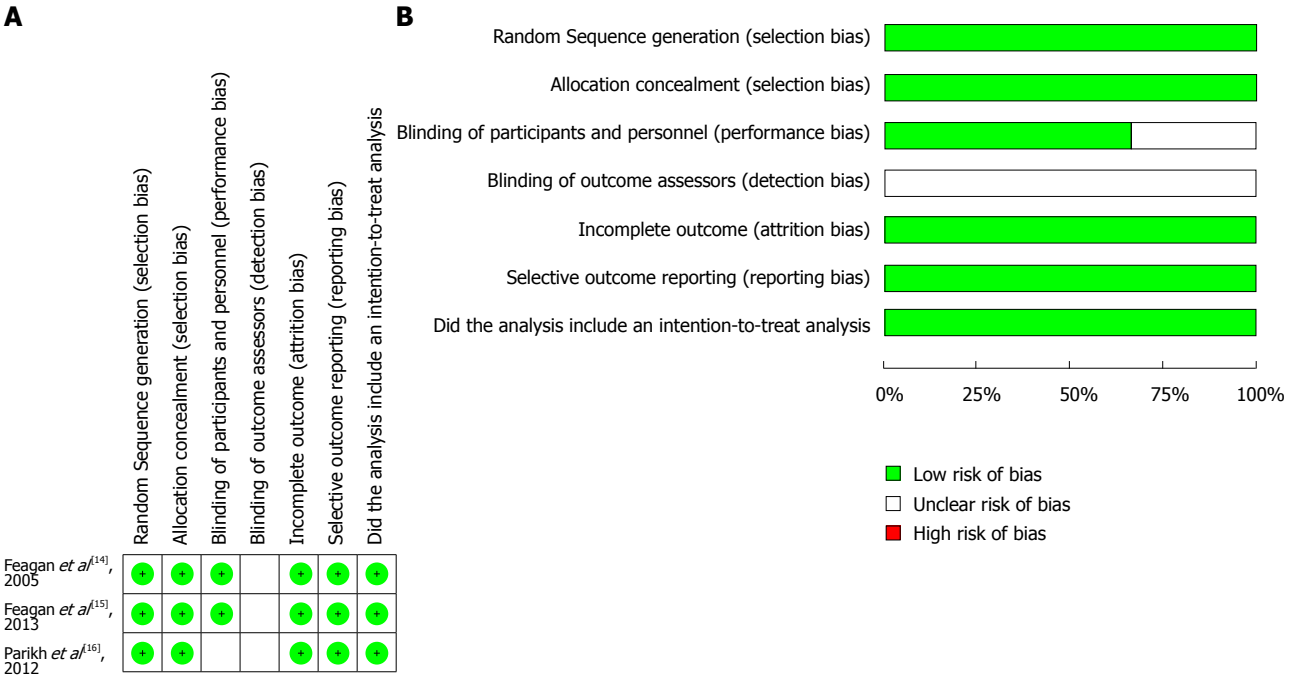

Figure 2 Quality assessments of included studies. A: Risk of bias summary; B: Risk of bias graph.

Patients who received 300 mg of vedolizumab in the study of Feagan *et al*[15] were included in the analysis of clinical response rate of patients who received 6 mg of drug per kilogram of body weight because 300 mg is approximately 6 mg of drug per kilogram of body weight. For the 2 mg per kilogram group, the clinical response rates of the intervention groups ranged from 50% to 53%,and of the control groups ranged from 33% to 33.3%. For the 6 mg per kilogram group, the clinical response rates of the intervention groups ranged from 47.1% to 63.3%, and of the control groups ranged from 25.5% to 33.3%. There was no evidence of significant heterogeneity when data from the 2 mg per kilogram studies were pooled (Q = 0.018, df = 1, P = 0.892, I^2 = 0%); therefore, a fixed-effect model was used for analysis of clinical response rate (Figure 3B). The overall analysis revealed the clinical response rate (2 mg) was significantly higher for patients who received vedolizumab as compared to control patients (P = 0.019). In addition, there was no evidence of significant heterogeneity when data from the 6 mg per kilogram studies were pooled (Q = 0.095, df = 1, P = 0.758, I^2 = 0%); therefore, a fixed-effect model was used for analysis of clinical response rate (Figure 3C). The overall analysis revealed the clinical response rate (6 mg) was significantly higher for patients who received vedolizumab as compared to control patients (P < 0.001).

Clinical remission rate: The clinical remission rates of the intervention groups ranged from 16.9% to 58%, and of the control groups ranged from 5.4% to 50% (Table 2). There was no evidence of significant heterogeneity when data from the studies were pooled (Q = 2.337, df = 2, P = 0.311, I^2 = 14.43%); therefore, a fixed-effect model was used for analysis of clinical remission rate (Figure 4). The overall analysis revealed the clinical remission rate was significantly higher for patients who received vedolizumab as compared to control patients (OR = 2.72, 95%CI:

Table 2 Clinical response and clinical remission rates of studies included in the meta-analysis

Ref.	Group	Drug	Definition of clinical response	Clinical response rate	Definition of clinical remission	Clinical remission rate
Feagan *et al*[14]	Intervention 1	MLN-02	An improvement of 3 points or more on the ulcerative colitis clinical score (modification of the Mayo Clinic Scoring system)	66% 59.3%[1]	Ulcerative colitis clinical score of 0 or 1 and a modified Baron score of 0 or 1 with no evidence of rectal bleeding	32.2%[1]
	Intervention 2			53%		
	Control	Placebo		33%		14.0%
Feagan *et al*[15]	Intervention	Vedolizumab	A reduction in the Mayo Clinic score of at least 3 points and a decrease of at least 30% from baseline, with an accompanying decrease in the rectal bleeding subscore of at least 1 point or an absolute rectal bleeding subscore of 0 or 1	47.1%	Mayo Clinic score of 2 or lower and no subscore higher than 1, and mucosal healing, defined as an endoscopic subscore of 0 or 1	16.9%
	Control	Placebo		25.5%		5.4%
Parikh *et al*[16]	Intervention 1	Vedolizumab	A decrease from baseline in the partial Mayo score (PMS) of ≥ 2 points and ≥ 25%, with an accompanying decrease in the subscore for rectal bleeding of ≥ 1 point or an absolute subscore for rectal bleeding of 0 or 1.	50% 56.8%[1]	PMS of ≤ 2 with no individual subscore > 1	58%[1]
	Intervention 2			63.3%		
	Intervention 3			53.3%		
	Control	Placebo		33.3%		50%

[1]Pooled of all intervention groups. MNL-02: Vedolizumab.

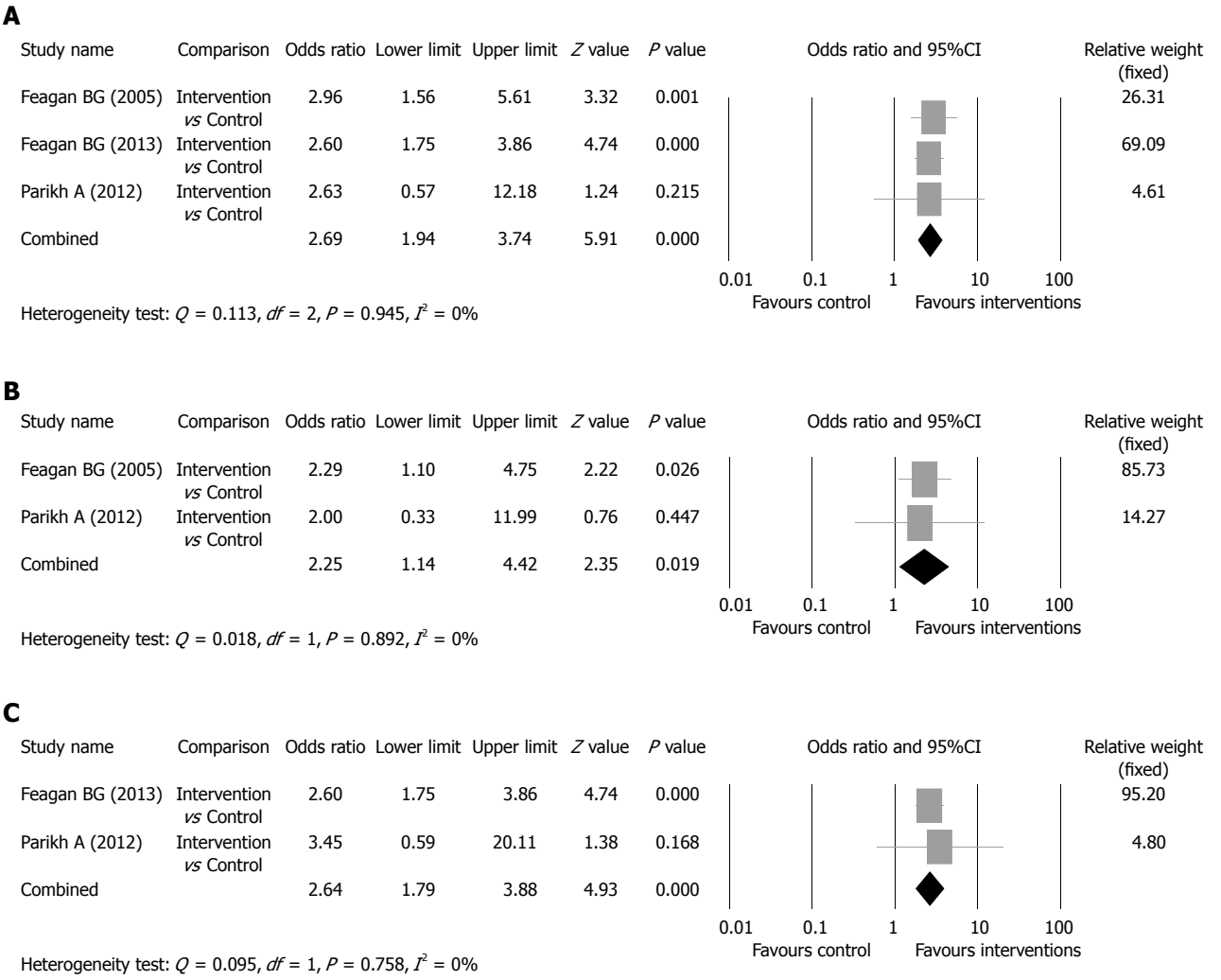

Figure 3 Forest plots of the meta-analysis of clinical response rate. A: Pooled of all intervention groups; B: Drug dose: 2 mg/kg; C: Drug dose: 6 mg/kg.

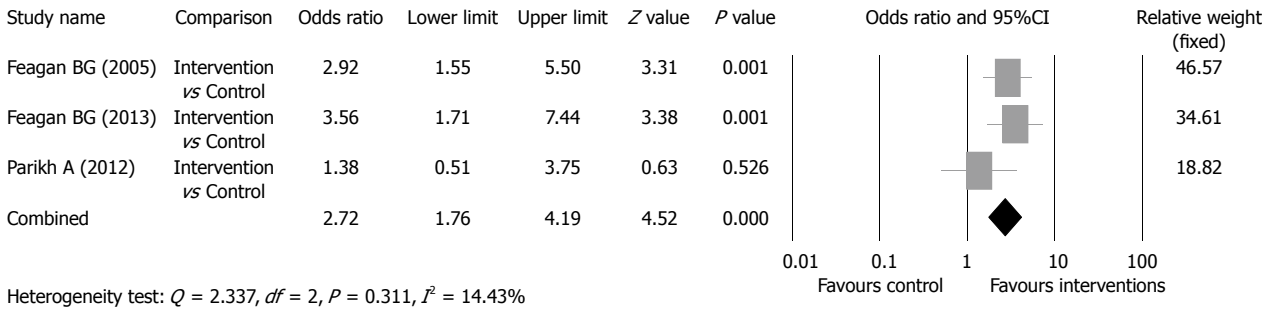

Figure 4 Forest plot of the meta-analysis of clinical remission rate.

Table 3 Summary of most common adverse events (occurring in 10% or more of patients in any group)

Ref.	Group	General adverse events[1]									
		Ulcerative colitis aggravated	Nausea/ vomiting	Headache	Frequent bowel movements	Fatigue	Upper respiratory tract infection	Abdominal pain/ tenderness	Arthralgia	Dizziness	Rash
Feagan *et al*[14]	Intervention 1	29 (50)	21 (36)	12 (21)	10 (17)	8 (14)	8 (14)	10 (17)	4 (7)	6 (10)	6 (10)
	Intervention 2	22 (37)	13 (22)	11 (18)	5 (8)	5 (8)	8 (13)	6 (10)	7 (12)	4 (7)	4 (7)
	Control	24 (38)	15 (24)	13 (21)	10 (16)	7 (11)	5 (8)	16 (25)	5 (8)	1 (2)	4 (6)
Feagan *et al*[15]	Intervention	97 (13)	38 (5)	80 (11)	NA	33 (4)	132 (18)	50 (7)	56 (8)	NA	NA
	Control	58 (39)	19 (13)	28 (19)	NA	10 (7)	47 (32)	10 (7)	25 (17)	NA	NA
Parikh *et al*[16]	Intervention 1	2 (17)	NA	2 (17)	NA	NA	4 (33)	NA	NA	1 (8)	NA
	Intervention 2	1 (7)	NA	3 (21)	NA	NA	3 (21)	NA	NA	0 (0)	NA
	Intervention 3	0 (0)	NA	2 (18)	NA	NA	1 (9)	NA	NA	0 (0)	NA
	Control	4 (44)	NA	1 (11)	NA	NA	4 (44)	NA	NA	1 (11)	NA
Feagan *et al*[14]	Intervention 1	6 (10)	NA	NA	NA	NA	NA	NA	6 (10)	18 (15)[2]	
	Intervention 2	3 (5)	NA	NA	NA	NA	NA	NA	12 (20)		
	Control	8 (13)	NA	NA	NA	NA	NA	NA	6 (10)		
Feagan *et al*[15]	Intervention	NA	35 (5)	13 (2)	NA	NA	NA	NA	77 (10)		
	Control	NA	16 (11)	36 (24)	NA	NA	NA	NA	37 (25)		
Parikh *et al*[16]	Intervention 1	NA	NA	0 (0)	0 (0)	0 (0)	1 (8)	0 (0)	1 (8)	2 (5)[2]	
	Intervention 2	NA	NA	2 (14)	2 (14)	2 (14)	0 (0)	0 (0)	0 (0)		
	Intervention 3	NA	NA	0 (0)	0 (0)	0 (0)	0 (0)	1 (9)	1 (9)		
	Control	NA	NA	0 (0)	0 (0)	0 (0)	1 (11)	1 (11)	0 (0)		

[1]Data reported as *n* (%); [2]Pooled of all intervention groups. NA: Not available.

1.76-4.19, $P < 0.001$).

Adverse event rate: A summary of general AEs and serious adverse events (SAEs) is shown in Table 3. The most common general AEs in all studies, and in both intervention and control groups was aggravation of UC, and the incidence in the intervention groups ranged from 0% to 50%, and in the control groups from 38% to 44%. Other common general AEs included nausea, headaches, fatigue, nasopharyngitis, and abdominal pain. A statistical analysis was only performed for SAEs. In the intervention groups, the frequency of SAEs ranged from 0% to 20%, and in the control groups ranged from 0% to 25%. There was evidence of significant heterogeneity when data from the studies were pooled ($Q = 9.07$, $df = 2$, $P = 0.011$, $I^2 = 77.94\%$); therefore, a random-effects model was used for analysis of SAEs (Figure 5). The overall analysis revealed the SAE rate was not significantly different for patients who received vedolizumab as compared with control patients ($P = 0.675$).

Sensitivity analysis

Figure 6 shows the results of the meta-analysis with one study removed-in-turn for the clinical response rate (pooled of all intervention groups); clinical remission rate; and SAE rate. For the clinical response rate (pooled of all intervention groups) and clinical remission rate, the direction and magnitude of the pooled estimate varied consistently, indicating good reliability. For the SAE rate, the pooled estimate was different when one study was left-out-in-turn, indicating poor reliability.

DISCUSSION

Summary of results

The results of this meta-analysis including three RCTs showed that the clinical response and remission rates were significantly higher for patients with UC treated with vedolizumab as compared to control patients, and SAEs were not more common in vedolizumab-treated patients than control patients.

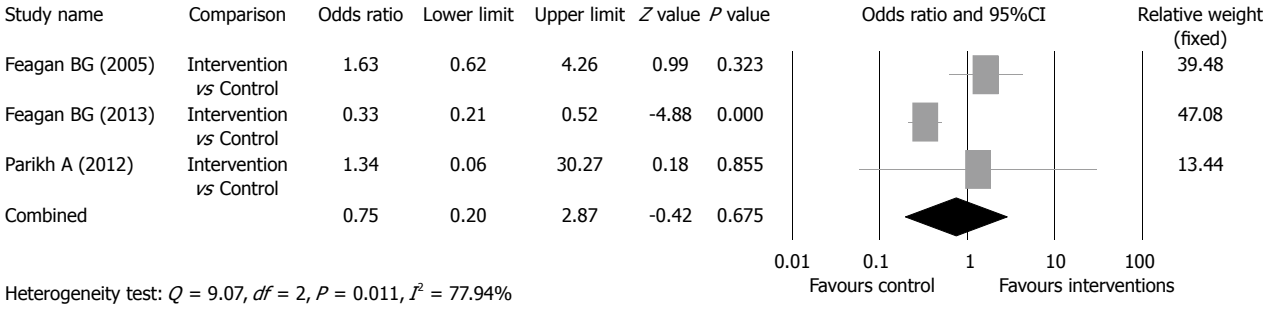

Figure 5 Forest plot of the meta-analysis of serious adverse events.

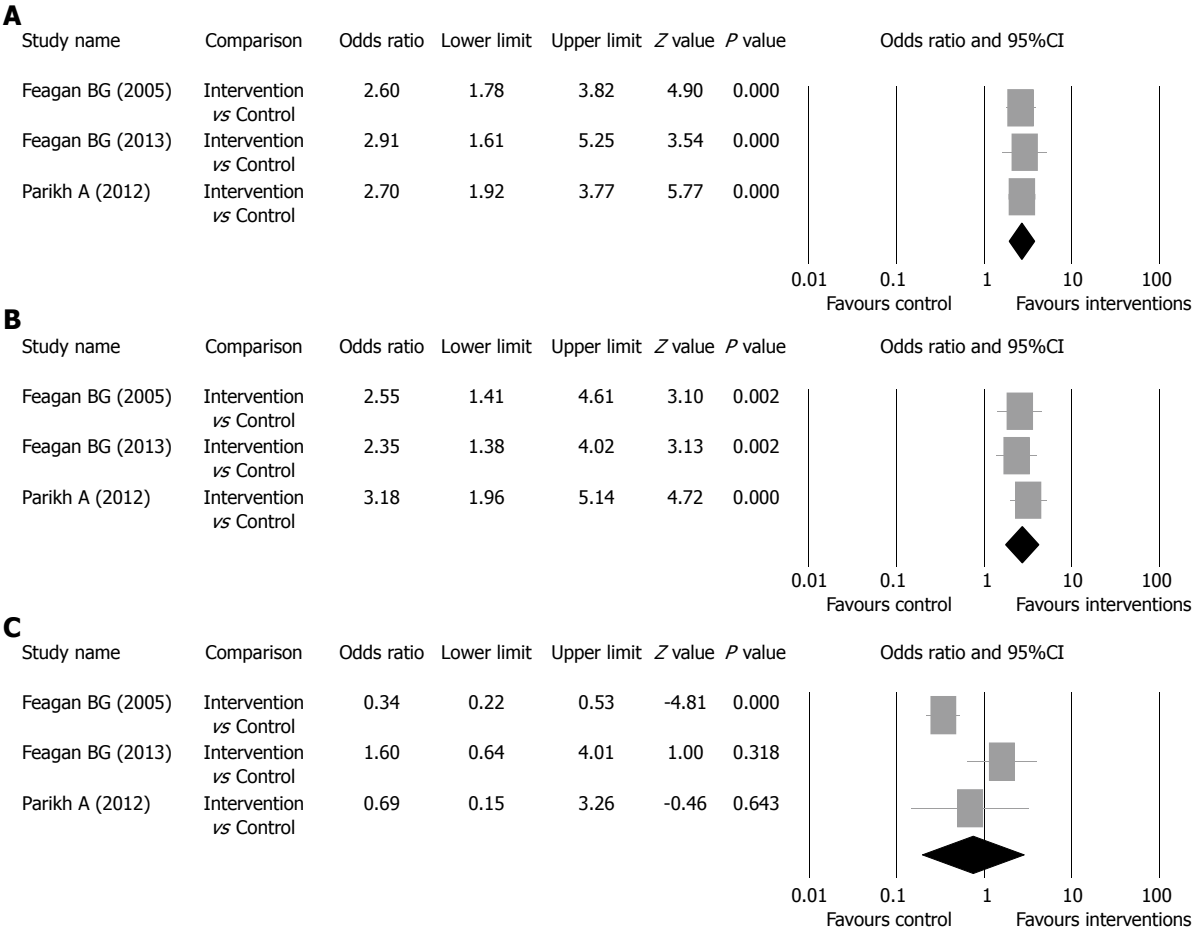

Figure 6 Sensitivity analysis for the influence of individual studies on pooled estimates as determined using the leave-one-out method. A: Clinical response rate; B: Clinical remission rate; C: Serious adverse event rate.

Explanations

UC is a relapsing disease that is difficult to treat, and a large percentage of patients are refractory to traditional medical management[1,2]. When medical treatment fails, the only recourse is colectomy. Since the discovery of integrins, a large amount of research has been devoted to elucidation of their functions, and they have been found to be viable therapeutic targets against thrombosis and inflammatory disease[6,7]. Because uncontrolled inflammation is the hallmark characteristic of UC, inflammatory mediators such as integrins have been investigated as therapeutic targets and shown promising results for the treatment of UC[4].

Integrin agonists block the lymphocyte-homing mechanism of T lymphocytes. In patients with IBD, there is recruitment of large numbers of T cells to the intestinal mucosa, and $\alpha4\beta7$ integrin, which is found on circulating T lymphocytes and is involved in their recruitment to the gastrointestinal tract[4,7]. The $\alpha4\beta7$ integrin is activated on the lymphocyte surface membrane, and binds with its glycosaminoglycan ligand [mucosal addressin-cell adhesion molecule-1 (MAdCAM-1)] located on the surface membrane of endothelial cells[9,17]. This binding results in lymphocytes migrating into the lamina propria and tissue, and subsequent inflammation[9,17]. Study has

shown that there are significantly higher levels of α4β7 integrin and MAdCAM-1 in the colons of IBD patients than patients with irritable bowel syndrome[18]. It has also been shown that there are lower numbers of t-lymphocytes with α4β7 integrin in the peripheral blood of patients with inflammation of the colon[19].

Initial studies which examined natalizumab, a humanized monoclonal antibody that inhibits α4 integrin, showed that it was effective in inducing remission in patients with CD[20,21]. However, studies suggested that its use in patients with CD receiving multidrug therapy was associated with the development of progressive multifocal leukoencephalopathy (PML)[22]. Unlike natalizumab, which inhibits both α4β1 and α4β7 integrin and thus affects multiple organs, vedolizumab is gut-specific[23,24], and no cases of PML have been reported with its use[25].

The initial phase Ⅱ trial of vedolizumab[14] showed that the drug was more effective than placebo for the induction of clinical and endoscopic remission in patients with active UC. Subsequent phase Ⅲ trials confirmed that vedolizumab was effective for the induction and maintenance of remission in patients with UC[15]. Parikh *et al*[16], also included in this meta-analysis, showed that a dose of vedolizumab up to 10 mg/kg body weight was well tolerated, and that more patients treated with vedolizumab achieved a clinical response as compared with those treated with placebo. Importantly, no significant safety issues, including significant infections, have been noted in any studies using vedolizumab to treat UC[14-16,25].

The aforementioned studies were relatively short-term. A longer-term phase Ⅲ study is currently being conducted in which patients who were involved in prior trials will have the option to enter a study in which they will receive vedolizumab every 4 wk for up to 100 wk[26].

While the three studies included in the meta-analysis did not all report the same general AEs, aggravation of UC as the most common AE was consistent between the studies as were other common AEs including nausea, headaches, fatigue, nasopharyngitis, and abdominal pain. These findings are consistent with those of other studies that have examined the use of vedolizumab and natalizumab for the treatment of UC[11,20,21].

Limitations

The primary limitation of this meta-analysis is the small number of RCTs available for inclusion. Also, the length of follow-up in the studies was not long enough to evaluate the effectiveness of clinical remission, and endoscopic outcomes were not evaluated in all studies. Thus, care should be used when interpreting the results of this meta-analysis.

In conclusion, the results of this meta-analysis showed that treatment with vedolizumab results in significantly higher clinical response and remission rates than placebo in patients with UC. Importantly, SAEs were not more common in vedolizumab-treated patients than control patients. This analysis supports the use of vedolizumab for the treatment of UC.

COMMENTS

Background

Inflammatory bowel disease (IBD) is an autoimmune disease in which the body's own immune system attacks elements of the digestive system. Ulcerative colitis (UC) is one form of IBD in which characteristic ulcers or open sores are restricted to the colonic mucosa. The main symptom of active disease is typically the gradual onset of constant diarrhea mixed with blood. To date there is no satisfactory treatment for UC, and in refractory cases colectomy is necessary.

Research frontiers

In patients with IBD, there is recruitment of large numbers of T cells to the intestinal mucosa, and alpha4beta7 (α4β7) integrin, which is found on circulating T lymphocytes, is involved in their recruitment to the gastrointestinal tract. Integrin antagonists are new class of agents that inhibit leukocyte adhesion and aim to selectively inhibit the inflammatory pathway by blocking the lymphocyte-homing mechanism of T lymphocytes.

Innovations and breakthroughs

Vedolizumab is a recombinant humanized IgG1 monoclonal antibody that inhibits adhesion and migration of leukocytes into the gastrointestinal tract by binding the integrin associated with their recruitment. Early animal and human studies have suggested that vedolizumab may be effective in reducing intestinal inflammation in patients with UC.

Applications

The initial phase Ⅱ trial of vedolizumab showed that the drug was more effective than placebo for the induction of clinical and endoscopic remission in patients with active UC. Subsequent phase Ⅲ trials confirmed that vedolizumab was effective for the induction and maintenance of remission in patients with UC. While the aforementioned studies were relatively short, a longer-term phase Ⅲ study is currently being conducted.

Terminology

Integrins are transmembrane receptors that mediate the attachment between a cell and its surroundings, and they interact with other proteins such as cadherins, immunoglobulin superfamily cell adhesion molecules, selectins, and syndecans to mediate cell-cell and cell-matrix interaction and communication. α4β7 integrin is found on circulating T lymphocytes, and is involved in the recruitment of leukocytes to the gastrointestinal tract. Integrin antagonists, which inhibit leukocyte adhesion, have shown promise in the treatment of UC.

Peer-review

The results of this meta-analysis showed that treatment with vedolizumab resulted in significantly higher clinical response and remission rates than placebo in patients with UC. Further, the authors found out that serious adverse events were not more common in vedolizumab-treated patients than control patients. The data, tables and figures are clear and easy to understand.

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META-ANALYSIS

Meta-analysis of subtotal stomach-preserving pancreaticoduodenectomy *vs* pylorus preserving pancreaticoduodenectomy

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Abstract

AIM: To investigate the differences in outcome following pylorus preserving pancreaticoduodenectomy (PPPD) and subtotal stomach-preserving pancreaticoduodenectomy (SSPPD).

METHODS: Major databases including PubMed (Medline), EMBASE and Science Citation Index Expanded and the Cochrane Central Register of Controlled Trials (CENTRAL) in The Cochrane Library were searched for comparative studies between patients with PPPD and SSPPD published between January 1978 and July 2014. Studies were selected based on specific inclusion and exclusion criteria. The primary outcome was delayed gastric emptying (DGE). Secondary outcomes included operation time, intraoperative blood loss, pancreatic fistula, postoperative hemorrhage, intraabdominal abscess, wound infection, time to starting liquid diet, time to starting solid diet, period of nasogastric intubation, reinsertion of nasogastric tube, mortality and hospital stay. The pooled odds ratios (OR) or weighted mean difference (WMD) with 95% confidence intervals (95%CI) were calculated using either a fixed-effects or random-effects model.

RESULTS: Eight comparative studies recruiting 650 patients were analyzed, which include two RCTs, one non-randomized prospective and 5 retrospective trial designs. Patients undergoing SSPPD experienced significantly lower rates of DGE (OR = 2.75; 95%CI: 1.75-4.30, $P < 0.00001$) and a shorter period of nasogastric intubation (OR = 2.68; 95%CI: 0.77-4.58,

$P < 0.00001$), with a tendency towards shorter time to liquid (WMD = 2.97, 95%CI: -0.46-7.83; $P = 0.09$) and solid diets (WMD = 3.69, 95%CI: -0.46-7.83; $P = 0.08$) as well as shorter inpatient stay (WMD = 3.92, 95%CI: -0.37-8.22; $P = 0.07$), although these latter three did not reach statistical significance. PPPD, however, was associated with less intraoperative blood loss than SSPPD [WMD = -217.70, 95%CI: -429.77-(-5.63); $P = 0.04$]. There were no differences in other parameters between the two approaches, including operative time (WMD = -5.30, 95%CI: -43.44-32.84; $P = 0.79$), pancreatic fistula (OR = 0.91; 95%CI: 0.56-1.49; $P = 0.70$), postoperative hemorrhage (OR = 0.51; 95%CI: 0.15-1.74; $P = 0.29$), intraabdominal abscess (OR = 1.05; 95%CI: 0.54-2.05; $P = 0.89$), wound infection (OR = 0.88; 95%CI: 0.39-1.97; $P = 0.75$), reinsertion of nasogastric tube (OR = 1.90; 95%CI: 0.91-3.97; $P = 0.09$) and mortality (OR = 0.31; 95%CI: 0.05-2.01; $P = 0.22$).

CONCLUSION: SSPPD may improve intraoperative and short-term postoperative outcomes compared to PPPD, especially DGE. However, these findings need to be further ascertained by well-designed randomized controlled trials.

Key words: Pancreaticoduodenectomy; Pylorus preserving Subtotal stomach preserving pancreaticoduodenectomy; Delayed gastric emptying; Pancreatic surgery; Meta-analysis

Core tip: As far as we know, pancreatoduodenectomy is one of the most complicated gastrointestinal operations and is associated with a number of serious postoperative complications. Modifications of standard operating techniques aim to reduce the incidence of complications and improve quality of life of patients while maintaining oncological effectiveness. Subtotal stomach-preserving pancreaticoduodenectomy (SSPPD) was specifically designed to reduce the incidence of delayed gastric emptying (DGE) and thus shorten recovery time in patients with pancreatic head and periampullary tumors. This study clarified that, compared to pylorus preserving pancreaticoduodenectomy (PPPD), SSPPD has a lower rate of DGE, shorter operation time and a shorter period of nasogastric intubation, albeit with no significant difference in pancreatic fistula and other postoperative complications. Therefore, SSPPD can improve intraoperative and short-term postoperative outcomes compared to PPPD for patients with pancreatic head and periampullary lesions.

Huang W, Xiong JJ, Wan MH, Szatmary P, Bharucha S, Gomatos I, Nunes QM, Xia Q, Sutton R, Liu XB. Meta-analysis of subtotal stomach-preserving pancreaticoduodenectomy *vs* pylorus preserving pancreaticoduodenectomy. *World J Gastroenterol* 2015; 21(20): 6361-6373 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6361.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6361

INTRODUCTION

In 1935, Whipple introduced a two-stage pancreaticoduodenectomy for patients with carcinoma of the ampulla of Vater[1]. In 1941, he reported a one-stage pancreaticoduodenectomy with resection of distal stomach and duodenum[2]. Soon afterwards, the first pylorus preserving pancreaticoduodenectomy (PPPD) was performed by Watson[3] in 1944. PPPD was also used by Traverso and Longmire[4] to preserve gastrointestinal function in 1978 and since then this procedure has been extensively applied to patients with tumors of the pancreatic head as well as periampullary malignancies.

Classic Whipple's and PPPD are now considered to be the most widely employed surgical procedures for the treatment of pancreatic head and periampullary tumors[5-8]. Whereas a classic Whipple's procedure includes resection of the pancreatic head, duodenum, gallbladder, distal common bile duct, partial jejunum and distal stomach, in a PPPD the proximal duodenum is transected 3 to 4 cm distal to the pylorus ring. While some randomized controlled trials (RCTs) and meta-analyses suggest that these two procedures are comparable in terms of postoperative complications, long-term survival rates and quality of life[6-9], other studies have reported that PPPD is superior to pancreaticoduodenectomy with antrectomy as it results in a reduced occurrence of dumping, diarrhea and bile reflux gastritis, thereby possibly affording patients with an improved nutritional status[10-12].

Delayed gastric emptying (DGE) is regarded as one of the most common postoperative complications of PPPD. This can potentially prolong the hospital stay, affecting patient quality of life and increasing hospital costs[12-16]. Decreasing the occurrence of DGE, therefore, is of particular importance in patients undergoing any type of pancreaticoduodenectomy. Subtotal stomach-preserving pancreaticoduodenectomy (SSPPD) was initially described during the 1990s in Japan. This procedure was intended to preserve the pooling ability of the stomach and minimize the occurrence of DGE[17,18]. It involves division of the stomach 2-3 cm proximal to the pylorus ring with resection of the entire duodenum distal to the site of transection, thereby removing the pylorus but retaining much of the body of the stomach compared to a classical Whipple's procedure. The rate of postoperative DGE after pancreaticoduodenectomy is controversial and whether SSPPD is able to reduce it and other postoperative complications compared to PPPD remains to be elucidated[19]. We, therefore, carried out a systematic review of the literature to investigate

this issue.

MATERIALS AND METHODS

Study selection

Major databases like PubMed (Medline), EMBASE and Science Citation Index Expanded and Cochrane Central Register of Controlled Trials (CENTRAL) in The Cochrane Library were searched for studies comparing SSPPD with PPPD from January 1978 to July 2014. The following medical search headings (MeSH) were used: "pancreaticoduodenectomy", "pancreatoduodenectomy", "Whipple", "pancreatoduodenal resection", "pylorus preserving pancreaticoduodenectomy", "PPPD", "subtotal stomach preserving pancreaticoduodenectomy", "SSPPD", "delayed gastric emptying" "pancreatic surgery", "comparative study" and combinations of them were used for word searches. References cited in the selected articles were also assessed to identify relevant studies in case studies were missed during the initial database searches. If needed, investigators and experts in the field of pancreatic surgery were contacted to ensure that all relevant studies were identified. Final inclusion of articles was determined by consensus of two researchers; when this failed, a third author adjudicated.

Inclusion and exclusion criteria

Two authors scrutinized potentially eligible studies using the following inclusion criteria: (1) English language full-text articles published in peer-reviewed journals; (2) human clinical trials comparing "PPPD" and "SSPPD"; (3) studies where DGE was mentioned; and (4) where multiple studies came from the same institute and/or authors using the same patient cohorts, the higher quality study was included in the analysis.

Studies were excluded if any of the following conditions existed: (1) abstracts, case reports, letters, editorials, expert opinions and reviews; (2) primary postoperative outcome unavailable; and (3) studies focused on long-term outcomes.

Outcomes of interest

DGE was the primary outcome of interest. Secondary outcomes including operation time, intraoperative blood loss, pancreatic fistula, postoperative hemorrhage, intraabdominal abscess, wound infection, time to starting liquid diet, time to starting solid diet, period of nasogastric intubation, reinsertion of nasogastric tube, mortality and hospital stay were also compared.

Data extraction and quality assessment

Data were collected by two independent researchers using standardized proformas and included: Study characteristics, surgical reconstructions, definition of DGE and postoperative management. Means of the outcomes were used for meta-analysis if not otherwise mentioned. If medians were used in some studies instead of means, the means were estimated using the following formula: (low end of range + median*2 + high end of range)/4 for a sample size smaller than 25. For a sample size greater than 25, means were estimated as the medians. When only a range was given, the standard deviations were estimated as range/4[20].

The qualitative assessment of the RCTs was based on the Jadad scoring system[21] which took into consideration the randomization and double blinding process and the description of withdrawals or dropouts. Note was also made of sample size calculation, sequence generation, allocation concealment and the definitions of outcome parameters. The non-randomized trials were assessed on the basis of the method described by McKay *et al*[22] which included assessment of the following parameters: prospective *vs* retrospective data collection; assignment to the PPPD group or the SSPPD group by means other than the surgeon's preference; and an explicit definition of DGE (studies were given a score of 1 for each of these areas; score 1-4).

Statistical analysis

Meta-analysis was performed using Review Manager Version 5.0 software (The Cochrane Collaboration, Oxford, United Kingdom). Continuous variables and categorical variables were expressed as weighted mean difference (WMD) and odds ratio (OR) with their respective corresponding 95% confidence interval (CI). Chi-square test was used to assess heterogeneity and a $P < 0.1$ was considered significant. I^2 values were used for the evaluation of statistical heterogeneity: an I^2 value of 50% or more was indicative of presence of heterogeneity[23]. The fixed-effects model was initially used for all outcomes[24], while the random-effects model was used if the test rejected the assumption of homogeneity of studies[25]. Descriptive methods were also used if the data were considered to be inappropriate for meta-analysis. Sensitivity analyses were performed by removing individual studies from the data set and analyzing the effect on the overall results, identifying sources of significant heterogeneity. Subgroup analyses were undertaken by including studies with the International Study Group of Pancreatic Surgery (ISGPS) definitions, reconstruction with pancreaticojejunostomy or pancreaticogastrostomy, RCTs or non-RCTs, and D1 or D2 lymph node dissection. Funnel plots[26] were constructed to evaluate potential publication bias based on the primary outcome - DGE.

RESULTS

Description of included trials in the meta-analysis

The search strategy initially identified 148 relevant

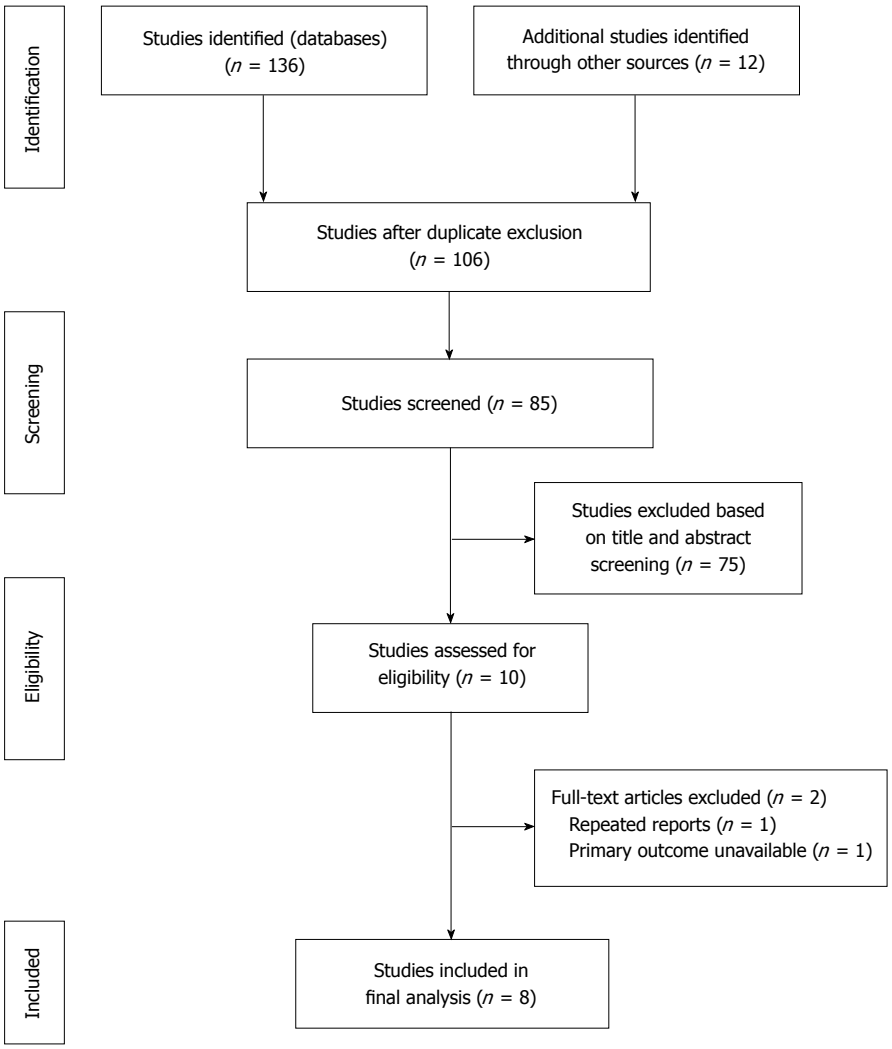


Figure 1 Flow diagram depicting the study selection process in accordance with PRISMA guidelines.

clinical trials. After filtering the studies using the inclusion criteria, ten studies[17,27-35] with full-text were identified to investigate the details. Of these, two studies[32,34] were excluded: one study[34] focused on the long-time outcomes of the same cohorts reported by their previous study; another study[32] had no data available. Finally, eight studies were identified for inclusion: two RCTs[29,35], one prospective non-randomized trials[27] and five retrospective studies[17,28,30,31,33]. Pancreaticojejunostomy was used in 6 studies[17,27,29,31,33,35], while pancreaticogastrostomy was used in two studies[28,30]. A total of 650 patients were included: 294 and 356 patients in the PPPD and SSPPD groups respectively. The ISGPS definition of DGE was used in six studies[28-31,33,35]. Indications for removal of nasogastric tube were reported in six studies[27-29,31,33,35]. All the included studies were from Japan. Figure 1 shows the process of selecting comparative studies included in our meta-analysis. The study characteristics and quality assessments are shown in Table 1. The surgical reconstruction, definition of DGE and postoperative management are listed in Table 2.

Results of meta-analysis

Results of the analyses are shown in Figures 2 and 3 and summarized in Table 3.

All included studies reported the occurrence rate of DGE. Patients in the SSPPD group had a lower incidence of DGE compared to those in the PPPD group (OR = 2.75, 95%CI: 1.75-4.30; $P < 0.00001$) and the period of nasogastric intubation was also shorter in the SSPPD group (WMD = 2.68, 95%CI: 0.77-4.58; $P = 0.006$). Furthermore, there was a tendency towards shorter time to liquid (WMD = 2.97, 95%CI: -0.46-7.83; $P = 0.09$) and solid diets (WMD = 3.69, 95%CI: -0.46-7.83; $P = 0.08$) as well as shorter hospital stay (WMD = 3.92, 95%CI: -0.37-8.22; $P = 0.07$), although the latter three did not reach statistical significance. PPPD was, however, associated with less intraoperative blood loss compared to SSPPD (WMD = -217.70, 95%CI: -429.77-(-5.63); $P = 0.04$). There were no differences in operating time (WMD = -5.30, 95%CI: -43.44-32.84; $P = 0.79$) or outcomes such as pancreatic fistula (OR = 0.91; 95%CI: 0.56-1.49; $P = 0.70$), postoperative hemorrhage (OR = 0.51; 95%CI: 0.15-1.74; $P = 0.29$), intraabdominal abscess (OR =

Table 1 Characteristics of the included studies

Ref.	Country	Year	Design	Group	*n*	Sex (M/F)	Age[1]	Benign/ malignant	Quality score
Hayashibe *et al*[17]	Japan	2007	Retro	PPPD	12	4/8	60.9 ± 8.5	0/12	1 (McKay)
				SSPPD	21	8/13	64.3 ± 9.5	1/20	
Akizuki *et al*[27]	Japan	2008	PNR	PPPD	34	20/14 18/12	66 (28-78)	15/19	2 (McKay)
				SSPPD	30		65 (39-79)	4/26	
Kurahara *et al*[28]	Japan	2010	Retro	PPPD	48	26/22 38/26	64.4	18/30	1 (McKay)
				SSPPD	64		66.8	11/53	
Oida *et al*[30]	Japan	2011	Retro	PPPD	25	21/4	66.2 ± 4.7	0/25	1 (McKay)
				SSPPD	42	30/12	65.8 ± 5.8	0/42	
Kawai *et al*[29]	Japan	2011	RCT	PPPD	64	33/31 38/28	68 ± 9	12/52	3 (Jadad)
				SSPPD	66		67 ± 9	14/52	
Fujii *et al*[31]	Japan	2012	Retro	PPPD	33	19/14	63.8 (35-83)	0/33	1 (McKay)
				SSPPD	56	28/28	64.6 (41-84)	0/56	
Nanashima *et al*[33]	Japan	2013	Retro	PPPD	28	21/7	68 ± 8	7/21	1 (McKay)
				SSPPD	27	15/12	66 ± 12	5/22	
Matsumoto *et al*[35]	Japan	2014	RCT	PPPD	50	29/21 35/15	66 ± 10	18/32	3 (Jadad)
				SSPPD	50		67 ± 9	21/29	

[1]Mean ± SD, standard deviation or Median and range. Retro: Retrospective observational study; PNR: Prospective nonrandomized observational study; RCT: Randomized controlled trial; SSPPD: Subtotal stomach-preserving pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy.

Table 2 Surgical reconstruction, definition of delayed gastric emptying and postoperative management

Ref.	Reconstruction	Definition of DGE	Indication for removing NGT	PPI	PA
Hayashibe *et al*[17]	Duct to mucosa and end-to-side pancreaticojejunostomy, end-to-side antecolic gastrojejunostomy, and side-to-side jejunojejunostomy (Braun anastomosis)	(1) NGT ⩾ POD 10 (2) inability to tolerate a solid diet ⩾ POD 14	Unknown	Unknown	Unknown
Akizuki *et al*[27]	Duct-to-mucosa and end-to-side pancreaticojejunostomy, end duodenal (or stomach)-to-side jejunal, Braun anastomosis was made	(1) NGT ⩾ POD 10 (2) inability to tolerate a solid diet ⩾ POD 14	The fluid of NGT < 500 mL per night, generally removed on POD 1	Yes	Yes
Kurahara *et al*[28]	End-to-side pancreaticogastrostomy with an internal stent, end-to-side duodenojejunostomy (PPPD) or gastrojejunostomy (SSPPD)	ISGPS	The fluid of NGT < 500 mL per night	Yes	Yes
Oida *et al*[30]	Pancreaticogastrostomy and end to end duodenojejunostomy (PPPD) Pancreaticogastrostomy and end to end gastrojejunostomy (SSPPD)	ISGPS	Unknown	Unknown	Unknown
Kawai *et al*[29]	Duct-to-mucosa, end-to-side pancreatojejunostomy with internal stent Duodenojejunostomy (PPPD), gastrojejunostomy (SSPPD)	ISGPS	All removed on POD 1	No	No
Fujii *et al*[31]	End-to-side pancreatojejunostomy and end-to-side antecolic gastrojejunostomy in SSPPD or a duodenojejunostomy in PPPD	ISGPS	Generally removed on POD 1, or on POD 2 if the fluid of NGT > 500 mL per night	Unknown	Unknown
Nanashima *et al*[33]	End-to-side with external stent pancreatojejunostomy	ISGPS	The fluid of the NGT < 300 mL per night	Yes	Unknown
Matsumoto *et al*[35]	End-to-side pancreatojejunostomy, end-to-side duodenojejunostomy (PPPD) and gastrojejunstomy (SSPPD)	ISGPS	The fluid of the NGT < 200 mL per night	Yes	Unknown

ISGPS: International Study Group on Pancreatic Surgery; SSPPD: Subtotal stomach-preserving pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; POD: Postoperative day; DGE: Delayed gastric emptying; NGT: Nasogastric tube; SSA: Somatostatin analogues; PPI: Proton pump inhibitors; PA: Prokinetic agents.

1.05; 95%CI: 0.54-2.05; P = 0.89), wound infection (OR = 0.88; 95%CI: 0.39-1.97; P = 0.75), reinsertion of nasogastric tube (OR = 1.90; 95%CI: 0.91-3.97; P = 0.09), mortality (OR = 0.31; 95%CI: 0.05-2.01; P = 0.22) and hospital stay.

Sensitivity and subgroup analysis

Sensitivity analyses were carried out by excluding each study out of each outcome measure. These exclusions did not alter the results obtained from cumulative analyses. The subgroup analyses were undertaken for all outcome measures by including studies with ISGPS definition or other definition, studies with pancreaticogastrostomy or pancreaticojejunostomy, RCTs or non-randomized trials, and D1 or D2 lymph node dissection. Results of the analyses are also

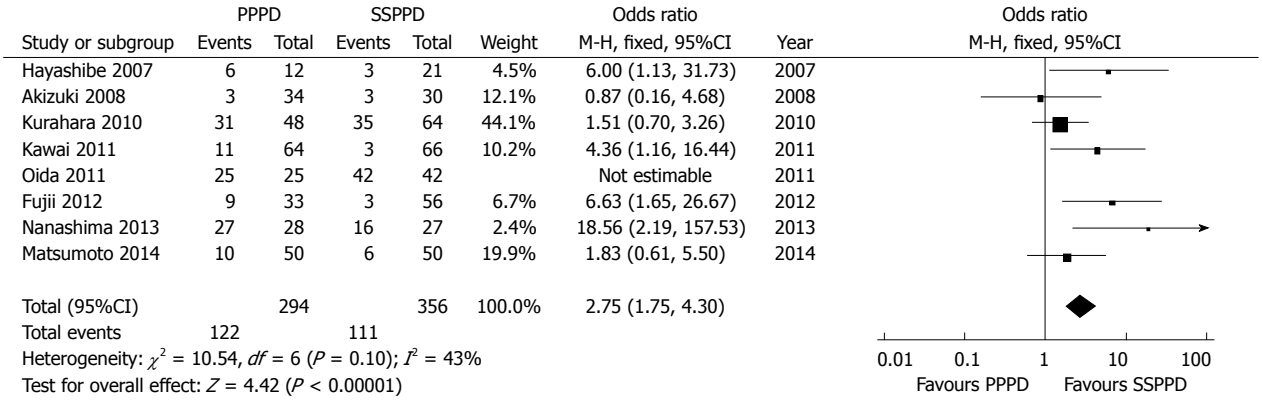


Figure 2 Forest plots demonstrating primary outcome. Forest plots illustrating results of delayed gastric emptying in the form of meta-analysis comparing PPPD with SSPPD. Pooled odds ratios (ORs) with 95% confidence intervals (CI) were calculated using the fixed-effects model. PPPD: Pylorus preserving pancreaticoduodenectomy; SSPPD: Subtotal stomach-preserving pancreaticoduodenectomy.

Table 3 Summary results for studies comparing pylorus-preserving pancreaticoduodenectomy and subtotal stomach-preserving pancreaticoduodenectomy

Outcome of interest	No. of studies	No. of patients	OR/WMD = (95%CI)	*P* value	Heterogeneity *P* value	I^2
Delayed gastric emptying	8	650	2.75 (1.75-4.30)	< 0.00001	0.10	43%
Operation time	8	650	-5.30 (-43.44-32.84)	0.79	0.0003	77%
Intraoperative blood loss	8	650	-217.70 [-429.77-(-5.63)]	0.04	0.004	68%
Pancreatic fistula	7	583	0.91 (0.56-1.49)	0.7	0.97	0%
Postoperative hemorrhage	5	461	0.51 (0.15-1.74)	0.29	0.95	0%
Intra-abdominal abscess	5	461	1.05 (0.54-2.05)	0.89	0.65	0%
Wound infection	5	394	0.88 (0.39-1.97)	0.75	0.88	0%
Time of start liquid diet	4	286	2.97 (-0.43-6.38)	0.09	0.001	82%
Time of start solid diet	4	316	3.69 (-0.46-7.83)	0.08	< 0.00001	91%
Time of nasogastric intubation	6	438	2.68 (0.77-4.58)	0.006	< 0.00001	96%
Reinsertion of nasogastric tube	4	349	1.90 (0.91-3.97)	0.09	0.58	0%
Mortality	6	471	0.31 (0.05-2.01)	0.22	1.00	0%
Hospital stay	4	255	3.92 (-0.37-8.22)	0.07	0.04	64%

WMD: Weight mean difference.

summarized in Table 4. The rate of DGE was also shown to be lower in the studies using the ISGPS definition (OR = 8.73; 95%CI: 2.09-36.56; *P* = 0.003), pancreaticojejunostomy (OR = 3.72; 95%CI: 2.12-6.56; *P* < 0.00001), RCTs (OR = 2.69; 95%CI: 1.17-6.17; *P* = 0.02) or non-RCTs (OR = 3.36; 95%CI: 1.25-9.08; *P* = 0.02).

Publication bias

The funnel plot based on the incidence of DGE is shown in Figure 4. None of the studies lies outside the limits of the 95%CI, indicating there was no evidence of publication bias.

DISCUSSION

Physiologically, gastric emptying requires coordination of the gastric antrum, pylorus and duodenum through paracrine messages and extrinsic stimulation from the vagus nerve[36]. DGE is one of the most common complications after pancreaticoduodenectomy and has been reported to occur in 1%-6% of patients[37]. While this is not a life-threatening complication of pancreatic surgery, it results in a reduced quality of life, impaired oral intake, increased hospital costs and the delayed initiation of adjuvant chemotherapy, where required. The pathogenesis of DGE after PPPD is thought to involve a number of factors, such as: gastric atony caused by vagotomy[38]; pylorospasm[39,40]; ischemia of the pylorus ring due to division of the right gastric artery[41]; congestion around the pylorus ring due to division of the left gastric vein[42]; and gastric dysrhythmia secondary to other complications such as pancreatic fisecon[43,44]. SSPPD was introduced in recent years as an alternative to PPPD to maintain the pooling ability of the stomach and reduce the incidence of DGE[17] by retaining most of the gastric body but resecting the pyloric complex itself. Whereas in a PPPD the proximal duodenum is divided 3 to 4 cm distal to the pylorus ring, in an SSPPD more than 95% of the stomach is preserved.

This meta-analysis of two RCTs and six non-randomized trials (prospective and retrospective) revealed a significant benefit of SSPPD compared with

Table 4 Sensitivity analysis performed for studies comparing pylorus-preserving pancreaticoduodenectomy and subtotal stomach-preserving pancreaticoduodenectomy

Outcome of interest	No. of studies	No. of patients	OR (95%CI)	*P* value	Heterogeneity *P* value	I^2
DGE with ISGPS definition						
DGE (ISGPS B/C)	6	553	8.73 (2.09-36.56)	0.003	< 0.0001	82%
Operation time	6	553	6.50 (-35.02-48.02)	0.76	0.0005	80%
Intraoperative blood loss	6	553	0.78 (-81.90-83.46)	0.99	0.20	33%
Pancreatic fistula	5	486	0.92 (0.54-1.55)	0.75	0.93	0%
Postoperative hemorrhage	4	397	0.57 (0.15-2.16)	0.41	0.91	0%
Intraabdominal abscess	4	397	1.07 (0.53-2.18)	0.85	0.49	0%
Wound infection	3	297	1.18 (0.45-3.09)	0.74	0.98	0%
Time of start liquid diet	2	189	3.62 (-2.82-10.06)	0.27	0.03	80%
Time of start solid diet	3	252	4.77 (-0.11-9.64)	0.06	< 0.00001	94%
Time of nasogastric intubation	4	341	3.32 (0.31-6.32)	0.03	< 0.00001	97%
Reinsertion of nasogastric tube	3	285	2.15 (0.93-4.96)	0.07	0.44	0%
Mortality	4	374	0.32 (0.03-3.18)	0.33	0.97	0%
Hospital stay	3	222	5.96 (3.46-8.46)	< 0.00001	0.55	0%
DGE with other definition						
DGE	2	97	2.29 (0.35-15.21)	0.39	0.11	61%
Operation time	2	97	-48.51 (-109. 22-12.20)	0.12	0.79	0%
Intraoperative blood loss	2	97	-588.29 [-898.10-(-278.49)]	0.0002	0.79	0%
Pancreatic fistula	2	97	0.86 (0.22-3.33)	0.83	-	-
Wound infection	2	97	0.44 (0.09-2.08)	0.30	0.88	0%
Time of start liquid diet	2	97	2.30 (-2.30-6.91)	0.33	0.006	87%
Time of NGD insertion	2	97	1.82 (-2.43-6.06)	0.40	0.02	
Mortality	2	97	0.29 (0.01-7.26)	0.45	-	-
Hospital stay	2	97	0.17 (-2.77-3.11)	0.91	0.24	27%
Reconstruction with pancreaticojejunostomy						
DGE	6	471	3.72 (2.12-6.56)	< 0.00001	0.18	35%
Operation time	6	471	-11.79 (-32.26-8.68)	0.26	0.21	30%
Intraoperative blood loss	6	471	-265.87 (-422.93-108.81)	0.0009	0.15	38%
Pancreatic fistula	6	471	0.90 (0.55-1.48)	0.67	0.94	0%
Postoperative hemorrhage	4	349	0.61 (0.16-2.38)	0.48	0.93	0%
Intra-abdominal abscess	4	349	1.06 (0.51-2.22)	0.88	0.48	0%
Wound infection	4	327	0.83 (0.34-2.03)	0.68	0.78	0%
Time of start solid diet	3	249	2.29 (-1.97-6.54)	0.29	0.06	64%
Time of nasogastric intubation	5	371	1.92 (0.38-3.47)	0.01	< 0.00001	89%
Hospital stay	3	188	1.23 (-1.64-4.09)	0.40	0.25	28%
Reconstruction with pancreaticogastrostomy						
DGE	2	179	1.51 (0.70-3.26)	0.29	-	-
Operation time	2	179	49.00 (30.41-67.59)	< 0.00001	-	-
Intraoperative blood loss	2	179	41.00 (-51.78-133.78)	0.39	-	-
Randomized controlled trial						
DGE	2	230	2.69 (1.17-6.17)	0.02	0.32	0%
Operation time	2	230	-16.69 (-42.68-9.30)	0.21	0.83	0%
Intraoperative blood loss	2	230	-120.23 (-364.94-124.48)	0.34	0.77	0%
Pancreatic fistula	2	230	1.08 (0.53-2.20)	0.82	0.91	0%
Postoperative hemorrhage	2	230	0.67 (0.11-4.11)	0.67	0.72	0%
Intra-abdominal abscess	2	230	0.75 (0.14-4.14)	0.75	0.15	51%
Wound infection	2	230	1.19 (0.38-3.70)	0.76	0.86	0%
Reinsertion of nasogastric tube	2	230	2.17 (0.84-5.59)	0.11	0.20	39%
Mortality	2	230	0.34 (0.01-8.46)	0.51	-	-
Non-randomized controlled trial						
DGE	6	420	3.36 (1.25-9.08)	0.02	0.05	58%
Operation time	6	420	-0.56 (-47.55-46.43)	0.98	0.007	72%
Intraoperative blood loss	6	420	-283.77 (-593.42-25.87)	0.07	0.001	78%
Pancreatic fistula	5	353	0.77 (0.39-1.53)	0.46	0.93	0%
Postoperative hemorrhage	3	231	0.41 (0.08-2.22)	0.30	0.78	0%
Intra-abdominal abscess	3	231	1.21 (0.47-3.13)	0.70	0.83	0%
Wound infection	3	164	0.64 (0.19-2.08)	0.45	0.73	0%
Time of start liquid diet	3	186	3.68 (-0.30, 7.66)	0.07	0.0006	87%
Time of start solid diet	3	186	5.06 (0.39-9.73)	0.03	0.04	69%
Time of nasogastric intubation	5	308	3.49 (0.82-6.16)	0.01	< 0.00001	95%
Hospital stay	3	155	4.28 (-0.56-9.12)	0.08	0.02	76%
Mortality	4	240	0.30 (0.03-2.94)	0.30	0.97	0%

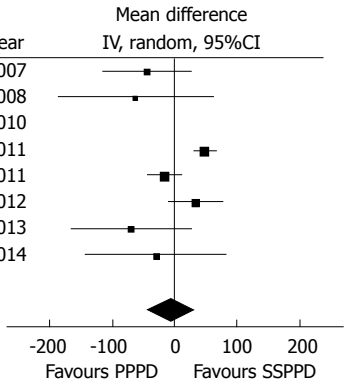
D1 lymph node dissection						
DGE	2	57	0.32 (0.07-1.48)	0.14	0.90	0%
D2 lymph node dissection						
DGE	2	119	3.07 (1.05-9.02)	0.04	0.53	0%

SSPPD: Subtotal stomach-preserving pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy; PD: Pancreaticoduodenectomy; WMD: Weighted mean difference.

A

	PPPD			SSPPD				Mean difference	
Study or subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, random, 95%CI	Year
Hayashibe 2007	450.5	63.8	12	494.5	138.9	21	13.4%	-44.00 (-113.51, 25.51)	2007
Akizuki 2008	457	236.3	34	520	268.3	30	6.7%	-63.00 (-187.60, 61.60)	2008
Kurahara 2010	460.6	0	48	545	0	64		Not estimable	2010
Oida 2011	464	39	25	415	35	42	22.8%	49.00 (30.41, 67.59)	2011
Kawai 2011	342	71	64	358	84	66	21.5%	-16.00 (-42.71, 10.71)	2011
Fujii 2012	488	98	33	455	107	56	18.3%	33.00 (-10.63, 76.63)	2012
Nanashima 2013	540	148	28	610	209	27	9.5%	-70.00 (-166.02, 26.02)	2013
Matsumoto 2014	523	285.3	50	552	290.3	50	7.7%	-29.00 (-141.82, 83.82)	2014
Total (95%CI)			294			356	100.0%	-5.30 (-43.44, 32.84)	

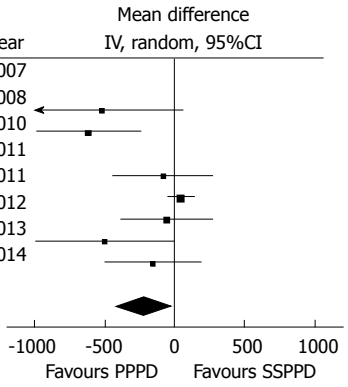
Heterogeneity: Tau^2 = 1573.21; χ^2 = 25.68, *df* = 6 (*P* = 0.0003); I^2 = 77%
Test for overall effect: *Z* = 0.27 (*P* = 0.79)



B

	PPPD			SSPPD				Mean difference	
Study or subgroup	Mean	SD	Total	Mean	SD	Total	Weight	IV, random, 95%CI	Year
Hayashibe 2007	1321.7	682	12	1843.2	987.7	21	8.7%	-521.50 (-1093.65, 50.65)	2007
Akizuki 2008	619	565	34	1235	882.5	30	13.8%	-616.00 (-984.50, -247.50)	2008
Kurahara 2010	1084.6	0	48	1779.5	0	64		Not estimable	2010
Kawai 2011	820	987	64	902	1075	66	14.3%	-82.00 (-436.59, 272.59)	2011
Oida 2011	600	196	25	559	172	42	22.7%	41.00 (-51.78, 133.78)	2011
Fujii 2012	1225	728	33	1284	821	56	15.1%	-59.00 (-387.53, 269.53)	2012
Nanashima 2013	1306	776	28	1810	1054	27	10.5%	-504.00 (-994.58, -13.42)	2013
Matsumoto 2014	826	645.8	50	981	1035	50	14.8%	-155.00 (-493.15, 183.15)	2014
Total (95%CI)			294			356	100.0%	-217.70 (-429.77, -5.63)	

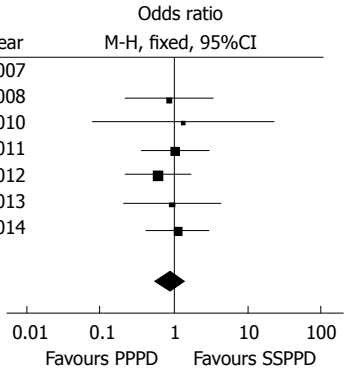
Heterogeneity: Tau^2 = 49231.24; χ^2 = 18.98, *df* = 6 (*P* = 0.004); I^2 = 68%
Test for overall effect: *Z* = 2.01 (*P* = 0.04)



C

	PPPD		SSPPD			Odds ratio	
Study or subgroup	Events	Total	Events	Total	Weight	M-H, fixed, 95%CI	Year
Hayashibe 2007	0	12	0	21		Not estimable	2007
Akizuki 2008	5	34	5	30	13.5%	0.86 (0.22, 3.33)	2008
Kurahara 2010	1	48	1	64	2.5%	1.34 (0.08, 21.99)	2010
Kawai 2011	8	64	8	66	20.6%	1.04 (0.36, 2.95)	2011
Fujii 2012	7	33	17	56	29.7%	0.62 (0.22, 1.70)	2012
Nanashima 2013	4	28	4	27	10.4%	0.96 (0.21, 4.29)	2013
Matsumoto 2014	11	50	10	50	23.3%	1.13 (0.43, 2.96)	2014
Total (95%CI)		269		314	100.0%	0.91 (0.56, 1.49)	
Total events	36		45				

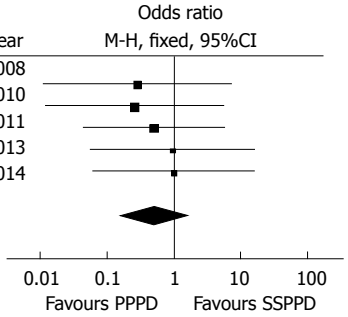
Heterogeneity: χ^2 = 0.90, *df* = 5 (*P* = 0.97); I^2 = 0%
Test for overall effect: *Z* = 0.38 (*P* = 0.70)



D

	PPPD		SSPPD			Odds ratio	
Study or subgroup	Events	Total	Events	Total	Weight	M-H, fixed, 95%CI	Year
Akizuki 2008	0	34	1	30	20.6%	0.29 (0.01, 7.26)	2008
Kurahara 2010	0	48	2	64	28.0%	0.26 (0.01, 5.49)	2010
Kawai 2011	1	64	2	66	25.5%	0.51 (0.04, 5.74)	2011
Nanashima 2013	1	28	1	27	12.9%	0.96 (0.06, 16.21)	2013
Matsumoto 2014	1	50	1	50	12.9%	1.00 (0.06, 16.44)	2014
Total (95%CI)		224		237	100.0%	0.51 (0.15, 1.74)	
Total events	3		7				

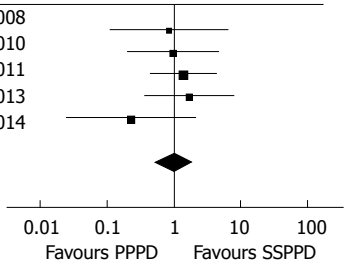
Heterogeneity: χ^2 = 0.73, *df* = 4 (*P* = 0.95); I^2 = 0%
Test for overall effect: *Z* = 1.07 (*P* = 0.29)



E

Study or subgroup	PPPD Events	PPPD Total	SSPPD Events	SSPPD Total	Weight	Odds ratio M-H, fixed, 95%CI	Year	Odds ratio M-H, fixed, 95%CI
Akizuki 2008	2	34	2	30	11.9%	0.88 (0.12, 6.63)	2008	
Kurahara 2010	3	48	4	64	19.1%	1.00 (0.21, 4.69)	2010	
Kawai 2011	8	64	6	66	30.7%	1.43 (0.47, 4.38)	2011	
Nanashima 2013	5	28	3	27	14.9%	1.74 (0.37, 8.12)	2013	
Matsumoto 2014	1	50	4	50	23.3%	0.23 (0.03, 2.18)	2014	
Total (95%CI)		224		237	100.0%	1.05 (0.54, 2.05)		
Total events	19		19					

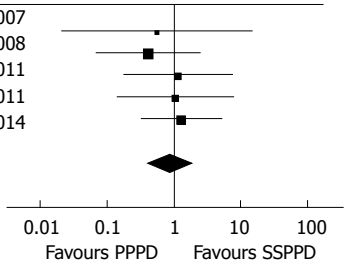
Heterogeneity: χ^2 = 2.48, *df* = 4 (*P* = 0.65); I^2 = 0%
Test for overall effect: *Z* = 0.14 (*P* = 0.89)



F

Study or subgroup	PPPD Events	PPPD Total	SSPPD Events	SSPPD Total	Weight	Odds ratio M-H, fixed, 95%CI	Year	Odds ratio M-H, fixed, 95%CI
Hayashibe 2007	0	12	1	21	8.5%	0.55 (0.02, 14.48)	2007	
Akizuki 2008	2	34	4	30	31.6%	0.41 (0.07, 2.40)	2008	
Oida 2011	2	25	3	42	16.3%	1.13 (0.18, 7.28)	2011	
Kawai 2011	2	64	2	66	15.1%	1.03 (0.14, 7.56)	2011	
Matsumoto 2014	5	50	4	50	28.5%	1.28 (0.32, 5.07)	2014	
Total (95%CI)		185		209	100.0%	0.88 (0.39, 1.97)		
Total events	11		14					

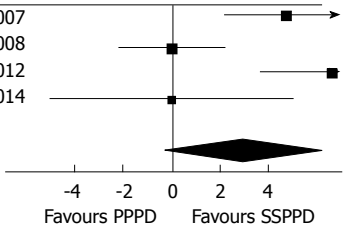
Heterogeneity: χ^2 = 1.19, *df* = 4 (*P* = 0.88); I^2 = 0%
Test for overall effect: *Z* = 0.31 (*P* = 0.75)



G

Study or subgroup	PPPD Mean	PPPD SD	PPPD Total	SSPPD Mean	SSPPD SD	SSPPD Total	Weight	Mean difference IV, random, 95%CI	Year	Mean difference IV, random, 95%CI
Hayashibe 2007	14.7	4.2	12	10	2	21	27.1%	4.70 (2.17, 7.23)	2007	
Akizuki 2008	5	4.8	34	5	4	30	28.2%	0.00 (-2.16, 2.16)	2008	
Fujii 2012	15.2	7.8	33	8.6	4.7	56	25.8%	6.60 (3.67, 9.53)	2012	
Matsumoto 2014	5	11.3	50	5	14	50	18.9%	0.00 (-4.99, 4.99)	2014	
Total (95%CI)			129			157	100.0%	2.97 (-0.43, 6.38)		

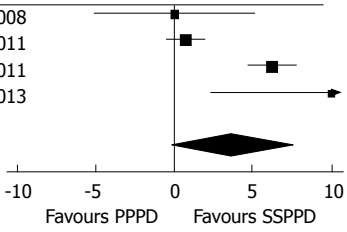
Heterogeneity: Tau^2 = 9.47; χ^2 = 16.31, *df* = 3 (*P* = 0.0010); I^2 = 82%
Test for overall effect: *Z* = 1.71 (*P* = 0.09)



H

Study or subgroup	PPPD Mean	PPPD SD	PPPD Total	SSPPD Mean	SSPPD SD	SSPPD Total	Weight	Mean difference IV, random, 95%CI	Year	Mean difference IV, random, 95%CI
Akizuki 2008	7	9.8	34	7	10.8	30	21.9%	0.00 (-5.08, 5.08)	2008	
Kawai 2011	6.3	3.7	64	5.6	3.3	66	31.7%	0.70 (-5.01, 1.91)	2011	
Oida 2011	16.4	3.6	25	10.2	2	42	31.1%	6.20 (4.66, 7.74)	2011	
Nanashima 2013	19	16	28	9	13	27	15.3%	10.00 (2.31, 17.69)	2013	
Total (95%CI)			151			165	100.0%	3.69 (-0.46, 7.83)		

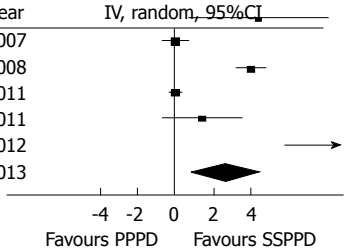
Heterogeneity: Tau^2 = 13.74; χ^2 = 35.01, *df* = 3 (*P* < 0.00001); I^2 = 91%
Test for overall effect: *Z* = 1.74 (*P* = 0.08)



I

Study or subgroup	PPPD Mean	PPPD SD	PPPD Total	SSPPD Mean	SSPPD SD	SSPPD Total	Weight	Mean difference IV, random, 95%CI	Year	Mean difference IV, random, 95%CI
Hayashibe 2007	10.5	6.4	12	6.1	1.3	21	11.6%	4.40 (0.74, 8.06)	2007	
Akizuki 2008	1	0.8	34	1	1.8	30	19.7%	0.00 (-0.70, 0.70)	2008	
Oida 2011	5.6	1.9	25	1.6	0.7	42	19.6%	4.00 (3.23, 4.77)	2011	
Kawai 2011	0.6	0.9	64	0.6	1	66	20.1%	0.00 (-0.33, 0.33)	2011	
Fujii 2012	2.7	6.1	33	1.3	0.7	56	16.3%	1.40 (-0.69, 3.49)	2012	
Nanashima 2013	15	7	28	6	5	27	12.9%	9.00 (5.79, 12.21)	2013	
Total (95%CI)			196			242	100.0%	2.68 (0.77, 4.58)		

Heterogeneity: Tau^2 = 4.67; χ^2 = 120.92, *df* = 5 (*P* < 0.00001); I^2 = 96%
Test for overall effect: *Z* = 2.75 (*P* = 0.006)



J

Study or subgroup	PPPD Events	PPPD Total	SSPPD Events	SSPPD Total	Weight	Odds ratio M-H, fixed, 95%CI	Year	Odds ratio M-H, fixed, 95%CI
Akizuki 2008	4	34	3	30	26.3%	1.20 (0.25, 5.85)	2008	
Kawai 2011	8	64	2	66	16.1%	4.57 (0.93, 22.43)	2011	
Nanashima 2013	4	28	2	27	16.3%	2.08 (0.35, 12.45)	2013	
Matsumoto 2014	6	50	5	50	41.2%	1.23 (0.35, 4.32)	2014	
Total (95%CI)		176		173	100.0%	1.90 (0.91, 3.97)		
Total events	22		12					

Heterogeneity: χ^2 = 1.97, *df* = 3 (*P* = 0.58); I^2 = 0%
Test for overall effect: *Z* = 1.71 (*P* = 0.09)

0.01 0.1 1 10 100
Favours PPPD Favours SSPPD

K

Study or subgroup	PPPD Events	PPPD Total	SSPPD Events	SSPPD Total	Weight	Odds ratio M-H, fixed, 95%CI	Year	Odds ratio M-H, fixed, 95%CI
Hayashibe 2007	0	12	0	21		Not estimable	2007	
Akizuki 2008	0	34	1	30	34.6%	0.29 (0.01, 7.26)	2008	
Kawai 2011	0	64	1	66	32.3%	0.34 (0.01, 8.46)	2011	
Fujii 2012	0	33	0	56		Not estimable	2012	
Nanashima 2013	0	28	1	27	33.1%	0.31 (0.01, 7.95)	2013	
Matsumoto 2014	0	50	0	50		Not estimable	2014	
Total (95%CI)		221		250	100.0%	0.31 (0.05, 2.01)		
Total events	0		3					

Heterogeneity: χ^2 = 0.01, *df* = 2 (*P* = 1.00); I^2 = 0%
Test for overall effect: *Z* = 1.23 (*P* = 0.22)

0.01 0.1 1 10 100
Favours PPPD Favours SSPPD

L

Study or subgroup	PPPD Mean	PPPD SD	PPPD Total	SSPPD Mean	SSPPD SD	SSPPD Total	Weight	Mean difference IV, random, 95%CI	Year	Mean difference IV, random, 95%CI
Hayashibe 2007	33.1	4	12	32.5	4.7	21	39.6%	0.60 (-2.43, 3.63)	2007	
Oida 2011	30	6.2	25	24.1	3.1	42	41.8%	5.90 (3.30, 8.50)	2011	
Nanashima 2013	42	18	28	31	26	27	10.4%	11.00 (-12.68, 14.68)	2013	
Matsumoto 2014	38	32.3	50	37	37.3	50	8.2%	1.00 (-12.68, 14.68)	2014	
Total (95%CI)			115			140	100.0%	3.92 (-0.37, 8.22)		

Heterogeneity: Tau^2 = 9.73; χ^2 = 8.37, *df* = 3 (*P* = 0.04); I^2 = 64%
Test for overall effect: *Z* = 1.79 (*P* = 0.07)

-4 -2 0 2 4
Favours PPPD Favours SSPPD

Figure 3 Forest plots demonstrating secondary outcomes. Forest plots illustrating results of operation time (A), intraoperative blood loss (B), pancreatic fistula (C), postoperative hemorrhage (D), intraabdominal abscess (E), wound infection (F), time to starting liquid diet (G), time to starting solid diet (H), period of nasogastric intubation (I), reinsertion of nasogastric tube (J), mortality (K), hospital stay (L) in the form of meta-analysis comparing PPPD with SSPPD. Pooled odds ratios (ORs) or weighted mean difference (WMD) with 95% confidence intervals (CI) were calculated using the fixed effects model or the random-effects model. PPPD: Pylorus preserving pancreaticoduodenectomy; SSPPD: Subtotal stomach-preserving pancreaticoduodenectomy.

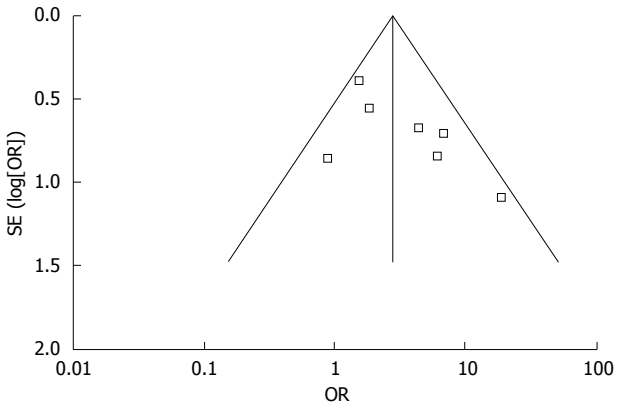


Figure 4 Funnel plot to investigate publication bias. Funnel plot on delayed gastric emptying basing on all studies. The funnel plot revealed no publication bias.

PPPD with regard to DGE and period of nasogastric intubation, albeit with greater intraoperative blood loss. Subgroup analysis specifically looking at trials using the ISGPS definition of DGE, reconstruction with pancreaticojejunostomy, RCTs or non-randomized trials also favored SSPPD with lower rates of DGE. This is the first complete pooled study to date comparing rates of DGE with the two surgical techniques (SSPPD *vs* PPPD). Apart from obvious potential benefits relating to hospital stay, nutrition and possible quality of life benefits, SSPPD may even lead to shorter postoperative recovery times and even earlier commencement of oral chemotherapy, where required. As a result, we consider SSPPD to have distinct short-term advantages over PPPD.

Due to the lack of an internationally accepted consensus definition for DGE in the past, the differences in reported DGE rates may have reflected differences in definitions rather than true differences in incidence. The ISGPS proposed definition of DGE, which includes a 3 tiered clinical grading system[45] based on clinical impact, allows more accurate comparisons. DGE grades B and C signify a prolonged hospital stay and increased costs. In our subgroup analysis, DGE grades B and C were lower in the SSPPD

group compared to the PPPD group.

Previous studies have often found associations between DGE and postoperative intraabdominal complications such as biliary fistula, pancreatic fistula, and intraabdominal abscess[16,29,42,46], although causality has never been clearly demonstrated. Our systematic review did not reveal any significant differences between SSPPD and PPPD in the incidence of pancreatic fistula or intraabdominal abscess rates, suggesting that these did not have a simple relationship with DGE.

Coordination of the antro-pyloric region is considered to be impaired after surgery involving lymph node dissection in the area of the hepatoduodenal ligament and can lead to a physiological derangement similar to that seen with truncal vagotomy[39]. In our subgroup analysis, however, degree of nodal dissection (D1 *vs* D2) did not influence rates of DGE, although the sample size was very small.

Importantly, our study found no statistically significant differences in mortality, post-operative hemorrhage, pancreatic fistula or wound infection rates between the two operative techniques. While time to commencing liquid and solid diet and hospital stay were also not statistically significant, there is a clear tendency favoring SSPPD in relation to these outcomes. It is notable, however, that there seems to be the greatest difference in the retrospective and non-randomized trials, raising the possibility of selection bias. One can envisage, for example, a surgical team introducing diet earlier in patients who underwent SSPPD *vs* PPPD. Furthermore, it is worth noting that there was no mortality at all in any of the included PPPD groups, whereas there were reports of single patient mortality in the SSPPD groups. While this difference did not reach statistical significance, one has to note that none of the studies were sufficiently powered to detect small differences in infrequent events such as death and this must be addressed in any future randomized controlled trial.

There are a number of limitations to this study. Firstly, all included studies originated from Japan, which may skew both the population under investigation as well as operative techniques. Also, most included studies were non-randomized and retrospective in design. Furthermore, there was significant variability in clinical parameters such as operative time, intraoperative blood loss, time of start of liquid or solid diet, time of nasogastric intubation and hospital stay. This was likely related to the differences in operative technique (pancreaticojejunostomy *vs* pancreaticogastrostomy, end-to-end *vs* end-to-side anastomoses, use of pancreatic stents). Due to a lack of detailed information in the included studies, it was not possible to perform a subgroup analysis based on various reconstruction approaches. The greatest shortcoming, however, is that the studies included provided us with insufficient information to conduct a sound comparison of long-term nutritional status, gastrointestinal function and quality of life. Clearly, if there were to be significant long-term complications such as increased rates in dumping syndrome, it would argue strongly against SSPPD as a technique.

In conclusion, this study suggests SSPPD is as safe as PPPD in the studied population and may be superior to PPPD with respect to DGE. However, there is an evident need for well-designed RCTs comparing SSPPD and PPPD with respect to quality of life and survival outcomes.

COMMENTS

Background

Currently, classic pancreaticoduodenectomy and pylorus preserving pancreaticoduodenectomy (PPPD) are considered to be the most widely used surgical procedures for the treatment of pancreatic head and periampullary tumors. Delayed gastric emptying (DGE) is regarded as one of the most common postoperative complications in these two surgical approaches. Recently, a new surgical method, subtotal stomach-preserving pancreaticoduodenectomy (SSPPD), was developed to reduce the incidence of DGE. However, whether this can be achieved in practice is not clear.

Research frontiers

To conduct a meta-analysis comparing perioperative outcomes, especially DGE, after PPPD and SSPPD for the first time.

Innovations and breakthroughs

Based on this meta-analysis, patients undergoing SSPPD had a significantly lower rate of DGE and a shorter period of nasogastric intubation despite being associated with greater intraoperative blood loss.

Applications

SSPPD can reduce DGE as well as improve short-term postoperative outcomes compared with PPPD. However, future randomized trials should compare the advantages between SSPPD and PPPD.

Peer-review

This report was of great interest because it seems to be the first complete pooled study to compare rates of DGE with the two surgical techniques, SSPPD *vs* PPPD. Of course there are limitations in that most of the studies included in this study were retrospective. However, this study can be the chart for deciding the surgical management for patients requiring pancreaticoduodenectomy and for further studies.

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META-ANALYSIS

Differential diagnosis of pancreatic cancer by single-shot echo-planar imaging diffusion-weighted imaging

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Abstract

AIM: To investigate the diagnostic ability of single-shot echo-planar imaging (EPI) diffusion-weighted imaging (DWI) to differentiate between malignant and benign pancreatic lesions.

METHODS: A computerized search was performed on PubMed, MEDLINE and EMBASE up to August 2014. Nine studies (10 sets of data) with a total of 304 malignant pancreatic lesions and 188 benign pancreatic lesions were included. The characteristics of each study included the study name, year of publication, magnetic resonance modalities used, patient population, strength of field, pulse time, repetition time, echo time (TE), maximum b factor, mean age, mean body weight, fat suppression, number of benign and malignant lesions, and true positive, true negative, false positive and false negative results. All analyses were performed using Meta-DiSc and Stata 11.0.

RESULTS: The pooled sensitivity and specificity of single-shot EPI DWI were 0.83 (95%CI: 0.79-0.87) and 0.77 (95%CI: 0.70-0.83), respectively. The positive likelihood ratio and negative likelihood ratio were 5.09 (95%CI: 2.19-11.84) and 0.23 (95%CI: 0.15-0.36), respectively. The P value for the χ^2 heterogeneity for all pooled estimates was < 0.05. From the fitted summary receiver operating characteristic curve, the area under the curve and Q* index were 0.89 and 0.82, respectively. Publication bias was not present ($t = 0.58$, $P = 0.58$). Meta-regression analysis indicated that fat suppression, mean age, TE, and maximum b factor were not sources of heterogeneity (all $P > 0.05$).

CONCLUSION: Single-shot EPI DWI is useful to differentiate between malignant and benign pancreatic lesions. Lesion size $\geqslant$ 2 cm is the limit for the diagnosis of early lesions.

Key words: Meta-analysis; Single-shot echo-planar imaging; Diffusion-weighted imaging; Pancreatic cancer; Differential diagnosis

Core tip: We performed a meta-analysis to investigate the diagnostic capability of single-shot echo-planar imaging (EPI) diffusion-weighted imaging (DWI) to differentiate between malignant and benign pancreatic lesions. Single-shot EPI DWI was useful to differentiate between malignant and benign pancreatic lesions. Lesion size ⩾ 2 cm was the limit for the diagnosis of early lesions.

Hong BZ, Li XF, Lin JQ. Differential diagnosis of pancreatic cancer by single-shot echo-planar imaging diffusion-weighted imaging. *World J Gastroenterol* 2015; 21(20): 6374-6380 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6374.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6374

INTRODUCTION

Pancreatic ductal adenocarcinoma accounts for 85%-90% of all solid pancreatic tumors and is the fourth leading cause of cancer-related death[1]. In 2014, it is estimated that there will be 46420 new cases of pancreatic cancer and an estimated 39590 people will die from this disease (http://seer.cancer.gov/). The only chance for a cure in pancreatic adenocarcinoma is surgery. Most of the time, by the time the tumor presents, it is already invasive. The 5-year survival rate of patients with pancreatic adenocarcinoma is dismal, being < 10%. At initial diagnosis, fewer than 10% of patients can undergo surgical resection, which is the only potential curative treatment[2]. Hence, early detection and characterization, followed by appropriate treatment, are currently the most effective strategies to reduce pancreatic cancer mortality[1,3-5].

Diffusion-weighted (DW) imaging is a magnetic resonance imaging technique that provides unique information related to the diffusion of water molecules in the tissue, and allows estimation of cellularity and tissue structure[6]. Recent reports have shown that the apparent diffusion coefficient (ADC) can be used to detect and characterize malignant and benign pancreatic lesions. Pancreatic cancer tissue has a significantly lower ADC value than that of normal pancreatic tissue, mass-forming focal pancreatitis, and autoimmune pancreatitis[7,8]. DWI of the upper abdomen is a technical challenge because of artifacts secondary to heart and bowel motion, and field inhomogeneity related to parenchyma-gas interfaces.

With EPI, the information in the k-space can be acquired in a single shot. The advantage of using single-shot EPI as a readout sequence is that only one excitation is necessary, and hence the DW images become less sensitive to subject motion[9]. The implementation of ultrafast imaging of single-shot EPI has made DWI of the upper abdomen a feasible option, and is useful to differentiate malignant from benign liver lesions[10-13]. The diagnostic ability of single-shot EPI DWI for the pancreas has not yet been defined. In the present study, we performed a meta-analysis to evaluate the diagnostic ability of single-shot EPI DWI to differentiate between malignant and benign pancreatic lesions.

MATERIALS AND METHODS

Search strategy

We performed a search of PubMed, MEDLINE and EMBASE up to August 2014. The following search terms were used: "pancreatic and diffusion-weighted imaging", "pancreatic and diffusion weighted imaging", "pancreatic and diffusion", "pancreas and diffusion-weighted imaging", "pancreas and diffusion weighted imaging", and "pancreas and diffusion". The search was limited to English language studies only.

Eligibility criteria for study selection

Studies were included in this analysis if: (1) single-shot EPI DWI data were obtained using either a 1.5 or 3.0 T MR scanner; (2) applied field strength was 1.5 or 3 T to represent common technical standards used for clinical pancreatic imaging; (3) the diagnostic criteria of the malignant or benign pancreatic lesions were clearly stated; and (4) data were available to fill out cross-tabs to assess true-positive (TP), true-negative (TN), false-positive (FP) and false-negative (FN) cases.

Data collection

The characteristics of each study, including study name, year of publication, MR modalities used, patient population, strength of field, pulse time, repetition time (TR), echo time (TE), maximum b factor, mean age, mean body weight, fat suppression, number of benign and malignant lesions, and TP, TN, FP and FN results, are shown in Tables 1 and 2.

Statistical analysis

The statistical methods of this study were reviewed by xiaoyuan from the Second Affiliated Hospital of Fujian Medical University. All analyses were performed using Meta-DiSc and Stata 11.0 (StataCorp, College Station, TX, United States). The DerSimonian-Laird random-effects model was used to pool together the final sensitivity, specificity, positive likelihood ratio (PLR) and negative likelihood ratio (NLR). Publication bias was evaluated. We also performed a meta-regression analysis by adding covariates to the summary receiver operating characteristic (SROC) curve using the Moses-Shapiro-Littenberg method[14,15]. For all tests, $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Study selection and data extraction

The initial database search of PubMed and EMBASE identified 170 relevant articles that were published up

Table 1 Diffusion-weighted imaging studies of pancreatic lesions

No.	Ref.	Year of publication	Patient population	MRI unit	Field (T)	TR (ms)	TE (ms)	Max b factor (s/mm^2)	Mean age (yr)	FS	Mean size (cm)
1	Muhi *et al*[21]	2011	Japanese	GE	1.5	8000-10000	73	1000	66.1	Yes	2.6
2	Lemke *et al*[20]	2009	Germany	Siemens	1.5	1300	60	800	65.1	Yes	2.8
3	Sandrasegaran *et al*[22]	2011	American	Siemens	1.5	1500	71	800	68.0	Yes	2.0
4	Sandrasegaran *et al*[23]	2013	American	Siemens	1.5	1500	71	800	66.2	Yes	3.6
5	Kartalis *et al*[17]	2009	Sweden	Siemens	1.5	4600	77	500	NA	Yes	NA
6	Lee *et al*[19]	2008	Korean	Siemens	1.5	2100	72	1000	57.4	NA	3.6
7	Ichikawa *et al*[2]	2007	Japanese	GE	1.5	8000-10000	73	1000	62.0	Yes	2.8
8	Huang *et al*[16]	2011	Chinese	GE	3.0	5700	55	1000	58.9	Yes	3.4
9	Klauss *et al*[21]	2011	Germany	Siemens	1.5	1300	60	800	62.8	Yes	3.5

TR: Repetition time; TE: Echo time.

Table 2 Diffusion-weighted imaging studies of pancreatic lesions

No.	Ref.	Benign lesions	Malignant lesions	TP	FP	FN	TN
1	Muhi *et al*[21]	10	54	52	0	2	10
2	Lemke *et al*[20]	14	23	17	2	6	12
3	Sandrasegaran *et al*[22]	45	25	20	26	5	19
4	Sandrasegaran *et al*[23]	23	13	9	4	4	19
5	Kartalis *et al*[17]	24	12	11	2	1	22
6a	Lee *et al*[19]	13	47	34	3	13	10
6b	Lee *et al*[19]	13	47	41	4	6	9
7	Ichikawa *et al*[2]	23	26	25	0.3	1	22.7
8	Huang *et al*[16]	14	37	31.7	1.9	5.3	12.1
9	Klauss *et al*[18]	9	20	13	0	7	9

TP: True-positive; TN: True-negative; FP: False-positive; FN: False-negative cases.

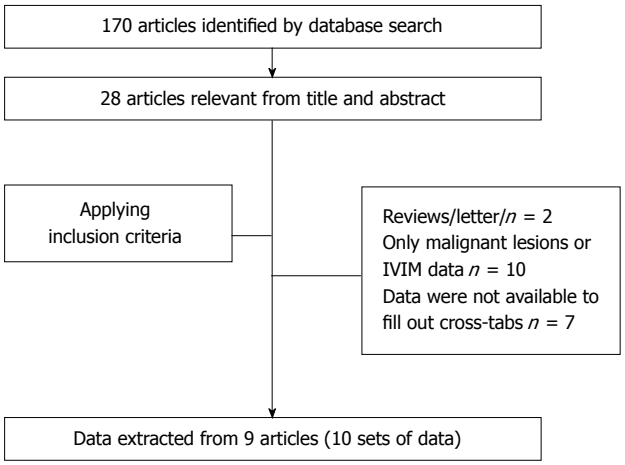

Figure 1 Selection process of the articles. Pooled analysis.

to August 2014. The initial screening by one reviewer reduced this to 28 articles. After applying the inclusion criteria, nine articles[2,16-23] were selected for data extraction (10 sets of data) (Figure 1).

Description of studies

The meta-analysis included 304 malignant and 188 benign pancreatic lesions from nine studies (10 sets of data) (Table 1).

Eight studies used a 1.5 T MRI scanner (Nos. 1-7 and 9) and the other (No. 8) used a 3 T scanner. Seven studies (Nos. 2-6, 8 and 9) used a DWI sequence with TR in the range of 1300-5700 ms, and two studies (Nos. 1 and 7) used a DWI sequence with TR of 8000-10000 ms. Typical acquisition parameters included TE of $\geqslant$ 55 ms (Nos. 1-9 range: 55-73 ms). Typical acquisition parameters included maximum b factor of 800 or 1000 ms (No. 1-4 and No. 6-9). One study (No. 6) did not provide information on the fat suppression technique used. One study (No. 5) did not provide information on the mean size of malignant tumors. The mean age of patients with malignant pancreatic lesions was 63.3 years (No. 1-4 and No. 6-9). The results of all analyses are reported in Tables 1 and 2.

Synthesis of general diagnostic parameters

Figure 2 shows the forest plots of sensitivity (Figure 2A), specificity (Figure 2B), PLR (Figure 2C) and NLR (Figure 2D), of DWI for differential diagnoses between malignant and benign pancreatic lesions.

The pooled sensitivity and specificity of single-shot EPI DWI were 0.83 (95%CI: 0.79-0.87) and 0.77 (95%CI: 0.70-0.83), respectively. PLR and NLR were 5.09 (95%CI: 2.19-11.84) and 0.23 (95%CI: 0.15-0.36), respectively. The *P* value for the χ^2 heterogeneity for all pooled estimates was < 0.05.

The overall accuracy was further explored by drawing SROC curves and finding the area under the curve (AUC) and *Q** index (Figure 3), which were 0.89 and 0.82, respectively, indicating good diagnostic

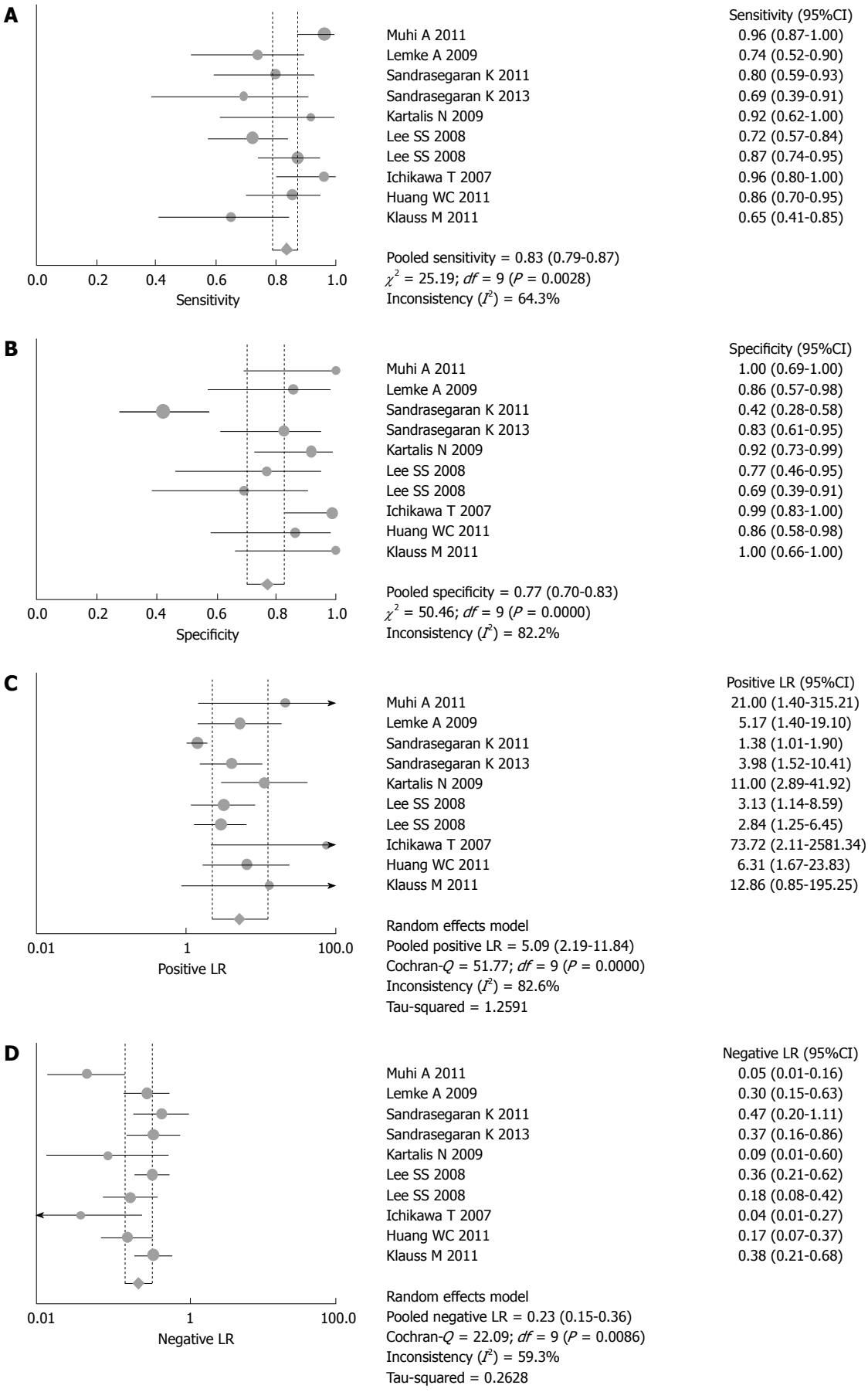

Figure 2 Forest plot of sensitivity (A), specificity (B), positive likelihood ratio (C) and negative likelihood ratio (D) with corresponding 95%CI of nine studies (10 sets of data). The random-effects model was used. The pooled sensitivity and specificity of DWI were 0.83 (95%CI: 0.79-0.87) and 0.77 (95%CI: 0.70-0.83), respectively. Positive likelihood ratio (PLR) and negative likelihood ratio (NLR) were 5.09 (95%CI: 2.19-11.84) and 0.23 (95%CI: 0.15-0.36), respectively.

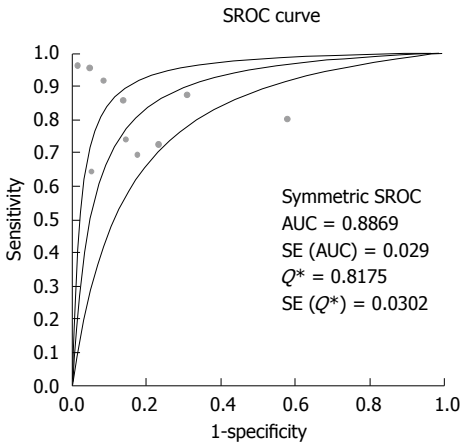

Figure 3 Summary receiver operating characteristic curve. Sensitivity and specificity are plotted in the receiver operating characteristic space for individual studies. The AUC and *Q** index were 0.89 and 0.82, respectively, indicating good diagnostic accuracy. SROC: Summary receiver operating characteristic.

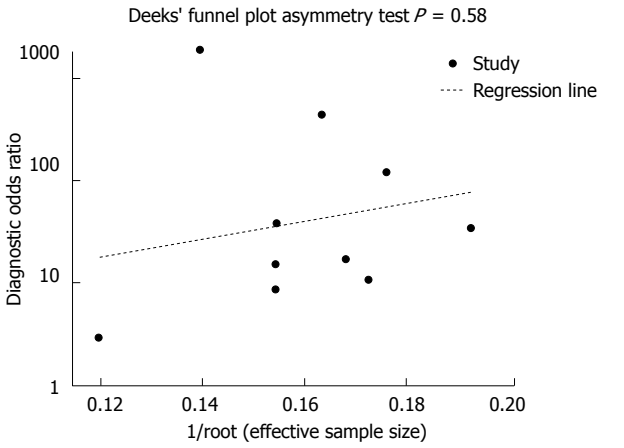

Figure 4 Publication bias was not present (*t* = 0.58, *P* = 0.577).

accuracy.

Publication bias was not observed (Figure 4; t = 0.58, P = 0.58).

The meta-regression analysis indicated that fat suppression, mean age, TE, and maximum b factor were not sources of heterogeneity (all $P > 0.05$).

DISCUSSION

Research concerning pancreatic DWI is rapidly expanding, and a growing amount of data are being published[5,16,24-28]. Fast imaging is important to avoid motion artifacts. The advantage of using single-shot EPI as a readout sequence is that only one excitation is necessary, and hence the DW images become less sensitive to subject motion[9].

Based on calculations of the relevant data available in the currently published articles, our systematic review and meta-analysis demonstrated that pancreatic single-shot EPI DWI was useful to differentiate between malignant and benign pancreatic lesions. The pooled sensitivity and specificity were 83% and 77%, respectively. PLR and NLR were 5.09 and 0.23, respectively. From the fitted SROC, AUC was 0.89 and *Q**, the point where sensitivity equals specificity, was 0.82. All these data indicated that the overall diagnostic performance of single-shot EPI to differentiate malignant from benign pancreatic lesions was high.

There was significant heterogeneity among the studies in our analysis; therefore, it is critical to investigate the source of heterogeneity to determine the potential impact factors. Publication bias is a common source of heterogeneity in meta-analyses. However, in the present analysis, the funnel plot suggested that there may not have been publication bias. Meta-regression analysis was performed to explore other sources of heterogeneity for pancreatic DWI.

There are many factors that affect the signal intensity on DWI and affect measured ADC values[29-32]. Meta-regression analysis indicated that fat suppression, mean age, TE, and maximum b factor were not sources of heterogeneity. The best acquisition strategies for DWI data in the focal pancreatic disease are still a matter of debate. There was considerable variation in the results, which indicated that more-detailed, high-quality prospective studies on pancreatic DWI should be carried out to establish the presence of heterogeneity.

Some limitations of the present study should be mentioned. First, as described above, there was notable heterogeneity among the studies. Evaluated covariates were the sources of the heterogeneity, which requires further study. Standardization of the acquisition protocol for pancreatic DWI across the multicenter studies is recommended. Lesion size $\geqslant$ 2 cm was the limit for the diagnosis of early lesions. Optimization of the DWI protocol includes appropriate b-value selection, sufficient signal-to-noise ratio, adequate fat suppression, and artifact reduction via shimming and parallel imaging[6]. The application of those techniques may be necessary to enhance the results of the present study.

In conclusion, single-shot EPI DWI was useful to differentiate between malignant and benign pancreatic lesions. The pooled sensitivity and specificity were 83% and 77%, respectively. PLR and NLR were 5.09 and 0.23, respectively. From the fitted SROC, AUC was 0.89 and *Q** was 0.82. Lesion size $\geqslant$ 2 cm was the limit for the diagnosis of early lesions. More-detailed, high-quality prospective studies on pancreatic DWI should be carried out to establish the presence of heterogeneity.

COMMENTS

Background

Diffusion-weighted imaging (DWI) provides tissue contrast based on the diffusion properties of water molecules in tissue, without using any contrast agents. The advantage of using single-shot echo-planar imaging (EPI) as a readout sequence is that only one excitation is necessary, and hence the DW

images become less sensitive to subject motion.

Research frontiers

There is no current consensus on the diagnostic ability of single-shot EPI DWI. We conducted a systematic review to investigate the diagnostic capability of single-shot EPI DWI to differentiate between malignant and benign pancreatic focal lesions.

Innovations and breakthroughs

Lesion size ⩾ 2 cm was the limit for the diagnosis of early lesions. More detailed, high quality prospective studies on pancreatic DWI should be carried out in the presence of heterogeneity.

Applications

Single-shot EPI DWI was useful to differentiate between malignant and benign pancreatic focal lesions.

Terminology

DWI provides tissue contrast based on the diffusion properties of water molecules in tissue. DWI has a potential role in the differentiation and evaluation of pancreatic masses on the basis of the high contrast between the lesion and normal tissue.

Peer-review

The paper discusses the prognostic value of single-shot EPI DWI in differentiation of benign *vs* malignant pancreatic masses. The meta-analysis is comprehensive and carefully done.

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CASE REPORT

Self-medication of achalasia with cannabis, complicated by a cannabis use disorder

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Author contributions: Luquiens A was involved in acquisition of data; analysis and interpretation of data; drafting of the manuscript and final approval of the version of the article to be published; Lourenco N, Benyamina A and Aubin HJ were involved in analysis and interpretation of data, making critical revisions related to important intellectual content of the manuscript and provided critical revision of the manuscript for important intellectual content and gave final approval of the version of the article to be published.
Ethics approval: Our research" Self-medication of achalasia with cannabis, complicated by a cannabis use disorder Cannabis in achalasia and manometry" was conducted in accordance with the Helsinki Declaration. We kindly ask for an exemption from providing the Institutional review board (institutional review board) statement, which cannot be available. The case report « Self-medication of achalasia with cannabis, complicated by a cannabis use disorder - Cannabis in achalasia and manometry » was written retrospectively, with the written patient's consent. No review board is required or available in our Institution for this kind of case report, as it has no impact on the care providing process.
Informed consent: The patient gave written informed consent for publication before the study enrollment.
Conflict-of-interest: No relevant conflict of interest for Lourenco N, Aubin HJ, Benyamina A; Luquiens A requested for patent application of cannabis use in achalasia.

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Abstract

Achalasia is a rare esophagus motility disorder. Medical, endoscopic and surgical treatments are available, but all endorse high relapse rates. No data has been published to date reporting a therapeutic effect of cannabis use neither in achalasia nor on its influence on manometric measurements. We report the case of a patient diagnosed with achalasia. He could benefit from a large panel of therapeutic interventions, but none of them was effective over the time. He first used cannabis at age 20 and identified benefits regarding achalasia symptoms. He maintained regular moderate cannabis use for 9 years, with minimal digestive inconvenience. A manometry performed without cannabis premedication was realized at age 26 and still found a cardiospasm. Cannabis use could explain the gap between functional symptoms assessment and manometry measurement. Further investigations are warranted to explore a therapeutic effect of cannabis in achalasia and possible influence on outcome measurements.

Key words: Achalasia; Cannabis; Treatment; Manometry; Addiction

Core tip: Achalasia is a rare esophagus motility disorder. Medical, endoscopic and surgical treatments are available, but all endorse high relapse rates. We report

the case of a patient diagnosed with achalasia who identified benefits from cannabis use on achalasia symptoms. Cannabis non-use before manometry could explain the gap between functional symptoms assessment and manometry measurement. Further investigations are warranted to explore a therapeutic effect of cannabis in achalasia and possible influence on outcome measurements. Particular attention should be given to cannabis addiction risk to vulnerable patients.

Luquiens A, Lourenco N, Benyamina A, Aubin HJ. Self-medication of achalasia with cannabis, complicated by a cannabis use disorder. *World J Gastroenterol* 2015; 21(20): 6381-6383 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6381.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6381

INTRODUCTION

Achalasia is a rare esophageal motility disorder characterized by the absence of peristalsis and impaired relaxation of the lower esophageal sphincter[1]. The main symptoms are dysphagia, regurgitation, heartburn, chest pain and weight loss. The effectiveness of medical treatment is inconsistent, and no trial could support the effectiveness of nitrates or calcium-channel blockers[2]. Endoscopic treatment, namely pneumatic dilation and botulinum toxin injection, requires frequent interventions. Surgical options, namely laparoscopic cardiomyotomy and esophagectomy, are proposed for end-stage achalasia[3] and have high relapse rates.

To our knowledge, no data have been reported on the therapeutic effect of cannabis in achalasia, nor on the influence of cannabis on manometric measurements.

Medical cannabis extract (Nabiximols) is approved in several countries for the treatment of refractory spasticity in multiple sclerosis[4]. Commercialized nabiximols is standardized to contain dronabinol and Cannabidiol (CBD) in a ratio of 1:1 and is sprayed under the tongue using a dose pump. Cannabidiol is the most important non-psychotropic cannabinoid found in the cannabis plant. It is not a cannabinoid (CB) receptor agonist. Dronabinol is the international non-proprietary name for (-)-trans-delta-9-tetrahydrocannabinol (THC). THC is used to refer to the naturally occurring (-)-trans-isomer of delta-9-tetrahydrocannabinol from the cannabis plant (*Cannabis sativa L.*). THC is a CB receptor agonist. THC is responsible for most of the pharmacological actions of cannabis, including the psychoactive effects. Medical cannabis showed positive results for spasticity in patients with multiple sclerosis[5,6] or paraplegia[4] and in the treatment of anorexia, nausea, and neuropathic pain.

CASE REPORT

A 31-year-old patient came to our addiction clinic seeking care for a cannabis use disorder. He was diagnosed with achalasia at the age of 17, with high disability linked to severe symptoms: Regurgitation of undigested food, dysphagia for solid food, heartburn. He initially benefited from nitrate medication but suffered from unacceptable headaches, that led to treatment discontinuation. At age 18, the symptoms were increasingly severe, resulting in a weight loss of 27% of body weight associated with food disgust. He then benefited from a laparoscopic cardiomyotomy, which improved symptoms for one year and a half before relapse. At age 20, a manometry confirmed relapse with impaired relaxation of the lower esophageal sphincter, and major diffuse esophageal dyskinesia. He first used cannabis and identified benefits regarding achalasia symptoms, specifically with food intake facilitation and weight intake. He maintained regular cannabis use of one joint a day for 9 years. During this time period, he did not lose weight, suffered from minimal inconvenience. A manometry realized at age 26 still found a cardiospasm that did not allow the passage of the catheter. This manometry was performed without cannabis premedication, whether the food intakes were pre-medicated by cannabis use at this time period. This could explain the gap between functional symptoms assessment and manometry measurement. He stopped cannabis use at age 29 for a short time period, but suffered from immediate relapse of previous digestive symptoms. At age 29 a first pneumatic dilatation at 30 mm did not allow to facilitate food intakes. He decided to handle his digestive symptoms by cannabis consumption, leading to their improvement, enduring no more weight loss and only sporadic dysphagia. At age 30, negative life events were followed by increased cannabis use that escalated to a severe cannabis use disorder.

Our research was conducted in accordance with the Helsinki Declaration, and the patient gave written informed consent for publication.

DISCUSSION

This spontaneous report of a therapeutic effect of cannabis on achalasia symptoms provides a promising and innovative therapeutic approach. The mechanism of action could involve smooth muscle relaxation of the lower esophageal sphincter, as the endogenous ligand of CB1, anandamide, is an effective antispasticity agent[7,8]. CB1 receptors are primarily presynaptic; their activation inhibits calcium influx and glutamate release and reduces neuronal excitability by activating somatic and dendritic potassium channels[9]. A relaxing effect on other smooth muscles has been reported[10]. CBD is also known for its anti-emetics properties[4]. Therapeutic effect of cannabis in achalasia could

then be due to both CBD and THC actions. Further investigations are warranted to explore a therapeutic symptomatic effect of cannabis in achalasia and the physiopathology of this potential effect. Use of cannabis should be taken into account to analyze outcome measurements, in particular manometric findings[11], and their correlates to functional symptoms.

Cannabis abuse potential is well documented[12]. Although smoked cannabis contains over 400 other chemicals (about 60 are cannabinoids), its reinforcing effects are known to be due to Delta-9-THC[13]. A ratio 1:1 of CBD and THC seems to be protective from addiction-onset risk[14]. Medical cannabis seems to have a much less addictive potential than those of smoked cannabis[15]. However, particular attention should be given to cannabis addiction risk to vulnerable patients.

COMMENTS

Case characteristics

A 31-years-old patient suffers from achalasia since 17 years old. Medical, endoscopic and surgical treatments failed. Symptoms were improved by cannabis consumption, which was complicated by a cannabis use disorder.

Clinical diagnosis

Main clinical symptoms of achalasia were dysphagia, heartburn and weight loss.

Differential diagnosis

Endoscopic exploration and two manometries confirmed achalasia.

Laboratory diagnosis

No laboratory blood test found any consequence of weight loss (no malnutrition or deficiency).

Imaging diagnosis

Manometry was performed two times after surgical cardiomyotomy, and confirmed esophageal dyskinesia.

Pathological diagnosis

Pathological examination was not performed.

Treatment

First line medical treatment (nitrites) and second line surgical treatment and pneumatic dilatation failed in preventing long time symptoms return; cannabis consumption was described by the patient as facilitating food and weight intake.

Related reports

This is the first case report of the effects of cannabis on achalasia symptoms. It provides a promising therapeutic approach in a field with limited medical, endoscopic and surgical therapeutics, which endorse high relapse rates.

Term explanation

The human endocannabinoid system comprises two major cannabinoid receptors (CB1 and CB2, expressed primarily in central nervous system tissues and immune cells, respectively) and their endogenous ligands, known as endocannabinoids.

Experiences and lessons

This is the first description of effects of cannabis on esophagus motility disorder probably involving smooth muscle relaxation of the lower esophageal sphincter, nevertheless other effects of cannabis on smooth muscles were previously reported.

Peer-review

The present manuscript presents a case study speaking to the efficacy of cannabis for the treatment of achalasia in a 31-year-old patient. Findings highlight the potential therapeutic effect of cannabis for this rare esophageal disorder.

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CASE REPORT

Metastasized pancreatic carcinoma with neoadjuvant FOLFIRINOX therapy and R0 resection

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Abstract

Patients with metastasized carcinoma of the pancreas have a very poor prognosis, and long-term survival cannot be expected. This case report describes two patients with an initial diagnosis of metastatic pancreatic cancer, both with hepatic metastases and one with an additional peritoneal carcinomatosis. Initially, both patients were treated intravenously with the FOLFIRINOX chemotherapy regimen, consisting of 5-FU, folinic acid, irinotecan and oxaliplatin. Surprisingly, the FOLFIRINOX treatment resulted in complete resolution of the hepatic metastases in both patients, with no lesions detectable by computed tomography scan. Furthermore, treatment response included decreased diameter of the primary tumor in the tail of the pancreas and disappearance of the additional peritoneal carcinomatosis. Both patients were discussed by our multidisciplinary tumor board, which recommended surgical resections of the carcinoma. The R0 resection of the primary tumor was successful in both cases and, interestingly, the resected tissues showed no evidence of the hepatic metastases intraoperatively. In the first case, the patient received a postoperative 6-mo course of adjuvant chemotherapy with gemcitabine. In the second case, the patient continued to receive the FOLFIRINOX regimen for an additional 6 mo postoperatively. At 12 mo after the operation, a nonresectable retroperitoneal lymph node metastasis was detected in the first patient, whereas the second patient remained in complete remission at the time of this report (5 mo after the adjuvant therapy was discontinued). This case report

is the first of its kind to describe two cases of hepatic metastatic pancreatic carcinoma that were resectable following treatment with FOLFIRINOX. Further studies are required to examine the role of FOLFIRINOX as a neoadjuvant treatment option in subgroups of patients with initially metastasized pancreatic carcinoma.

Key words: FOLFIRINOX; Neoadjuvant treatment of pancreatic neoplasms; Chemotherapy; Metastatic pancreatic neoplasm; Curative operation

Core tip: The FOLFIRINOX regimen is a promising treatment option for metastatic pancreatic carcinoma. This case report describes two cases in which treating metastatic pancreatic carcinoma with neoadjuvant FOLFIRINOX therapy prior to R0 resection was possible. The positive outcomes for these patients provide hope that metastatic pancreatic neoplasms may be cured in certain cases.

Schneitler S, Kröpil P, Riemer J, Antoch G, Knoefel WT, Häussinger D, Graf D. Metastasized pancreatic carcinoma with neoadjuvant FOLFIRINOX therapy and R0 resection. *World J Gastroenterol* 2015; 21(20): 6384-6390 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6384.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6384

INTRODUCTION

It is generally accepted that the prognosis of pancreatic cancer is very poor, with 5-year survival rates of 8%[1]. Since the disease onset manifests few symptoms, diagnosis of pancreatic carcinomas is frequently made after the cancer has already metastasized. Currently, the only curative option is radical surgery for patients with non-metastatic and locally resectable lesions. However, only 10% to 20% of the patients who undergo surgical intervention are cured[2]. In the palliative setting, gemcitabine (as a monotherapy or in combination with nab-paclitaxel or erlotinib) is available as a treatment option[3-6]. In 2011, a new and effective form of chemotherapy was described for treating metastatic pancreatic cancer patients; this chemotherapy regimen, FOLFIRINOX (consisting of 5-FU/folinic acid, irinotecan and oxaliplatin), was reported to achieve an average life extension of 11.1 mo compared to the extension of 6.8 mo that was achieved using gemcitabine monotherapy[7]. Since then, several studies have reported interesting findings from neoadjuvant treatment with FOLFIRINOX in patients with borderline resectable pancreatic cancer[2,8,9]. In terms of locally advanced pancreatic cancers, neoadjuvant therapy also provides an opportunity for resection[8]. This report describes two patients with metastatic pancreatic neoplasms that became secondarily resectable following treatment with FOLFIRINOX.

CASE REPORT

Patient 1

A routine check-up by ultrasound detected suspicious lesions in the liver of a 65-year-old Caucasian female, whose medical history included a case of pneumonia in 1985, as well as cervical cancer and subsequent hysterectomy in 1994, high blood pressure, hip arthrosis and the effects of chronic nicotine abuse. The patient was entirely symptom-free and had an unremarkable clinical examination. The patient had been taking medication to treat high blood pressure. Subsequent computed tomography (CT)-, magnetic resonance imaging (MRI)-scans and an endoscopic ultrasound (Figure 1A, C, E) performed in August of 2012 indicated a carcinoma in the tail of the pancreas with metastasis to the liver. Liver biopsies confirmed the suspected diagnosis and showed liver metastases of a ductal pancreatic adenocarcinoma (G2). Tumor histological findings included a positive dye reaction to antibodies against cytoceratin 7, MUC1 (indicting partial coexpression) and cytoceratin 20 (indicating coexpression in individual cells). Furthermore, there was a negative dye reaction with antibodies against CDX2, CA19-9 and TTF1. While there was no observed elevated level of the CA19-9 biomarker for pancreatic neoplasms, the carcinoembryonic antigen (CEA) tumor marker was slightly elevated (4.7 μg/L; normal: < 3.4 μg/L). The patient was treated with FOLFIRINOX (85 mg/m^2 of oxaliplatin, 180 mg/m^2 of irinotecan, 400 mg/m^2 of folinic acid, 400 mg/m^2 bolus of 5-FU, with 2400 mg/m^2 > 46 h of 5-FU; 5 cycles of 100% dose) from October 2012 to January 2013.

Subsequent to this treatment, the CT scan showed that the pancreatic carcinoma had decreased in size (Figure 1F) and no signs of liver metastases (Figure 1B and D). The multidisciplinary tumor board recommended resection of the tail of the pancreas as an individual therapeutic strategy. The surgery was performed in February of 2013 and included a resection of the tail of the pancreas as well as of the spleen, a radical dissection of the lymph nodes, a gall bladder resection, and a non-anatomical resection of liver segments Ⅱ, Ⅵ and Ⅶ (1.4 cm × 1.2 cm × 0.9 cm, 2.7 cm × 1.8 cm × 1.3 cm, 3.8 cm × 4.4 cm × 2.3 cm). The pathological stage was ypT3 ypN0 (0/16), L0, V0, Pn1 G2 R0 M0 (Figure 2A). The resected liver tissue showed scars and fibrosis without any evidence of persistent cancer cells. Adjuvant treatment with gemcitabine was administered from April to October, 2013. The patient was cancer-free in subsequent check-ups with CT, the last of which was performed in November of 2013 (Figure 1G and H). The patient returned in April of 2014, due to a complaint of pain

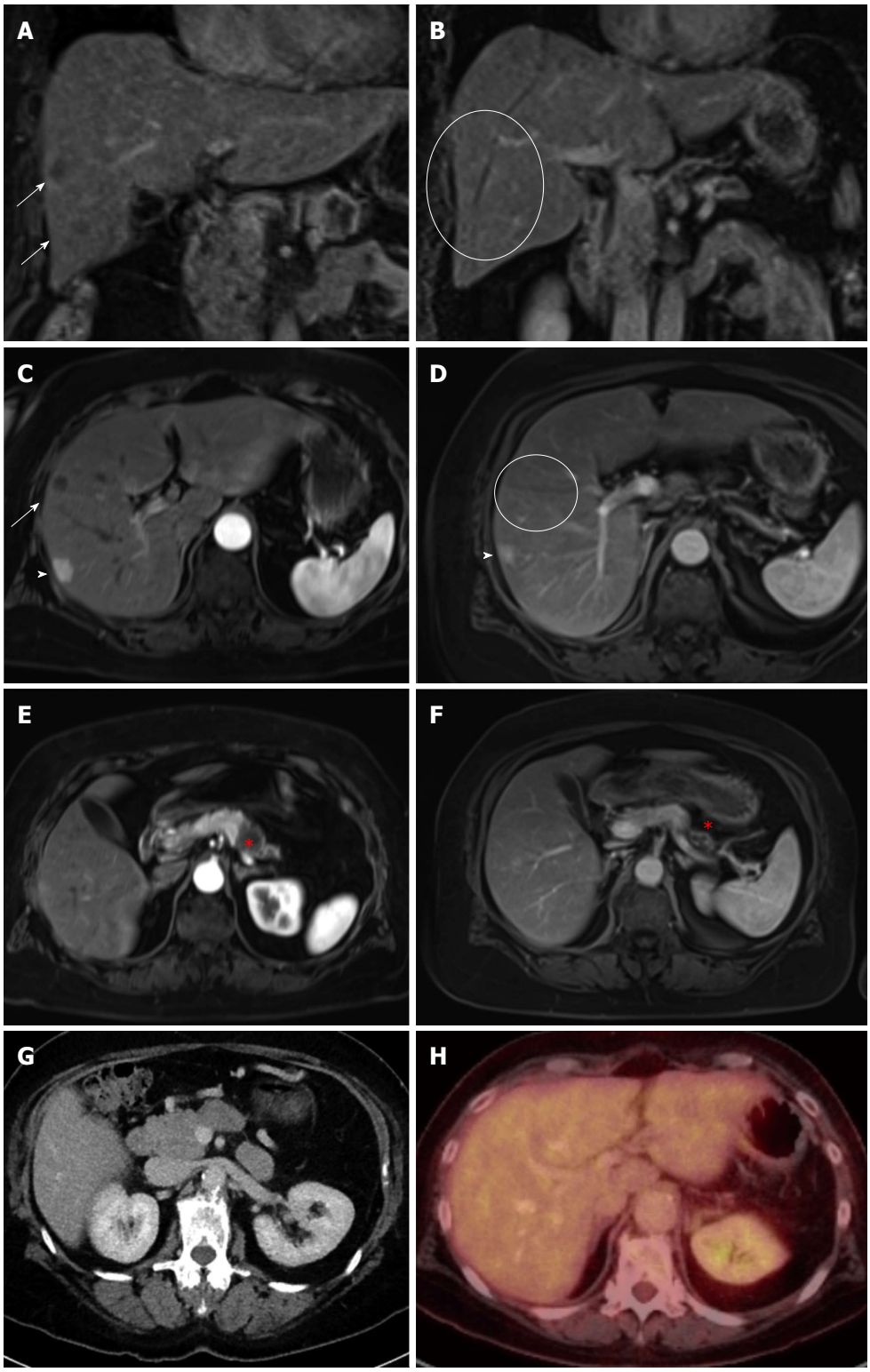

Figure 1 Computed tomography, magnetic resonance imaging and positron emission tomography images of patient 1. Coronal (A, B) and transversal (C-F) T1w contrast-enhanced MRI images demonstrated regredient primary malignancy (star) in the tail of pancreas (E, F) and regredient hepatic metastases (arrow, cycle) in the right liver lobe (A, B, C, D). An additional hemangioma (arrowhead) was observed. Postoperative CT (G) and PET-CT (H) showed complete resection of the primary tumor and no visible metastasis. CT: Computed tomography; MRI: Magnetic resonance imaging; PET: Positron emission tomography.

in her lower left side. CT scan showed metastases in a retroperitoneal lymph node and muscles of the back. The FOLFIRINOX chemotherapy regimen was resumed in May of 2014. The most recent CT scan (performed in August of 2014) showed partial remission of the retroperitoneal lymph node metastasis. No liver metastases have been detected since the initial diagnosis in January of 2013 (Table 1).

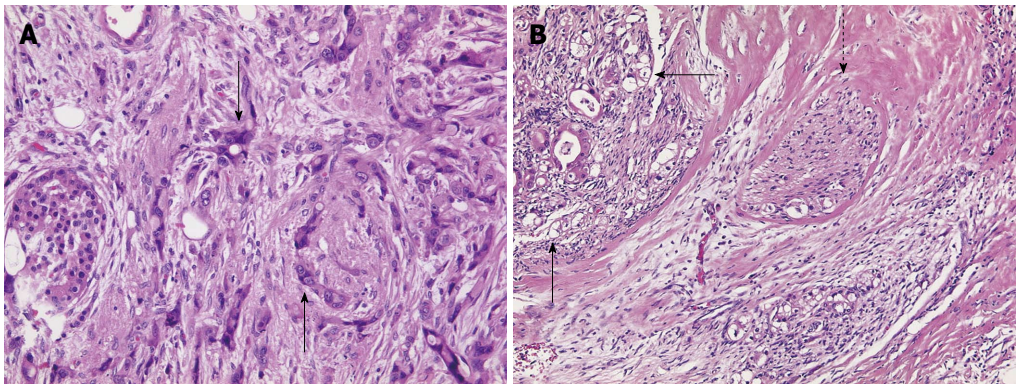

Figure 2 Resected carcinoma of the pancreas after neoadjuvant treatment from patient 1 (A) and patient 2 (B). Hematoxylin and eosin-stained sections are shown. A: 200 × magnification; B: 100 × magnification. Tumor tissue is indicated by the solid arrow and the perineurium is indicated by the dotted arrow.

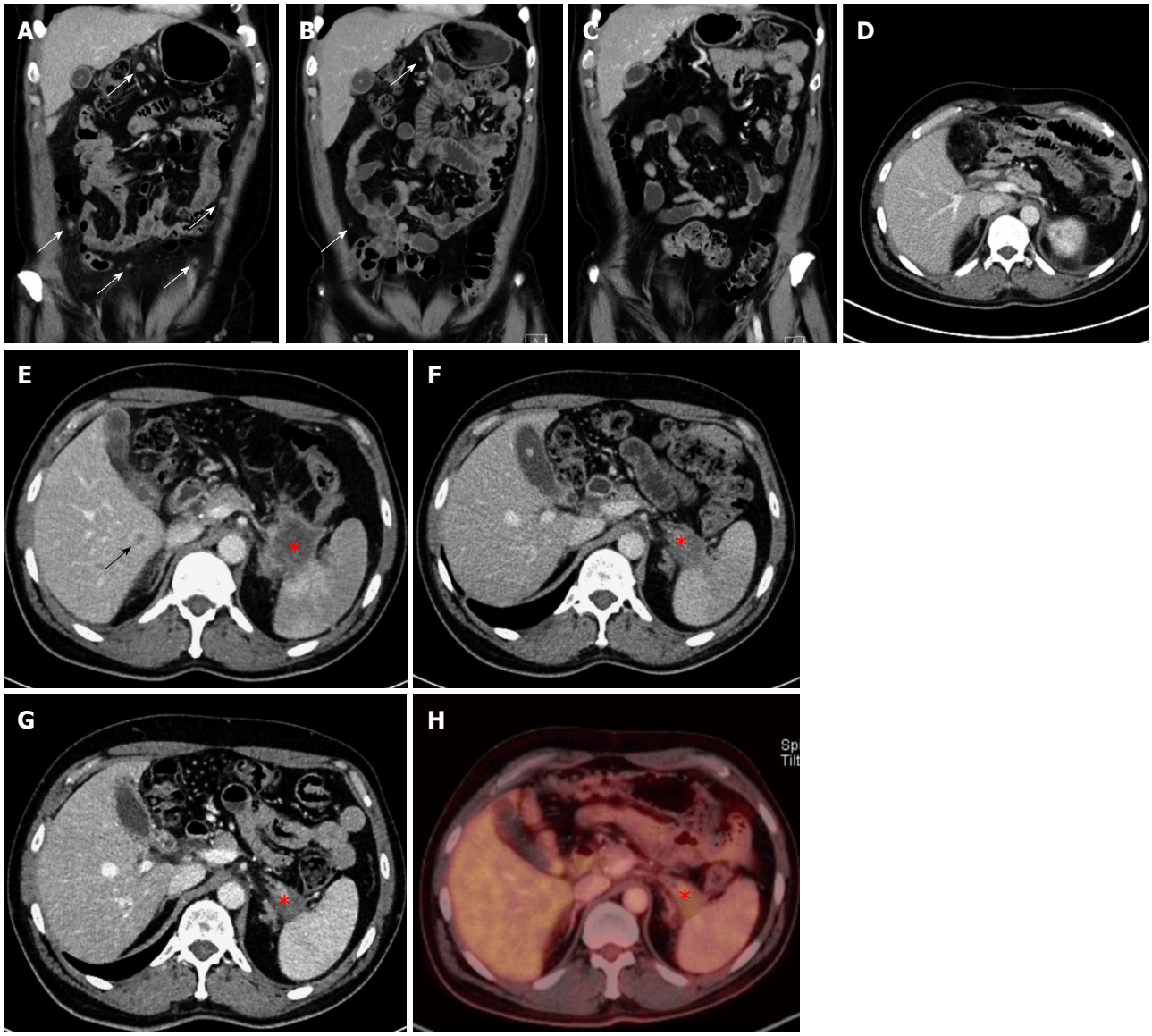

Figure 3 Computed tomography and positron emission tomography images of patient 2. Coronal (A, B, C) contrast-enhanced CT images demonstrated complete regression of multiple peritoneal metastases (white arrow). Axial CT images (E, F, G) and PET-CT image (H) show a regredient mass (star) in the tail of the pancreas. Liver metastasis (black arrow) was seen only in the initial CT-examination (E). Post-operative CT (D) showed complete resection of the primary tumor in the pancreatic tail and no visible metastases. CT: Computed tomography; PET: Positron emission tomography.

Table 1 Case timelines

Course	Patient 1	Patient 2
First diagnosis	08/2012	01/2013
Start of neoadjuvant therapy	10/2012	02/2013
Length of neoadjuvant therapy	3 mo	6 mo
Operation	02/2013	09/2013
Start of adjuvant therapy	04/2013	11/2013
Length of adjuvant therapy	6 mo	6 mo
Relapse	04/2014	-

Patient 2

A 45-year-old Caucasian male was admitted to the hospital *via* the emergency room in January of 2013. The patient's symptoms included increasing dyspnea, nausea and abdominal pain, and chronic pain in the left shoulder. The patient had been diagnosed with non-Hodgkin lymphoma in 1996, which had been resolved by treatment with rituximab and the standard cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP) regimen. Epigastric pain was the only pathological clinical finding during the clinical exam. The patient reported unintentional weight loss (2 kg) within the last 2 mo, during which time he had been taking the following standard medications: Tilidin/Naloxon (at 100 mg/8 mg; extended release tablet 1-0-1), ibuprofen (at 600 mg; 1-0-1), pantoprazol (at 40 mg; 0-0-1), metamizol as needed (at 4 × 40°) and bromazepam as needed daily (1/4 of a 3 mg tablet). The patient presented with elevated levels of C-reactive protein (CRP: 2.3 mg/dL; normal: < 0.5 mg/dL), gamma-glutamyl transferase (99 U/L; normal: < 55 U/L), β2-microglobulin (2.01 mg/L; normal: 0.8-1.8 mg/L), CEA (3.8 μg/L; normal: < 3.4 μg/L), and D-dimer (1.61 mg/dL; normal: < 0.50 mg/L). However, the CA19-9 marker level was within normal range (16.9 U/mL; normal: < 27 U/mL). A chest CT ruled out pulmonary embolism, but showed an area in the tail of the pancreas with possibility as a neoplasm, as well as positive lymph nodes and suspicious peritoneal areas. Subsequent ultrasound showed a tumor in the tail of the pancreas that was in contact with the splenic artery. An abdominal CT confirmed the presence of a 4.3 cm × 5.4 cm neoplasm in the tail of the pancreas with infiltration of the spleen (Figure 3F and 3G), as well as multiple hepatic (Figure 3E), nodal and peritoneal metastases (Figure 3A and B). An endoscopic ultrasound indicated the stage was T4, N1, Mx. Ultimately, fine needle biopsy of the lesion *via* oral endoscopic ultrasound led to the diagnosis of a ductal pancreatic adenocarcinoma (G2) in the tail of the pancreas. Immunohistochemistry analysis of the biopsied tissue showed a significant positive reaction to cytokeratin 7 antibodies, as well as a positive focal reaction to CA19-9 antibodies. Immunoreactivity to MIB1 antibodies showed expression in approximately 50% of the tumor tissue, and immunoreactivity to p53 antibodies showed moderate to strong expression throughout. In February of 2013, the patient was started on the FOLFIRINOX regimen (85 mg/m^2 of oxaliplatin, 180 mg/m^2 of irinotecan, 400 mg/m^2 of folinic acid, 400 mg/m^2 bolus of 5-FU, and 2400 mg/m^2 > 46 h of 5-FU; 6 cycles of 100% dose). The patient suffered from complications during chemotherapy, including pneumonia in the left lung and a deep vein thrombosis with pulmonary embolism.

After 3 mo of the chemotherapy treatment, a decline in tumor growth was observed and the decision to continue treatment with FOLFIRINOX was made (6 cycles of 75%-100% dose). A subsequent CT scan performed in August of 2013 showed neither liver nor peritoneal metastases (Figure 3C), and these findings were confirmed by positron emission tomography (PET)-CT (Figure 3G and H). In September of 2013, the patient underwent surgery with a multivisceral resection of the tail and body of the pancreas, an adrenal gland resection on the left side, a resection of the spleen, a large bowel resection on the left side with transverso-descendostomy, a resection of the cranial part of the kidney on the left side, a gall bladder resection, an atypical liver resection segment Ⅴ (1.7 cm × 1.5 cm × 0.7 cm), an aortic lymph nodal dissection and a celiac lymph node dissection. Postoperatively, the patient's stage was ypT3, ypN0 (0/10), V1, L0, Pn0, R0 and Dworak TRG 3 (Figure 2B). As there were no previous cases for adjuvant treatment involving initial metastatic pancreatic carcinoma and an initially advanced metastatic stage, the decision was made in November of 2013 to continue treatment with FOLFIRINOX. The chemotherapy regimen, however, was delivered at 75% of the full dose to address the patient's frequent presentation of leukopenia in the adjuvant phase. The FOLFIRINOX treatment was discontinued in May of 2014 (Table 1), and the last CT scan (performed in June of 2014) showed no metastatic lesions (Figure 3D).

DISCUSSION

The prognosis of carcinoma of the pancreas is very poor, especially when metastasis has occurred. One-year survival rates of patients with metastatic pancreatic carcinoma treated with gemcitabine are between 18% and 20%[3]. In recent years, new treatment options, such as FOLFIRINOX[7,10] or gem-citabine/nab-paclitaxel, have been introduced and have provided life-extending benefits for patients with metastasized pancreatic carcinomas[4]. The FOLFIRINOX chemotherapy regimen extends life by an average of 11.1 mo and prolongs progression-free survival rate to 6.4 mo longer than the gemcitabine therapy[7].

This report describes two patients with hepatic metastases who achieved complete remission following treatment with FOLFIRINOX and a subsequent R0 resection of the primary tumors in the tail of

the pancreas. For the first case, the patient is still alive 26 mo after the initial diagnosis; although the patient developed a new retroperitoneal lymph node metastasis at 1 year after the surgical resection. For the second case, the patient was still in complete remission at the time of this report (22 mo after the initial diagnosis). Surgical resection may have provided a complete cure, but this conclusion is subject to the findings that will come from future clinical follow-ups. Interestingly, to date neither patient has shown recurrent hepatic metastases, though hepatic resections have not been taken.

A previously published case reported similar findings for a patient with pancreatic neoplasia and pathological findings in the lymph nodes near the superior mesenteric artery, as well as a paraaortal lesion[11]. Among the few studies on borderline resectable or nonresectable locally advanced carcinomas in response to neoadjuvant chemotherapy or radiochemotherapy that have been described in the literature[2,8,9,12], none investigated the potential resectability for cases of pancreatic carcinomas diagnosed with hepatic metastases.

Both of the cases described herein involved carcinomas in the tail of the pancreas. A study by Lorgis *et al*[13] examined the influence of tumor localization, as it relates to the effectiveness of chemotherapy, and found that carcinomas in the head of the pancreas are not as responsive to FOLFIRINOX treatment as carcinomas in other locations. In terms of the embryonic development of the pancreas, it is well known that development can be divergent between the head, body and tail, arguably resulting in varying malignant potential among the various kinds of cells present in each section[14]. Therefore, varying responses to chemotherapy regimens may be expected depending on the location of the carcinoma within the pancreas. Independent of this feature, Lau *et al*[15] analyzed data from the Surveillance, Epidemiology and End RESULTS Program (SEER) of the National Cancer Institute in the United States and found that the incidence rates of pancreatic cancer in the body and tail of the pancreas are increasing, while the incidence rate of pancreatic cancer in the head of the pancreas has remained stable over time. Such a trend may lead to larger incidences of metastasizing carcinomas in the tail of the pancreas, which may respond positively to chemotherapy in a neoadjuvant setting.

In summary, both patients described in this case report may be representative of a wider subgroup of pancreatic carcinoma patients with a better prognosis. Further studies must be undertaken to analyze the impact of tumor localization, biomarkers and the stage and progression of this disease, and to determine whether FOLFIRINOX as a neoadjuvant therapy with subsequent resection is a suitable treatment option for all patients within this subset.

COMMENTS

Case characteristics

A symptom-free 65-year-old patient with suspected hepatic lesions (Case 1) and a 45-year-old male patient with increasing dyspnea, stomach pain and shoulder pain (Case 2).

Clinical diagnosis

The clinical examination was unremarkable for Case 1. Case 2 presented with pathological epigastric pain.

Differential diagnosis

There were no differential diagnoses pertaining to the unremarkable clinical examination for Case 1, who presented with no symptoms. For Case 2, the differential diagnoses were pulmonary embolism, atypical pneumonia, and lymphoma.

Laboratory diagnosis

For Case 1, the level of CA19-9 was unremarkable, but the level of carcinoembryonic antigen (CEA) was elevated (4.7 μg/L). Case 2 showed elevated levels of C-reactive protein (CRP; 2.3 mg/dL), gamma-glutamyl transferase (99 U/L), CEA (3.8 μg/L) and D-dimer (1.61 mg/L), but the level of CA19-9 was unremarkable.

Imaging diagnosis

For Case 1, computed tomography (CT), endoscopic ultrasound (EUS), magnetic resonance imaging (MRI) and contrast-enhanced ultrasound were performed. The findings showed distension (2 cm) in the tail of the pancreas, enlargement of the distal pancreatic duct, three suspected metastatic lesions in segments Ⅴ and Ⅵ, and three hepatic hemangiomas. For Case 2, the CT, EUS and contrast-enhanced ultrasound studies showed an invasive, growing carcinoma in the tail of the pancreas, with a size of approximately 45 mm × 34 mm and with hepatic metastases (8-11 mm in size) and peritoneal metastases.

Pathological diagnosis

For Case 1, liver biopsy showed moderately differentiated ductal adenocarcinoma (G2). For Case 2, the EUS-guided fine needle biopsy showed moderately differentiated ductal adenocarcinoma (G2).

Treatment

Case 1 was given neoadjuvant treatment with FOLFIRINOX (85 mg/m^2 of oxaliplatin, 180 mg/m^2 of irinotecan, 400 mg/m^2 of folinic acid, a 400 mg/m^2 bolus of 5-FU and 2400 mg/m^2 > 46 h of 5-FU) followed by resection surgery with subsequent adjuvant treatment with gemcitabine (1000 mg/m^2). The FOLFIRINOX treatment continued after recurrence. Case 2 was given neoadjuvant treatment with FOLFIRINOX and subsequent multivisceral resection that was followed by adjuvant treatment with FOLFIRINOX.

Related reports

This case report highlights the consideration of whether a subgroup of initially metastasized patients with pancreatic carcinoma may be treated effectively with chemotherapy in a neoadjuvant setting in order to achieve secondary resectability.

Term explanation

Pancreatic neoplasms have very poor prognosis since the disease onset is frequently asymptomatic, and diagnosis usually occurs after metastasis. FOLFIRINOX (5-FU, folinic acid, irinotecan and oxaliplatin) is a new and promising chemotherapy regimen that has been shown to increase life expectancy in patients with metastatic pancreatic neoplasms. Neoadjuvant treatments are administered with the aim of shrinking a tumor prior to administration of the primary treatment. Curative surgery involves the radical removal of a partial or entire organ in which a cancer has originated.

Experiences and lessons

This case report indicates that the treatment of pancreatic carcinomas may be at a turning point following decades of poor prognoses for patients. New treatment options have resulted in new clinical outcomes that could result in improved prognoses.

Peer-review

The authors reported two cases of hepatic metastasized pancreatic carcinoma, both of which were resectable after neoadjuvant treatment with FOLFIRINOX. This finding emphasizes the importance of considering whether carcinomas in the tail of the pancreas differ from those with other localizations in the pancreas, especially in regards to their response to FOLFIRINOX.

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CASE REPORT

Radiofrequency ablation for treatment of hypersplenism: A feasible therapeutic option

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Author contributions: Martins GLP, Herman P and Menezes MR designed the report; Martins GLP, Bernardes JPG, Rovella MR and Andrade RG collected the patient's clinical data; Martins GLP, Viana PCC and Menezes MR analyzed the data and wrote the paper; Cerri GG and Menezes MR revised the paper.

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Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest: No potential conflicts of interest relevant to this article were reported.

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Abstract

We present a case of a patient with hypersplenism secondary to portal hypertension due to hepato-splenic schistosomiasis, which was accompanied by severe and refractory thrombocytopenia. We performed spleen ablation and measured the total spleen and ablated volumes with contrast-enhanced computed tomography and volumetry. No major complications occurred, thrombocytopenia was resolved, and platelet levels remained stable, which allowed for early treatment of the patient's underlying disease. Previous work has shown that splenic radiofrequency ablation is an attractive alternative treatment for hypersplenism induced by liver cirrhosis. We aimed to contribute to the currently sparse literature evaluating the role of radiofrequency ablation (RFA) in the management of hypersplenism. We conclude that splenic RFA appears to be a viable and promising option for the treatment of hypersplenism.

Key words: Portal hypertension; Thrombocytopenia; Hypersplenism; Percutaneous radiofrequency ablation; Splenic ablation

Core tip: The role of splenic radiofrequency ablation (RFA) in the management of hypersplenism is still under study, and the current literature is sparse. This case report not only presents splenic RFA as an attractive alternative treatment for hypersplenism induced by liver cirrhosis, but also shows that it appears to be a viable, safe and promising option for these patients.

Martins GLP, Bernardes JPG, Rovella MS, Andrade RG, Viana PCC, Herman P, Cerri GG, Menezes MR. Radiofrequency ablation for treatment of hypersplenism: A feasible therapeutic option. *World J Gastroenterol* 2015; 21(20): 6391-6397 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6391.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6391

INTRODUCTION

Schistosomiasis mansoni, a chronic parasitic disease, is the most prevalent tropical liver disease in northeastern Brazil. Approximately 200 million individuals are affected by *Schistosoma mansoni* (*S. mansoni*) worldwide, with 600 million exposed to the infection. Approximately 5%-7% of patients infected by *S. mansoni* progress to hepatosplenic schistosomiasis, which is the most severe form of the disease. These patients exhibit periportal fibrosis, portal hypertension, splenomegaly and cytopenia and have a risk of developing upper digestive tract bleeding[1-6].

Hypersplenism is a consequence of massive splenomegaly and commonly occurs in chronic liver diseases. In schistosomiasis, hypersplenism results from hyperplasia of the reticuloendothelial system and the subsequent venous congestion caused by portal hypertension. Studies have reported a correlation between increased splenic size and drops in blood cell count, mainly platelets. These findings depend on the severity of portal hypertension, as some studies have shown that thrombocytopenia is more common in hepatosplenic schistosomiasis, especially after episodes of digestive tract bleeding[7-9].

There are many treatment modalities for hypersplenism secondary to portal hypertension, including splenectomy and embolization of the splenic artery[10-12]. These treatments are effective in the prevention of bleeding and in the correction of thrombocytopenia, but they are associated with high morbidity[13,14], as portal vein thrombosis, pain, splenic abscess and even rupture can occur in cases of splenic embolization[14-17].

Splenectomy remains a popular choice for the treatment of patients with spleen diseases and hypersplenism. However, it has been shown that the preservation of at least 25% of the splenic parenchyma ensures the maintenance of organ function in the short and long term[18], thereby avoiding post-splenectomy infection[19,20] and reducing immunologic induction of thrombocytopenia[21]. Therefore, preservation of splenic function by less invasive therapies has gained a place in the current therapeutic context.

Thermal ablation represents an important technological advance, with radiofrequency ablation (RFA) being the most commonly used technique. RFA is a minimally invasive and well-accepted method used mainly in the treatment of solid tumors of various organs such as the kidneys, liver and lungs[22,23]. The literature on splenic RFA is sparse, and the main indication is local control of neoplasms, but this technique has also been used for the treatment of infected hydatid cysts, hypersplenism and hemostasis in trauma[15,22-27].

There are no standardized criteria for splenic RFA in patients with hypersplenism. In some studies, this minimally invasive treatment has been shown to be safe and less expensive than conventional therapies; however, more studies are needed to determine the effectiveness of splenic ablation in patients with this condition[15,18,23,28].

Here, we report a case of a patient with hypersplenism and severe thrombocytopenia secondary to schistosomiasis. To receive systemic chemotherapy, splenic RFA is indicated to treat the resulting thrombocytopenia, as every other therapeutic possibility was contraindicated.

CASE REPORT

A 60-year-old male patient with a diagnosis of hepatosplenic schistosomiasis since 1989 presented with portal hypertension, esophageal varices, persistent thrombocytopenia secondary to hypersplenism (between 37000 and 44000/mm³ platelets), and chest pain. A CT scan showed a mass in the mediastinum, and a biopsy led to the diagnosis of a moderately differentiated thymoma (type B3) (Figure 1). The patient also suffered from comorbidities (*i.e.,* severe hypertension, dyslipidemia and diabetes), which were difficult to control. Chemotherapy was proposed to treat the patient´s thymoma; however, it was necessary to improve the thrombocytopenia before the treatment could commence.

Because of the high morbidity associated with splenectomy, splenic artery embolization was the first choice of treatment. However, the CT scan showed a critical stenosis of the celiac trunk, which precluded the use of this procedure (Figure 1). Therefore, once informed consent was obtained from the patient, we chose to perform splenic RFA, which we present here as an alternative treatment option for hypersplenism.

The patient was positioned obliquely, with his right side down on the CT table, and the ablation was conducted under general anesthesia with prophylactic antibiotics. For treatment planning, a non-enhanced CT scan (Philips Brilliance 40) was acquired. Images in axial, sagittal, and coronal planes were reconstructed,

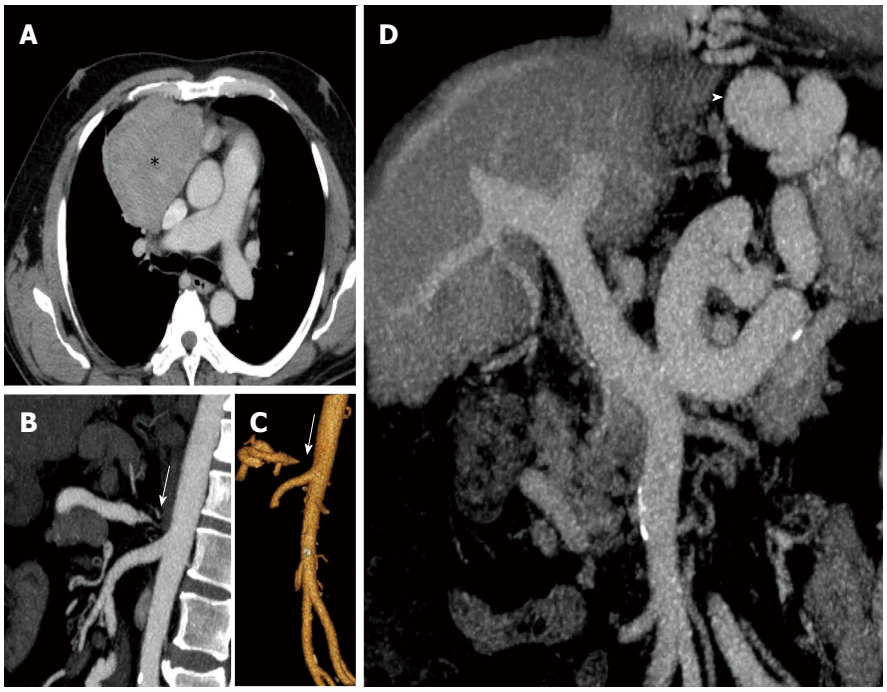

Figure 1 Axial, coronal and sagittal reconstructions of enhanced-computed tomography scan of the patient. A: Shows the anterior mediastinal mass (Asterisk) compressing and displacing the adjacent structures (after biopsy, the patient was diagnosed with thymoma). To reverse the thrombocytopenia to initiate chemotherapy treatment, the first option was splenic artery embolization, but this was contraindicated due to severe stenosis of the celiac trunk [arrows in (B) and (C)]; D: Cirrhotic liver, and the large portal trunk caliber, all characteristics of severe portal hypertension secondary to schistosomiasis. Also of note, the large sized collateral circulation (arrowhead in D) common to this profile.

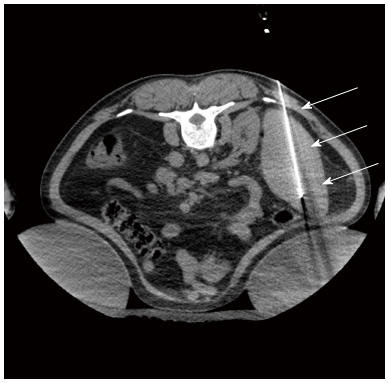

Figure 2 Axial non-enhanced computed tomography scan guiding the splenic radiofrequency ablation, with the patient in the prone position. Note the "single" probe (arrow) in the middle/ lower third of the spleen. where the ablation zones were concentrated.

and an experienced interventional radiologist chose the optimal puncture approaches and sequences. A single 3 cm internally cooled electrode (Cool-Tip; Covidien, Mansfield, Massachusetts) was percutaneously introduced into the spleen under CT fluoroscopic guidance and was repositioned multiple times to cover most of the parenchyma (mainly the middle and lower thirds, avoiding central and subcapsular regions) to ablation of more than 50% of the splenic parenchyma (Figure 2). Using an impedance control algorithm, RF energy was applied during internal cooling of the electrode, with a maximum power of 150 W for 6-12 min in each punctured site, for a total procedure time of 4 h.

A contrast-enhanced CT scan confirmed that the ablated area was concentrated to the middle and lower thirds of the spleen and represented approximately 70% of the spleen parenchyma (initial volume of 1296.2 cc, leaving only 359.5 cc) (Figure 3).

After the procedure, the patient developed mild hematuria associated with acute renal failure, which was characterized by moderate elevation of blood urea and creatinine. This complication was completely resolved after ten days of hospitalization, without the need for dialysis.

The platelet count increased to 225000/mm^3 three weeks after the procedure. A post-procedure leukocytosis, which was most likely secondary to systemic inflammatory response syndrome (commonly observed in patients undergoing ablation of large volumes of tissue), also completely resolved after one week. Chemotherapy was started one month after ablation.

DISCUSSION

In the context of liver disease and portal hypertension, where an enlarged spleen often leads to thrombocytopenia, the need for chemotherapy can present special problems. Cytotoxic chemotherapeutic regimens often induce bone marrow suppression, which can also result in thrombocytopenia. When

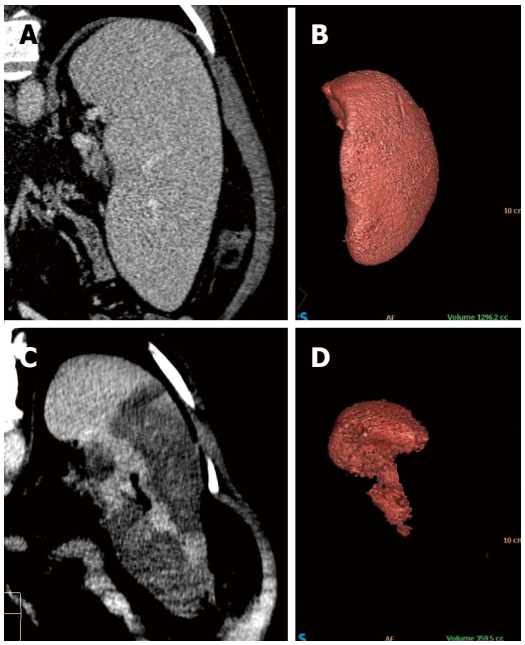

Figure 3 Coronal reformatted contrast-enhanced computed tomography and 3D volumetric reconstruction of the spleen before (A and B) and after (C and D) radiofrequency ablation. Observe the homogeneous splenic parenchyma (A) showing a volume of 1296.2 cc (B) before treatment. After ablation, note the heterogeneity of the spleen (C), with multiple areas of coagulative necrosis mainly concentrated in the middle and lower thirds, saving the splenic hilum and its upper third. After ablation, the volumetric 3D reconstruction confirmed treatment success, leaving approximately 30% of viable parenchyma (volume of 359.5 cc) (D).

this condition occurs, many patients must stop their treatment because serious and potentially lethal side effects may occur due to severe leucopenia and thrombocytopenia[29].

Patients with thrombocytopenia who are offered a splenectomy must present good functional status, preferably with thrombocytopenia as the only factor limiting surgical treatment. By using these stringent preoperative criteria prior to splenectomy, perioperative morbidity and mortality can be minimized[29]. Splenectomy can eliminate hypersplenism, but morbidity ranges from 9.6% to 26.6%, when considering both laparoscopic and open approaches[30-33]. Perioperative complications include bleeding, atelectasis, portal vein thrombosis (range: 1.6% to 11%) and subphrenic abscess formation[15,28,34]. The major long-term risk after splenectomy is overwhelming sepsis (up to a 30-fold increase when compared to the normal population). Other complications are thrombophilia, pulmonary hypertension, and death[28,30,34].

Patients who are not good candidates for splenectomy may opt for splenic artery embolization, as this treatment can also potentially reverse hypersplenism-induced thrombocytopenia[35]. Embolization is considered to be the second line of treatment because it can lead to significant postoperative pain and splenic abscesses[36]. In a large series, Zhu *et al*[30] found that in partial splenic embolization, the splenic infarction rate should be limited to 50%-70% to ensure long-term efficacy in alleviating hypersplenism and thrombocytopenia, as assessed by good outcome at the 5-year follow-up. In their series, the most common complications were post-embolization syndrome, followed by transient pleural effusion and/or ascites, as well as small left-sided atelectasis[30]. Portal vein thrombosis[37], severe bacterial peritonitis[38] and splenic abscess resulting in death have also been reported[37,38].

Because of severe comorbidities, high surgical risk, significant stenosis of the celiac trunk, and the need to prevent splenic embolization (first and second lines of treatment), our patient underwent splenic ablation as a last resort to revert thrombocytopenia.

There are no standardized selection criteria for the use of splenic RFA to treat hypersplenism; therefore, the indication of this procedure is individualized and case-based. In several published studies, the indication was based on the individual authors' experience, which included severe disease with persistent thrombocytopenia (between 15000 and 100000 platelets), persistent leucopenia, splenic volumes of less than 1500 mL, absence of esophageal varices and/or previous treatment of the varices, absence of portal vein or hepatic veins thrombosis, and prothrombin time less than 22 s[15,23,26,39].

The RFA ablation can be performed percutaneously, laparoscopically or with open surgery. The percutaneous approach is preferred because it is minimally invasive, results in a shorter recovery time, and allows for good visualization of the entire needle and the insertion site. Ultrasound or computed tomography may be used for guidance, and multiple regions of the spleen can be ablated or multiple needles can be used simultaneously, keeping in mind that the needle should be positioned at a certain distance from the splenic capsule and the vessels of the hilum. Although radiofrequency electrodes can be inserted into any portion of the spleen, the middle and lower thirds may be the best sites to avoid thermal damage to surrounding organs and reduce postoperative complications (particularly pleural effusion)[15,16,25,26,39]. Because of its superior resolution, we chose to use a percutaneous CT-guided approach, and we concentrated the ablation zones to the middle and lower thirds of the spleen, avoiding the central and upper portions, as previously described in the literature.

There is still no consensus regarding how much parenchyma should be ablated to ensure the resolution of thrombocytopenia while also avoiding complications. It has been shown that splenic ablation can lead to good outcomes in the short-term; however, the long-term results depend on the volume of ablated parenchyma[39]. Liang *et al*[39] divided patients into 3 groups based on the volume of ablation performed (less than 20%, 20%-40% and over 40%) and observed that larger ablation volumes (> 40%) yielded better clinical results, which was similar to the findings by Liu *et al*[25], who observed positive results after ablation of $\geqslant$ 40% of the spleen. Moreover, Feng *et al*[15] reported that patients with more than 50% of ablated parenchyma showed

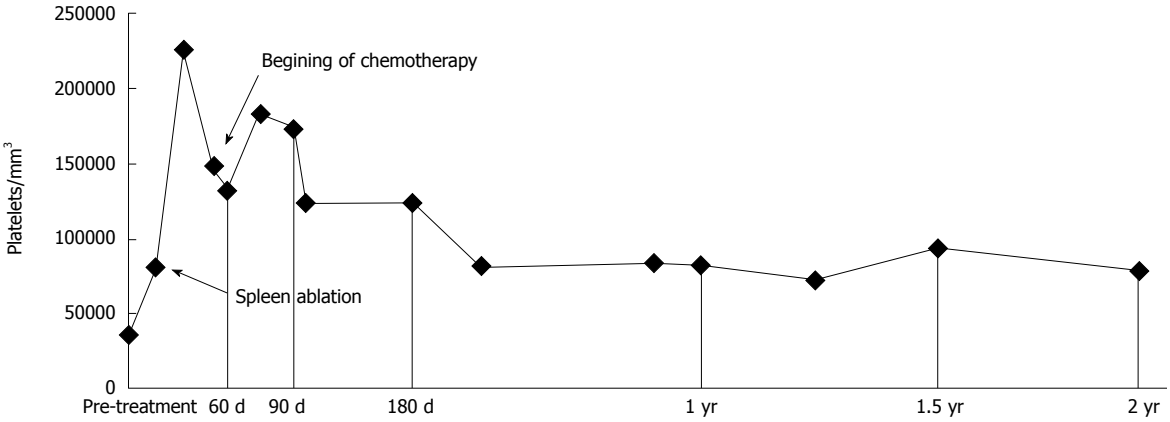

Figure 4 Graph above shows good recovery of platelet levels in a short period of time following the procedure, maintaining adequate levels during follow-up, and allowing chemotherapy to be conducted.

better clinical control than patients with smaller ablated volumes and that the results were even better when the ablation volume was greater than 70%. After 5 years of follow-up, these authors concluded that the ablation volume should ideally be between 50% and 70%. In the present case, resolution of thrombocytopenia in the short and medium terms was essential for the systemic treatment indicated for the patient. After ablation, the platelet count reached 225000 mm³ in 3 wk and remained between 80000 and 120000/mm³ at the 24-mo follow-up (Figure 4).

The procedure lasted 4 h, and only one single radiofrequency probe was used. Therefore, to achieve adequate ablation volumes with single electrodes, multiple overlapping sessions are required[40,41]; in clinical practice, repositioning electrodes is time consuming and even technically challenging. Several types of electrodes and even different techniques have been developed to overcome this limitation and achieve greater ablation zones, including clustered and multipolar electrodes[42,43], monopolar radiofrequency ablation with a multiple-electrode switching system[44,45] and even the use of microwave ablation[39,46]. Some studies have demonstrated that all of these techniques are promising and safe, sometimes allowing more energy delivery, with the advantage of being more efficient in creating a larger and confluent coagulation zone within a clinically acceptable and lower time frame than with conventional consecutive radiofrequency ablation, but with an increased cost.

The patient developed mild hematuria associated with acute renal failure after the procedure, which was characterized by moderate elevation of blood urea and creatinine and change in urine color. A literature search revealed that RFA can induce hemolysis in the experimental setting[47], which might be due to thermal injury of erythrocytes, leading to release of hemoglobin into the circulating blood[48], once blood hemolysis occurs when warmed up to 50 ℃[49]. In addition, the procedure had an overall duration of 4 h, and patient positioning and prolonged immobilization are normally associated with rhabdomyolysis in surgical patients; unfortunately, no serum markers were measured in this case[50]. Nevertheless, there are no proven factors that can predict acute renal failure in cases such as this, but patients who require larger ablation volumes and longer operating times, and those with low-flow state should be closely monitored for this complication[50]. In conclusion, we believe that the most likely cause of transient acute renal failure in our case was hemolysis from prolonged and large volume radiofrequency ablation and that measures such as intravenous hydration and alkalinization of urine should be adopted to avoid it when indicated[50].

The most common complications described in the literature associated with splenic RFA are transient low-grade fever, symptomatic pleural effusion (some patients required thoracentesis) and mild abdominal and left shoulder pain[15,25,26,39]. Less common complications include mild transient hemoglobinuria and mild hematuria[25,39], skin bruises[25], portal vein thrombosis[39] and intra-abdominal hemorrhage[39]. Splenic rupture, refractory ascites, thermal injury to adjacent organs (including stomach, pancreas and colon), liver dysfunction and acute pancreatitis are other life-threatening complications that could potentially occur but have not been reported in the literature.

In summary, splenic ablation was the last-resort chosen to resolve this patient's thrombocytopenia. Despite the occurrence of a major complication that was clinically monitored and no further observed consequences, in this case percutaneous splenic RFA was successful in managing hypersplenism thrombocytopenia in a safe, effective, and minimally invasive manner, which makes it a treatment option in patients who are not candidates for surgery or an endovascular intervention. Nevertheless, more studies are needed to determine the best technique for each case, taking into consideration the success and complication rates associated with each procedure.

COMMENTS

Case characteristics

This is a 60-year-old male patient with a diagnosis of hepatosplenic schistosomiasis and persistent thrombocytopenia, who presented with a moderately differentiated thymoma.

Clinical diagnosis

Dullness to percussion and pain on palpation of the left hypochondrium, associated with an increased spleen.

Differential diagnosis

Hypersplenism, abdominal tumor in the left hypochondrium.

Laboratory diagnosis

WBC 1.81 k/uL; HGB 14.2 gm/dL; Platelets: between 37000 and 44000/mm^3; metabolic panel and liver function test were within normal limits.

Imaging diagnosis

Thoracic CT scan showed an anterior mediastinal mass measuring approximately 9.2 cm × 7.3 cm. Abdominal imaging showed signs of chronic liver disease associated with portal hypertension and an estimated spleen volume of 1296.2 cc.

Pathological diagnosis

Biopsy revealed a moderately differentiated thymoma (type B3). Hypersplenism had no pathological diagnosis.

Treatment

The patient was treated for hypersplenism with radiofrequency ablation (initial volume of 1296.2 cc, leaving only 359.5 cc) to revert thrombocytopenia.

Related reports

Chemotherapy was proposed to treat the patient´s thymoma; however, it was necessary to revert thrombocytopenia to initiate the treatment, as cytotoxic chemotherapeutic regimens often induce bone marrow suppression, and serious and potentially lethal side effects may occur due to severe leucopenia and thrombocytopenia.

Term explanation

Thermal ablation showed great progress with new technological advances, and radiofrequency ablation (RFA) is the most used among our group and has attracted much attention because it is minimally invasive and well accepted. RFA is based on the principle of coagulative necrosis of tumors at temperatures above 50 ℃, generating a tissue dehydration and protein denaturation.

Experiences and lessons

This case report presents radiofrequency ablation as an alternative treatment for hypersplenism, proving to be not only a safe procedure, but also effective in controlling thrombocytopenia, which makes it a viable option especially when surgery or an endovascular intervention are contraindicated.

Peer-review

The authors have described one case of thrombocytopenia secondary to hypersplenism that needed to be reverted to initiate chemotherapy for the patient. The article highlights RFA as an alternative option for treating hypersplenism and provides some technical aspects and clinical outcome after spleen radiofrequency ablation.

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CASE REPORT

Gastric subepithelial lesion complicated with abscess: Case report and literature review

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Abstract

Gastric abscess is a localized pyogenic inflammation of the gastric wall, which is a rare form of suppurative gastritis. The rarity of gastric abscess may be associated with the difficulty of early diagnosis and high mortality as a result. In general, subepithelial lesions (SELs) of the stomach are incidentally detected during the course of upper endoscopy without specific clinical symptoms and signs. However, some gastric SELs present rarely as a form of hemorrhage, obstruction, perforation, and abscess. Here we report a 45-year-old man with gastric SEL presenting as a gastric abscess, which was diagnosed as an ectopic pancreas of the stomach, along with a review of the literature. Although gastric SEL presenting as an abscess is known as a serious and life-threatening lesion, the patient made a complete recovery through surgical resection as well as medical treatment.

Key words: Suppurative gastritis; Gastric abscess; Subepithelial lesion; Ectopic pancreas; Endoscopic ultrasound

Core tip: Suppurative gastritis is a rare disorder characterized by a purulent, exudative inflammatory process involving the submucosa that may extend to involve the entire gastric wall and may be divided into two categories: phlegmonous, or diffuse type; and localized, gastric abscess type. Predisposing conditions of phlegmonous gastritis or gastric abscess include alcoholism, diabetes mellitus, decreased gastric acidity, and immunosuppression, such as HIV infection. High mortality rate of suppurative gastritis, between 37% and 84%, has been reported. Medical treatment, such as endoscopic drainage and antibiotics, and surgical resection of the mass should be considered.

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INTRODUCTION

Gastric abscess is a localized pyogenic inflammation of the gastric wall, which is a rare form of suppurative gastritis, and is known to be life-threatening. The rarity of gastric abscess may be associated with sufficient blood supply to the stomach and the bactericidal effect of gastric acid[1]. In addition, the infrequency of gastric abscess makes early diagnosis difficult. As a result, gastric abscess has high mortality.

Subepithelial lesions (SELs) of the stomach are incidentally detected during performance of routine upper endoscopy. Although a previous study reported that gastric SEL was found in about 0.36% of cases by upper gastrointestinal endoscopy, the prevalence of SELs of the stomach is increasing as a result of the high frequency of endoscopy for health examination[2]. Most gastric SELs are regarded as benign lesions, however, an estimated 13% are likely to be malignant[3]. Most SELs of the stomach are observed without specific clinical symptoms and signs. However, a few gastric SELs may rarely present as gastric abscess accompanied by fever and chill[4-9].

Here, we report a 45-year-old man with gastric SEL presenting as an acute gastric abscess, which was completely cured through surgical resection after medical treatment. We also provide a short review of the literature on the rare condition of gastric SEL presenting as a gastric abscess.

CASE REPORT

A 45-year-old man visited a local private clinic because of intermittent febrile or chilling sensation, indigestion, and vague abdominal pain for 5 d. Upper endoscopy, abdominal ultrasonography, and contrast-enhanced computed tomography (CT) were performed to investigate the cause of fever and abdominal pain. On endoscopic examination, a large mass measuring about 5 cm, with superficial ulcers and a fistula of the gastric lumen was noted at the anterior wall of the distal antrum of the stomach. A small amount of milky discharge was observed to flow from the fistula. The endoscopist at the local clinic strongly pushed on the wall of the mass using a biopsy forceps, and more pus-like yellowish discharge spewed out from the opening of the fistula (Figure 1). An inhomogeneous, hypoechoic mass with vague contours was also observed at the anterior wall of the stomach through ultrasonographic examination. In addition, on abdominal CT, a heterogeneously enhanced mass measuring approximately 5 cm × 4 cm with concentric elevation and an overhanging edge was detected on the anterior wall of gastric antrum (Figure 2). Therefore, the patient was referred to the Gastroenterology Center of Yeungnam University Hospital for further evaluation and treatment of a bizarre-shaped mass of the stomach.

The patient, who did not have a significant past medical history, presented with a 5-d history of low-grade fever and intermittent abdominal pain. The abdominal pain worsened after eating meals and was accompanied by indigestion. Past medical history was only remarkable in that he had received dental implantation due to traumatic dental injury during the previous few days, and he already knew that SEL of the stomach through upper endoscopy had been detected 2 years ago. Because there were no specific clinical symptoms of the gastric SEL, no further evaluation or treatment was performed. The patient was not taking any specific medication, including proton-pump inhibitors and nonsteroidal

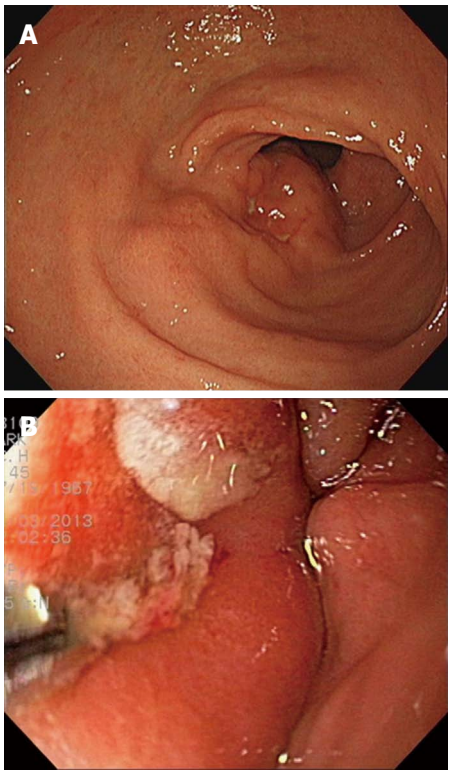

Figure 1 Endoscopic views. A: A mass with multiple ulcers measuring about 5 cm was noted on the anterior wall of the antrum of the stomach; B: Pus-like discharge spewed out from the fistula of the mass.

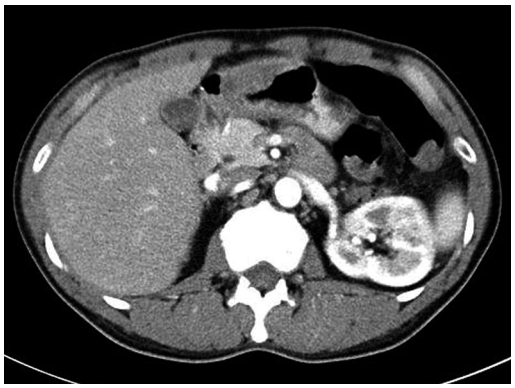

Figure 2 Contrast-enhanced abdominal computed tomography scan showed a heterogeneously enhanced mass measuring 5 cm × 4 cm with concentric elevation on the anterior wall of the stomach.

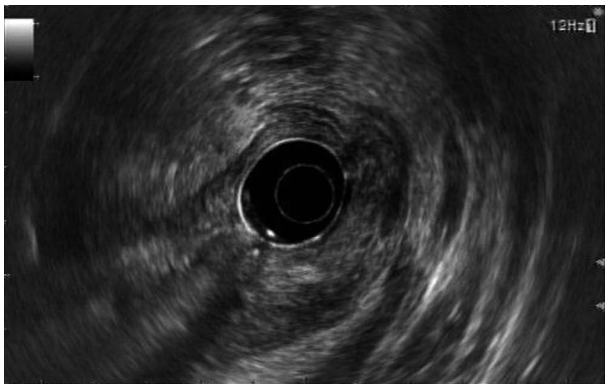

Figure 3 Endosonographic views. A lobulated mass measuring about 5 cm with mixed echogenicity and an irregular margin was observed at the gastric antrum.

anti-inflammatory drugs. Regarding family history, his father died of lung cancer. He was an ex-smoker with 15 pack-years, and was not an alcohol drinker.

Relative appearance of well-being and alertness of mental status were noted. Physical examination of the abdomen revealed a soft and non-tender point with no palpable mass and normoactive bowel sounds. Initial vital signs included blood pressure 110/60 mmHg, heart rate 89 beats/min, respiration rate 20 breaths/min, and body temperature 38.1 ℃. The initial laboratory evaluation showed white blood cell count of 11580 cells/μL (neutrophils: 74.5%), hemoglobin 15.4 g/dL, platelet count 1.70 × 10^5 cells/μL, total bilirubin 3.36 mg/dL, total protein 6.31 g/dL, albumin 3.47 g/dL, aspartate aminotransferase 116 IU/L, alanine aminotransferase 136 IU/L, alkaline phosphatase 618 IU/L, γ-glutamyl transpeptidase 289 IU/L, lactate dehydrogenase 457 IU/L, prothrombin time 10.2 s (international normalized ratio: 0.94), amylase 58 U/L, lipase 18U/L, blood urea nitrogen 10.09 mg/dL, creatinine 1.1 mg/dL, Na^+ 135 mEq/L, K^+ 3.6 mEq/L, Cl^- 100 mEq/L, erythrocyte sedimentation rate (ESR) 42 mm/H, and C-reactive protein (CRP) 18.866 mg/dL. Viral markers for chronic hepatitis B and C were unremarkable. Anti-HIV antibody test was negative. Of tumor markers, only carbohydrate antigen (CA) 19-9 level was elevated to 145.42 U/mL (reference range: 0-37 U/mL). On urine analysis, pyuria (WBC: > 10/HPF) was detected.

Plain chest and abdominal X-rays were non-specific. On follow-up abdominal contrast-enhanced CT, shrinkage of the heterogeneously enhanced mass of the stomach with central necrosis and air-fluid level was observed. Upper endoscopy and endosonography for the unusual mass were performed. On follow-up endoscopy, a bulging mucosa measuring about 5 cm, with multiple external ulcers and erosions, was still noted on the anterior wall of the gastric antrum, however, no pus-like discharge or fistula was observed.

On endoscopic ultrasonography (EUS), a heterogeneous, loculated mass measuring about 5 cm, with mixed echogenicity (partial hypoechogenicity as well as iso- and hyperechogenicity) and an irregular margin, was observed at the antrum of the stomach. In addition, the mass originated from the third layer or submucosal layer of the stomach with air shadows and fluid densities within the mass (Figure 3). According to endosonography of the mass, the lesion was comparable to ectopic pancreas. Considering the irregular contours and mixture of air shadows and fluid densities, internal inflammatory change, such as an abscess or necrosis, might have been combined with ectopic pancreas.

Our initial impression for symptoms and signs of the patient could be summarized as a systemic inflammatory response syndrome due to gastric abscess overlapping with an SEL or urinary tract infection. First, the patient was empirically started on intravenous antibiotics, including third-generation cephalosporin and metronidazole for severe infection. However, body temperature fluctuated to as high as 38.6 ℃ for 3 d. Due to high mortality of gastric abscess, surgical resection for the gastric SEL combined with abscess was considered in the case of no alleviation of fever. Fortunately, on admission day 4, the fever gradually subsided and his general condition also showed improvement. No growth was observed on the initial blood and sputum culture. Only *Klebsiella pneumoniae* with extended-spectrum β lactamase negativity was cultured in the initial urine culture test. Empirical intravenous antibiotics were administered continuously for 7 d. After replacement of intravenous with oral antibiotics, the patient showed good tolerance, and was in good condition at discharge. Oral antibiotics were administered over 1 wk in the outpatient clinic. The white cell count and liver function tests returned to normal after endoscopic drainage of the abscess through the fistula and intravenous administration of antibiotics. ESR and CRP level declined, but remained over the upper normal limit in spite of antibiotic therapy.

Three weeks after discharge, the patient was admitted again, and underwent laparoscopic wedge resection for the gastric SEL at the Department of Surgery because of concern over relapse of the gastric abscess and lack of complete remission of ESR and CRP level. The size of the specimen removed from the stomach by wedge resection was 5.6 cm × 3.5 cm × 2.4 cm. On opening, a submucosal tumor measuring 2.3 cm × 1.2 cm around necrotic tissue was identified. On microscopic view, multifocal benign acini around ductal structures were observed (Figure 4). In addition, hypertrophied muscularis mucosa, Brunner's gland hyperplasia, and gastritis cystica profunda were noted throughout certain parts of the resected specimen. The final pathological result of the gastric SEL was confirmed as an ectopic pancreas of Heinrich type Ⅲ observed only in the acinus and duct. The mass in the stomach was finally diagnosed as an ectopic pancreas accompanied by a gastric

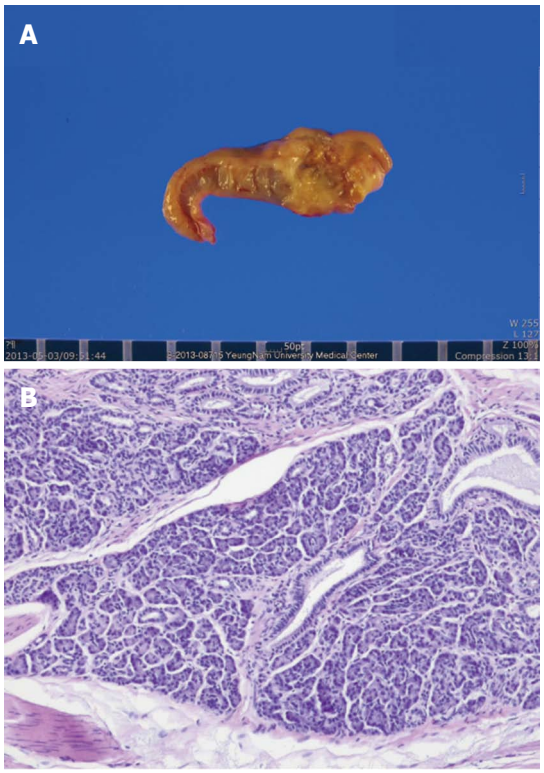

Figure 4 Macroscopic finding of the resected specimen (A) and microscopic view showed benign ductal structures lined by cuboidal epithelium and acini as an ectopic pancreas of Heinrich type Ⅲ. Aggregation of leukocytes around the pancreatic tissue was noted (B) (hematoxylin and eosin, 100 ×).

abscess. After surgical resection, the patient recovered without specific complications in 10 d. In addition, on postoperative follow-up laboratory tests, ESR was 3 mm/h, CRP 0.053 mg/dL, and CA 19-9 6.03 U/mL, which were normalized.

DISCUSSION

Suppurative gastritis is a rare disorder characterized by a purulent, exudative inflammatory process involving the submucosa that may extend to involve the entire gastric wall. The bactericidal effect of gastric acid is partially responsible for the rarity of this condition[1]. In general, suppurative gastritis may be divided into two categories based on the extension of pathology: the phlegmonous, or diffuse type; and the localized, gastric abscess type. The localized form of suppurative gastritis or gastric abscess occurs less frequently. The pylorus and antrum are the most frequently involved areas for gastric abscess, however, abscess may develop anywhere in the stomach. Although the pathogen of gastric abscess is unknown, it most commonly involves infection with oral microflora. Although streptococci are the most common pathogens isolated in culture from gastric abscess, accounting for about 70% of cases, a variety of aerobic and anaerobic bacteria, including *Escherichia coli, Proteus vulgaris, Clostridium perfringens, Clostridium welchi, Pseudomonas aeruginosa, Proteus mirabilis, Staphylococcus* and *Bacillus subtilis*, as well as various fungi, have been implicated[7,9,10-14]. In this case, the gastric abscess was located at the antrum. The culture from the mass or pus could not be obtained directly, and no growth was observed on the blood culture test.

Pathogenic mechanisms of gastric abscess include either direct invasion by microorganisms secondary to gastric mucosal trauma/injury or hematogenous spreading by metastasis of distant infection[7,14]. In our case, the patient had undergone dental implantation several days before. Through more detailed history taking to investigate the cause of gastric abscess, it was revealed that abdominal pain and febrile sensation occurred after eating fried chicken. Poor oral hygiene associated with the dental implantation process and gastric mucosal injury by chicken bone intake related to abnormal masticatory movement might lead to formation of an inflammatory mass in the stomach. In addition, urinary tract infection by *K. pneumoniae* might be associated with a hematogenous origin of the gastric abscess. Thus, in our case, involvement of both the pathogenic mechanisms of gastric abscess might be a possibility.

Predisposing conditions of phlegmonous gastritis or gastric abscess include alcoholism, diabetes mellitus, decreased gastric acidity, and immunosuppression, such as HIV infection[15,16]. In our case, there were no predisposing conditions of gastric abscess. The patient had no significant past medical history, including diabetes mellitus. He did not drink alcohol, and took no medication such as proton pump inhibitors and antacids. Anti-HIV antibody test was also negative. In addition, according to a review of English-language case reports, it was confirmed that all patients with gastric SELs presenting as gastric abscesses had no predisposing conditions (Table 1). Most patients developed gastric abscesses subacutely over an average of 6-8 wk. However, rarely, as in our case, formation of a gastric abscess took only 5 d. As shown in Table 1, Nozawa *et al*[8] reported that abscess formation in a gastrointestinal stromal tumor following endoscopic biopsy occurred within 1 wk. Thus, in spite of no predisposing conditions and acute manifestations, if the patient with gastric SEL complains of vague epigastric pain concomitant with fever, attention should be paid to development of gastric abscess accompanied with SEL.

A high mortality rate of suppurative gastritis, between 37% and 84%, has been reported[11,13,17]. Miller *et al*[11] reported a mortality rate of 100% in patients treated medically, compared with 18% mortality in patients treated with gastric resection and antibiotics. Thus, in all of the case reports, including our present case, surgical resection and medical treatment, such as endoscopic drainage and intravenous antibiotics, were performed on all patients with gastric SEL combined with gastric abscess (Table 1). In our case, although clinical signs and symptoms

Table 1 Summary of the cases of patients with a gastric subepithelial lesion combined with a gastric abscess

Ref.	Age/ sex	Predisposing conditions	Onset	ESR or CRP	Site/Layer	Pathogens of gastric abscess	Size of mass (Max.)	Treatment	Pathological diagnosis
Kaneda *et al*[5]	29/ female	None	Subacute (8 wk)	Elevated	Antrum (GC)/ SM	Unknown	3 cm	Partial gastrectomy	Ectopic pancreas
Honda *et al*[6]	78/ female	None	Subacute (8 wk)	Elevated	Antrum (LC)/MP	Alpha Streptococci, Gram (-) anaerobes	9 cm	Partial gastrectomy	GIST (GI autonomic nerve tumor)
Seidel *et al*[7]	50/ female	None	Subacute (6 wk)	Unknown	Angle (LC)/ MP	Unknown	6 cm	Endoscopic drainage and wedge resection of the mass	Leiomyosarcoma
Nozawa *et al*[8]	74/ male	None	Acute (7 d)	Unknown	Fundus to body (AW)/ NA	Clostridium perfringens	13 cm	Proximal partial gastrectomy	GIST
Osada *et al*[9]	74/ male	None	Subacute (8 wk)	Elevated	Fundus/ NA	Streptococcus intermedius	12 cm	Endoscopic drainage, antibiotics and proximal partial gastrectomy	GIST
Present case	45/ male	None	Acute (5 d)	Elevated	Antrum (AW)/SM	Unknown	5.6 cm	Endoscopic drainage, antibiotics and wedge resection of the mass	Ectopic pancreas

ESR: Erythrocyte sedimentation rate; CRP: C-reactive protein; GI: Gastrointestinal; GIST: Gastrointestinal stromal tumor; NA: Not available.

were improved through medical therapy, ESR and CRP as serological markers indicated that inflammatory processes were not normalized. For complete remission of gastric inflammation accompanied with SEL, gastric wedge resection was performed, and ESR and CRP level showed a normal range after resection.

Most patients with an ectopic pancreas have no clinical symptoms, and, if present, the symptoms are non-specific. However, specific clinical symptoms of heterotopic pancreas are presented by either mass effect or underlying pathology[5]. Clinical symptoms revealed due to mass effect depend on the site of an ectopic pancreas: intussusception (small bowel), obstructive jaundice (biliary tract), and pyloric obstruction (prepyloric region). Clinical manifestations according to underlying pathology are related to almost all of the changes arising from the pancreas itself. Underlying pathology includes exocrine parts such as pancreatic cancer, cystic formation, acute pancreatitis, and abscess formation, as well as endocrine parts such as insulinoma, gastrinoma, and growth hormone-producing tumor. In the current case, among many tumor markers, CA 19-9 was exclusively elevated to approximately four times the upper limit of normal. CA 19-9 is used as a screening test for cancer, particularly pancreatic cancer. In general, elevated CA 19-9 can occur in many types of gastrointestinal cancer, including colorectal cancer, esophageal cancer, and hepatocellular carcinoma. Apart from cancer, elevated level of CA 19-9 may also occur in pancreatitis, cirrhosis, and many diseases of the bile ducts, including obstruction of the bile ducts[18,19]. In other words, CA 19-9 can be elevated in the case of occurrence of inflammation of the pancreatobiliary tract. Although the initial levels of amylase and lipase were within the normal range, elevation of CA 19-9 in our case may be associated with pancreatic inflammation such as pancreatitis of the ectopic pancreas arising from gastric abscess or pancreatic tissue itself, and not pancreatic cancer. This hypothesis is supported by the fact that CA 19-9 was normalized at 6.03 U/mL after wedge resection of the gastric SEL, and leukocytes were mildly aggregated around the pancreatic tissue on microscopic examination of the resected specimen.

In conclusion, despite its rarity, SEL of the stomach can present with gastric abscess. Therefore, attention should be paid to patients with gastric SEL who develop vague abdominal pain, fever, and elevation of ESR or CRP. Further and thorough evaluation, including abdominal CT and EUS should be performed carefully due to the potential risk of infection. Medical treatment, such as endoscopic drainage and antibiotics, and surgical resection of the mass should be considered.

COMMENTS

Case characteristics

A 45-year-old man presented with intermittent febrile or chilling sensation, indigestion, and vague abdominal pain for 5 d.

Clinical findings

Physical examination on the abdomen revealed unremarkable findings and initial body temperature was 38.1 ℃.

Differential diagnosis

Systemic inflammatory response syndrome secondary to gastric abscess overlapping with a subepithelial lesion (SEL) or other causes of infection, such as urinary tract infection.

Laboratory findings

Initial laboratory findings were elevated for neutrophil count (11580 cells/μL), erythrocyte sedimentation rate (42 mm/h), C-reactive protein (18.866 mg/dL), aspartate aminotransferase (116 IU/L), alanine aminotransferase (136 IU/L), and on urinalysis, pyuria (WBC: > 10/HPF) was detected.

Imaging diagnosis

Endoscopic ultrasonography showed a heterogeneous, loculated mass measuring about 5 cm, with mixed echogenicity and an irregular margin at the antrum of the stomach. Abdominal computed tomography showed a heterogeneously enhanced mass measuring about 5 cm × 4 cm with concentric

elevation and an overhanging edge on the anterior wall of the gastric antrum.

Pathological diagnosis

Histological examination showed multifocal benign acini around ductal structures and the final pathological diagnosis of the gastric SEL was confirmed as ectopic pancreas of Heinrich type Ⅲ, observed only in the acinus and duct.

Treatment

The patient received intravenous antibiotics and underwent laparoscopic wedge resection for the gastric SEL.

Related reports

Only five cases of gastric SEL complicated with abscess have been reported in the literature.

Term explanation

Suppurative gastritis is a rare disorder characterized by a purulent, exudative inflammatory process involving the submucosa that may extend to involve the entire gastric wall and may be divided into two categories: phlegmonous, or diffuse type; and localized, gastric abscess type.

Experiences and lessons

This case report presents the clinical characteristics and treatment of gastric SEL complicated with abscess. We recommend that medical and surgical treatment should be considered according to response.

Peer-review

This is a well written case report concerning a diagnosis and possibility of surgical resection of gastric subepithelial lesion of the stomach presenting as an acute gastric abscess by a 45-year-old male patient.

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CASE REPORT

Gastrointestinal stromal tumor solitary distant recurrence in the left brachialis muscle

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Author contributions: Jin SS designed and wrote the case report; Jeong HS, Noh HJ, Choi WH, Choi SH, Won KY, Kim DP, Park JC, Joung MK and Kim JG revised the report critically for important intellectual content; Sul HJ provided the figures and discussion of the pathology; and Lee SW approved the final version to be published.
Ethics approval: The case report was reviewed and approved by the Daejeon Catholic Medical Center Institutional Review Board.
Informed consent: Informed consent was waived because the patient is deceased.
Conflict-of-interest: There are no potential conflicts of interest for any of the authors.

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Abstract

Gastrointestinal stromal tumors (GISTs) are mesenchymal tumors of the gastrointestinal tract that are most commonly found in the stomach. Although GISTs can spread to the liver and peritoneum, metastasis to the skeletal muscle is very rare and only four cases have previously been reported. These cases involved concurrent skeletal metastases of primary GISTs or liver metastases. Here, we report the first case of a distant recurrence in the brachialis muscle after complete remission of an extra-luminal gastric GIST following a wedge resection of the stomach, omental excision, and adjuvant imatinib therapy for one year. Ten months after therapy completion, the patient presented with swelling and tenderness in the left arm. Magnetic resonance imaging revealed a large mass in the brachialis muscle, which showed positivity for c-kit and CD34 upon pathologic examination. This is the first reported case of a solitary distant recurrence of a GIST in the muscle after complete remission had been achieved.

Key words: Brachialis muscle; Distant recurrence; Gastrointestinal stromal tumor; Skeletal metastasis; Tyrosine kinase inhibitor

Core tip: This report presents the first case of the solitary distant recurrence of a gastrointestinal stromal tumor in skeletal muscle after complete remission had been achieved. This case, along with previous reports,

indicates that an extended period of tyrosine-kinase inhibitor therapy may reduce metastasis and recurrence in patients with gastrointestinal stromal tumors.

Jin SS, Jeong HS, Noh HJ, Choi WH, Choi SH, Won KY, Kim DP, Park JC, Joung MK, Kim JG, Sul HJ, Lee SW. Gastrointestinal stromal tumor solitary distant recurrence in the left brachialis muscle. *World J Gastroenterol* 2015; 21(20): 6404-6408 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6404.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6404

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common malignant mesenchymal tumors of the gastrointestinal (GI) tract, accounting for 1% to 3% of all malignant GI tumors. These tumors can develop from very early forms of interstitial cells of Cajal in the wall of the GI tract[1]. Although GISTs can develop anywhere along the GI tract, 60%-70% are well-circumscribed lesions within the wall of the stomach, and 20%-30% arise in the small intestine[2-4]. Approximately 5% of all GIST cases arise from outside the GI tract, and occur in the mesentery, omentum, and retroperitoneum[5].

The most common metastatic sites for GISTs are the liver and peritoneum, and are less frequently found in bone or lung, and rarely in skeletal muscle[6]. Only four cases of GIST metastasis to the skeletal muscle have been reported in the English literature[7-10]. Here we present a rare case of primary GIST with a distant recurrence in the skeletal muscle following complete remission.

CASE REPORT

An 80-year-old woman with Parkinson's disease and hypertension was admitted for pain and swelling of the left arm. She had undergone omental excision with a wedge resection of a large (23 cm × 18 cm × 5 cm) pedunculated extra-luminal gastric GIST that was attached to the stomach three years previously. Sectioning revealed that the specimen had solid, myxoid features with necrosis and hemorrhage. Pathology showed CD117 (c-kit) positivity in the excised portion of the stomach and the omental mass. There were < 5 mitoses per 50 high-power fields (HPFs). She was subsequently diagnosed with a high-risk gastric GIST with omental invasion. She received adjuvant imatinib therapy for one year and complete remission was confirmed. She was observed regularly thereafter as an outpatient.

Ten months after the termination of imatinib therapy, she was admitted to our center after developing pain and swelling of the left arm. On physical examination, the sensory and motor functions of the distal part of the left arm were normal, and an X-ray did not indicate any bony abnormalities. The symptoms persisted for an additional three months, with increasing tenderness of the arm. Upon readmission, laboratory findings for aspartate aminotransferase (23 IU/L), alanine aminotransferase (19 IU/L), and creatine phosphokinase (137 IU/L) were within normal ranges, whereas the hemoglobin level (10.6 g/dL) was below normal and the lactate dehydrogenase (492 IU/L) was high. Although another X-ray failed to show an abnormality, a huge mass in the brachialis muscle was revealed by magnetic resonance imaging (Figure 1). Positron-emission tomography showed an area of increased metabolic activity in the left arm with irregular uptake (Figure 2). Further imaging did not reveal other abnormalities at other sites indicative of metastases.

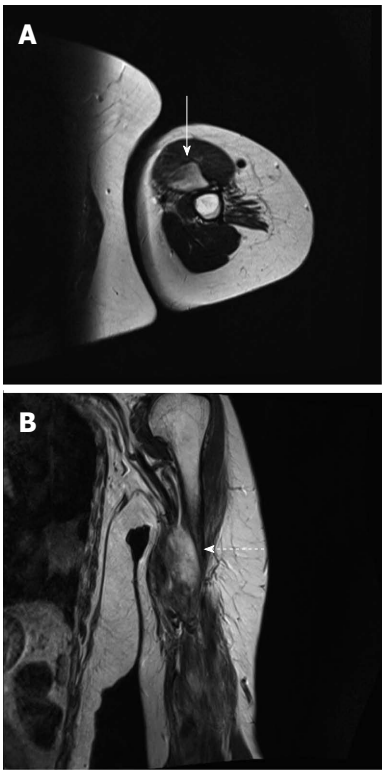

Figure 1 Magnetic resonance imaging of the left arm. A: Axial T2-weighted image showing a huge, ill-defined, high-signal-intensity mass (solid arrow); B: Coronal T2-weighted image showing a high-signal-intensity mass (dotted arrow).

An incisional biopsy of the left brachialis muscle was performed, revealing spindle-shaped cells positive for c-kit and CD34 (Figure 3). She was diagnosed with muscle recurrence of GIST and was again treated with imatinib (400 mg daily). Surgical resection of the solitary metastatic muscle mass was not performed due to her poor condition and high-risk GIST. Magnetic resonance imaging after three months of imatinib therapy showed that the mass was slightly decreased in size. However, she discontinued imatinib two months later because of severe nausea and vomiting. Three months after discontinuing imatinib therapy, her

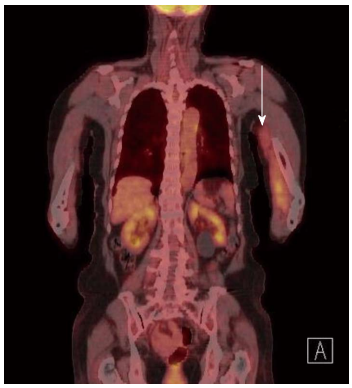

Figure 2 Positron emission tomography-computed tomography. The scan shows an area of increased metabolic activity with irregular uptake (arrow) in the left brachialis muscle.

general condition deteriorated, and she was discharged to another healthcare facility for conservative management.

DISCUSSION

GISTs rarely metastasize to the soft tissue, bone, or skeletal muscle[11], which is particularly resistant to metastatic cancer. Mechanical injury is one of the mechanisms responsible for this resistance. For example, deformation of the cancer cells by microvessels near the muscle ultimately destroys the cells once a critical level of negative pressure is reached[12,13]. Additionally, muscle contraction kills cells trapped within the muscle capillaries, whereas cancer cell survival is greatest in denervated relaxed muscle[14]. Most cancer cells die after hematogenous spread to muscle because of the unfavorable metabolic and mechanical environment in normal muscles[13]. However, injured muscle tissue may provide a more favorable mechanical or metabolic environment for metastatic cancer cell survival[7,13].

Rare metastasis to skeletal muscle was reported by Pasku *et al*[7] in a case involving bilateral gluteal muscles and lung metastasis of intrapelvic GISTs. The patient was successfully treated with tumorectomy and imatinib for one year. Bashir *et al*[8] reported a case with upper back muscle, adrenal gland, and cardiac metastases of small intestine GISTs; the patient underwent surgical resection and received imatinib treatment. The effect of treatment was not reported. Suzuki *et al*[9] reported metastasis of small intestine GISTs to the left buttock muscle in a patient who was treated only with chemotherapy because of a misdiagnosed leiomyosarcoma. Small intestine GISTs were later detected and the patient received surgical resection and imatinib treatment, though he died from GI bleeding six months after the initial diagnosis. The fourth case of skeletal metastasis was reported by Cichowitz *et al*[10], which described a patient with liver metastasis after resection of small intestine GISTs. Following an extended right hepatectomy, an adductor longus muscle metastasis of the small intestine GISTs was detected. Unlike these previous reports, the patient in the present case did not develop concurrent skeletal metastasis. This is the first known report of a distant recurrence in skeletal muscle after achieving complete remission with adjuvant imatinib therapy.

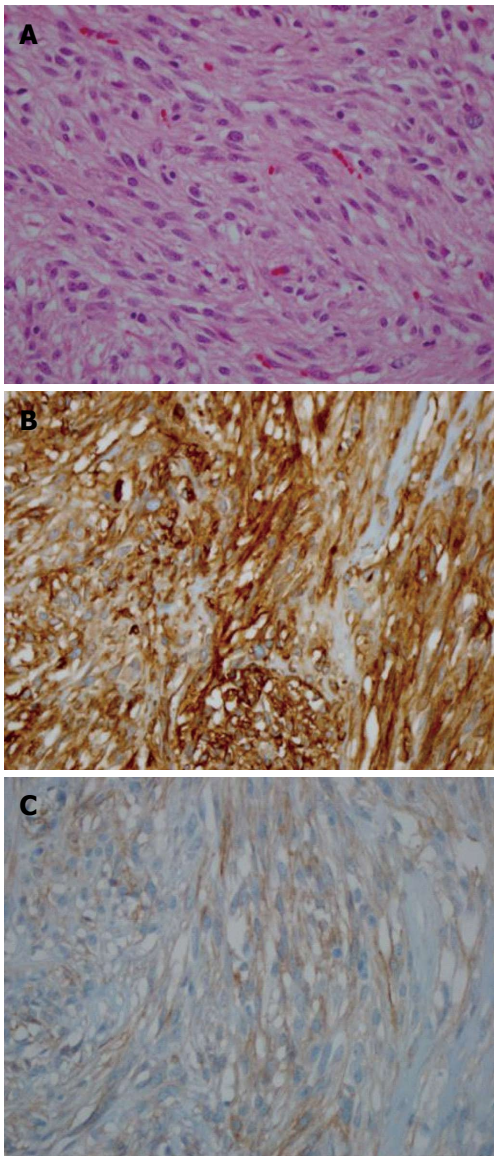

Figure 3 Histopathologic findings and immunohistochemical staining of the left brachialis muscle mass. A: Hematoxylin and eosin staining shows whorls of uniform spindle-shaped cells with elongated blunt nuclei and eosinophilic cytoplasm; B: Immunohistochemical staining for c-kit; C: Immunohistochemical staining for CD34 (magnification × 200).

Interestingly, three of the five known cases of muscle metastasis were derived from GISTs of the small intestine. Although most GISTs occur in the stomach, overtly malignant behavior is less commonly seen in gastric tumors[15]. In contrast, tumors of small-bowel origin tend to have more aggressive behavior, and thus a worse prognosis, than of tumors originating in other gastrointestinal sites[15,16]. According to the criteria of the National Institutes of Health, tumor size and the number of mitoses per HPF are predictive of

GIST recurrence[11,17]. Moreover, > 5 mitoses/50 HPF and a tumor size > 10 cm are associated with an increased risk of recurrence[3]. The patient described in this report therefore had a very aggressive GIST with a high probability of recurrence, with a mitotic count < 5/50 HPF but a tumor size > 10 cm.

Almost all of the cases of skeletal metastasis of GISTs were treated with imatinib, which is a tyrosine kinase inhibitor (TKI). Prior to the discovery of the c-kit tyrosine kinase receptor in GISTs and the antitumor effects of imatinib, surgical removal was the only viable treatment option, as conventional chemotherapy and radiation were largely ineffective[17]. When used as adjuvants following complete surgical resection, molecular-targeted therapies, such as imatinib, can reduce the frequency of recurrence[4,17]. Furthermore, Joensuu *et al*[18] showed that prolonged treatment (three years *vs* one year) with imatinib results in longer recurrence-free and overall survival in GIST patients. Indeed, the median duration of survival of patients with advanced GIST increases with the use of TKIs[19]. Thus, it is important to continue imatinib for at least three years to prevent metastasis or recurrence of GISTs in high-risk patients. Unfortunately, the imatinib therapy for the patient in the present case was limited to one year by her medical insurance. However, medical insurance in South Korea has recently been changed to allow for three years of imatinib treatment for patients with a GIST.

In conclusion, this report presents a rare case of GIST metastasis to skeletal muscle, and the first known case of solitary distant recurrence of GIST in the brachialis muscle after complete remission. Similar diagnoses should be considered in patients presenting with suggestive symptoms and signs, even if the site of metastasis is unusual. Moreover, maintenance of TKI therapy for a minimum of three years should be recommended for patients with GISTs.

COMMENTS

Case characteristics

An 80-year-old woman who had previously undergone wedge resection of an extra-luminal gastric gastrointestinal stromal tumor (GIST) presented with pain and swelling of the left arm.

Clinical diagnosis

On physical examination, swelling of the left arm was observed, which persisted for three months with increasing tenderness.

Differential diagnosis

Leiomyoma; leiomyosarcoma; schwannoma.

Laboratory diagnosis

The patient was anemic (hemoglobin, 10.6 g/dL) and had an elevated level of lactate dehydrogenase (492 IU/L).

Imaging diagnosis

T2-weighted magnetic resonance imaging revealed a huge, ill-defined high-signal-intensity mass while positron-emission tomography showed an area of increased metabolic activity in the left brachialis muscle with irregular uptake.

Pathological diagnosis

Histopathologic findings of the left brachialis muscle mass showed spindle-shaped cells with elongated blunt nuclei and eosinophilic cytoplasm; immunohistochemical staining showed positivity for c-kit and CD34.

Treatment

The patient had a wedge resection of the gastric GIST with adjuvant imatinib therapy; after the distant recurrence of left brachialis muscle, she was again treated with imatinib (400 mg daily).

Related reports

Only four cases of GIST with metastasis to the skeletal muscle have been reported in the literature, however, this is the first report of a distant muscle recurrence.

Term explanation

GISTs are mesenchymal tumors of the gastrointestinal tract that are most commonly found in the stomach.

Experiences and lessons

This is the first reported case of a solitary distant recurrence of GIST in the brachialis muscle after complete remission and we recommend that similar diagnoses should be considered in patients presenting with suggestive symptoms and signs, even if the site of metastasis is unusual.

Peer-review

This article highlights the distant recurrence of a GIST in the muscle after complete remission with adjuvant imatinib, and highlights the importance of prolonged TKI therapy (≥ 3 years) to prevent recurrence and metastasis.

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CASE REPORT

Liver transplantation for a giant mesenchymal hamartoma of the liver in an adult: Case report and review of the literature

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Author contributions: Shen ZY, Cai JZ and Guo QJ performed transplant surgery and provided the intellectual content; Li JJ summarized the clinical data; Sun XY performed the follow-up; Hu ZD contributed to the pathology; Li J performed a literature review and wrote the initial manuscript; Cooper DKC contributed critical comments and revised the manuscript; all authors read, contributed to, and approved the final manuscript.
Supported by National Natural Science Foundation of China, No. 81400680; and the National High Technology Research and Development Program of China, No. 2012 AA021001.
Ethics approval: Approval from the Ethics Committee of the Tianjin First Central Hospital was obtained for this study.
Informed consent: The patient gave her written informed consent before entering the study and gave consent to the study protocol.
Conflict-of-interest: We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work. There is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript.

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Abstract

Mesenchymal hamartomas of the liver (MHLs) in adults are rare and potentially premalignant lesions, which present as solid/cystic neoplasms. We report a rare case of orthotopic liver transplantation in a patient with a giant MHL. In 2013, a 34-year-old female sought medical advice after a 2-year history of progressive abdominal distention and respiratory distress. Physical examination revealed an extensive mass in the abdomen. Computed tomography (CT) of her abdomen revealed multiple liver cysts, with the diameter of largest cyst being 16 cm × 14 cm. The liver hilar structures were not clearly displayed. The adjacent organs were compressed and displaced. Initial laboratory tests, including biochemical investigations and coagulation profile, were unremarkable. Tumor markers, including levels of AFP, CEA and CA19-9, were within the normal ranges. The patient underwent orthotopic liver transplantation in November 2013, the liver being procured from a 40-year-old man after cardiac death following traumatic brain injury. Warm ischemic time was 7.5 min and cold ischemic time was 3 h. The recipient underwent classical orthotopic liver transplantation. The recipient operative procedure took 8.5 h, the anhepatic phase lasting for 1 h without the use of venovenous bypass. The immunosuppressive regimen included

intraoperative induction with basiliximab and high-dose methylprednisolone, and postoperative maintenance with tacrolimus, mycophenolate mofetil, and prednisone. The recipient's diseased liver weighed 21 kg (dry weight) and measured 41 cm × 32 cm × 31 cm. Histopathological examination confirmed the diagnosis of an MHL. The patient did not experience any acute rejection episode or other complication. All the laboratory tests returned to normal within one month after surgery. Three months after transplantation, the immunosuppressive therapy was reduced to tacrolimus monotherapy, and the T-tube was removed after cholangiography showed no abnormalities. Twelve months after transplantation, the patient remains well and is fulfilling all normal activities. Adult giant MHL is extremely rare. Symptoms, physical signs, laboratory results, and radiographic imaging are nonspecific and inconclusive. Surgical excision of the lesion is imperative to make a definite diagnosis and as a cure. Liver transplantation should be considered as an option in the treatment of a non-resectable MHL.

Key words: Liver; Mesenchymal hamartoma; Adult; Organ donor; After cardiac death; Transplantation

Core tip: Mesenchymal hamartoma of the liver is a rare disease in adults. Only 45 patients with this condition have been reported worldwide. This report presents a rare case of adult giant mesenchymal hamartoma of the liver that could not be treated by partial hepatectomy. Orthotopic liver transplantation relieved compression of other organs and avoided the risk of malignant change. Liver transplantation should be considered as an option in the treatment of non-resectable benign hepatic tumors.

Li J, Cai JZ, Guo QJ, Li JJ, Sun XY, Hu ZD, Cooper DKC, Shen ZY. Liver transplantation for a giant mesenchymal hamartoma of the liver in an adult: Case report and review of the literature. *World J Gastroenterol* 2015; 21(20): 6409-6416 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i20/6409.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i20.6409

INTRODUCTION

Mesenchymal hamartoma of the liver (MHL) was first described by Edmondson in 1956[1]. It is a rare mesenchymal tumor affecting almost exclusively infants and children in the first two years of life, with a slight male predilection. Its occurrence in children older than five years is rare (about 5% of cases) and is extremely rare in adults[2-4]. MHL is a potentially premalignant lesion that presents as a solid/cystic neoplasm. The patient's symptoms are typically nonspecific, though abdominal pain is the most common. Laboratory results are noncontributory and radiographic imaging is variable and inconclusive. Needle biopsy is rarely diagnostic and surgical excision of symptomatic or enlarging lesions is recommended to exclude the possibility of malignancy and to establish a diagnosis[5].

CASE REPORT

A 34-year-old, previously healthy, woman presented in 2011 with abdominal fullness and loss of appetite. She took no medications, had no history of liver disease, and denied alcohol and drug use, including the use of anabolic steroids. She presented to our hospital with increasing abdominal girth, abdominal pain, and vomiting. Physical examination revealed a grossly distended abdomen without evidence of ascites, a firm and massively enlarged liver extending below the umbilicus, and tenderness in the upper quadrant. Contrast enhanced computed tomography (CT) of the abdomen revealed near replacement of the liver with diffuse cystic masses of low density (Figure 1). Initial laboratory test results were unremarkable. Hematological, biochemical investigations and the coagulation profile were within normal limits. Tumor markers, including levels of α-fetoprotein, and carcinoembryonic antigen, carbohydrate antigen 19-9, were within the normal ranges. Serology for hepatitis B virus, hepatitis C virus and human immunodeficiency virus was negative. The extensive hepatic involvement precluded resection, and so she was evaluated and placed on the waiting list for liver transplantation.

The patient underwent orthotopic liver transplantation in November 2013. Our techniques of organ procurement and preservation have been previously described[6,7]. The liver graft was procured from a 40-year-old male donor after cardiac death. The liver graft was preserved in 4℃ UW solution. The warm ischemia time was 7.5 min and cold ischemia time was 3 h.

The native diseased liver filled about 80% of the abdominal cavity and displaced the normal vascular anatomy. The excised diseased native liver weighed 20 kg (dry weight) and measured 41 cm × 32 cm × 31 cm (Figure 2). The recipient operation was conducted according to the classical orthotopic liver transplantation procedure[8]. The whole transplant procedure took 8.5 h and the total blood volume loss was 5500 mL. A blood reinfusion system replaced 3000 mL, and an additional 10 units of packed RBC and 1000ml of plasma were infused. The anhepatic phase lasted for 1 h without the use of venovenous bypass. After release of the vascular clamps, Doppler ultrasound demonstrated the liver graft to be well perfused (Figure 3). The patient was extubated on the second day after surgery.

The immunosuppressive regimen included intra-operative induction with basiliximab and high-dose methylprednisolone, and postoperative maintenance with tacrolimus, mycophenolate mofetil and prednisone. No acute rejection episode was documented. The

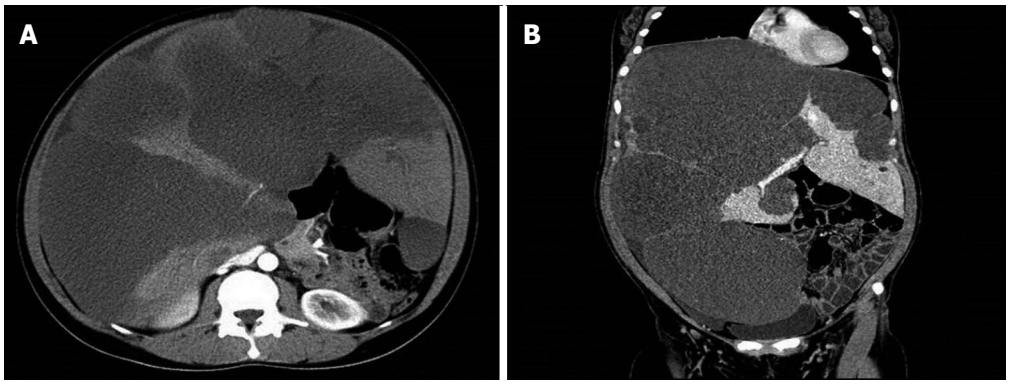

Figure 1 Contrast enhanced computed tomography of the abdomen revealed the near replacement of the liver with diffuse cystic masses of low density. A: Enhanced computed tomography scan shows the near replacement of the liver with diffuse cystic masses, leaving only small amounts of liver parenchyma. The portal vein and inferior vena cava are obviously compressed; B: The massively enlarged liver essentially occupies the entire abdominal cavity, with other abdominal organs being compressed and displaced.

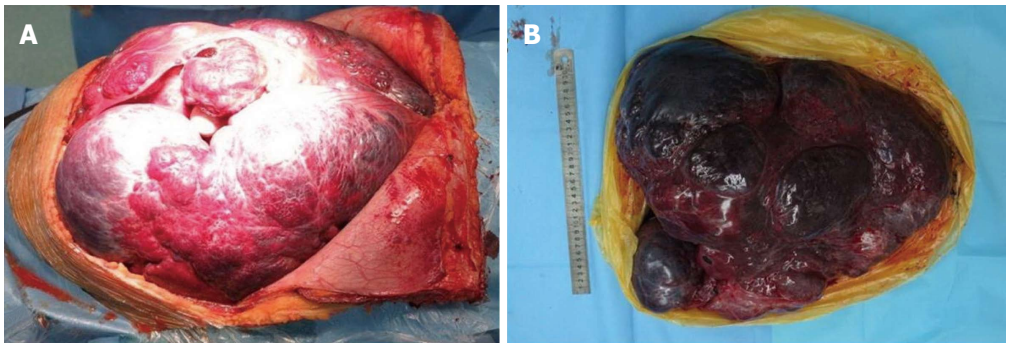

Figure 2 Intraoperative view of the tumor mass (A) and the excised liver (B).

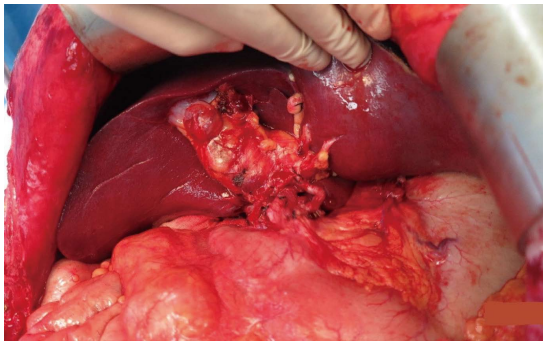

Figure 3 View of the operative field after liver transplantation, demonstrating the well-perfused liver graft.

patient was discharged home on postoperative day 20, at which time all laboratory tests were within normal limits. Three months after the operation, the immunosuppressive regimen was reduced to tacrolimus monotherapy, and the T-tube was removed after cholangiography showed no abnormalities. After 12 mo, the patient remains well and is carrying out all normal activities.

Pathologic examination of the excised diseased native liver was carried out. It contained multiple well-circumscribed masses, ranging in diameter from 2-16 cm. All masses were cystic in the central portion and contained 20-50 mL of muddy yellowish or bloody fluid. The liver mass contained dilated bile ducts with connective tissue forming multiple cysts. Histologically, corresponding to the cystic areas noted grossly, myxoid stroma and spindle cells showed smooth muscle differentiation, confirmed by positive staining for vimentin and smooth muscle actin. Benign dilated bile ducts were confirmed by positive staining for cytokeratin 7. In peripheral areas, only small amounts of liver tissue remained, with a lack of lobular architecture. There was a clear boundary between the liver parenchyma and proliferating connective tissue (Figure 4). The diagnosis of MHL was based on the typical morphological appearance, as described above.

DISCUSSION

MHL was first reported by Maresch in 1903[9]. Until relatively recently, this disease was known by different names, such as cavernous lymph adenomatoid tumor, bile cell fibroadenoma and benign mesenchymoma. The first definitive description of MHL was provided by Edmondson[1]. While the precise pathogenesis of MHL is uncertain, the most common theory relates to aberrant mesenchymal development in the portal tract, likely related to the bile ducts[10,11].

The clinical presentation of MHL appears to depend

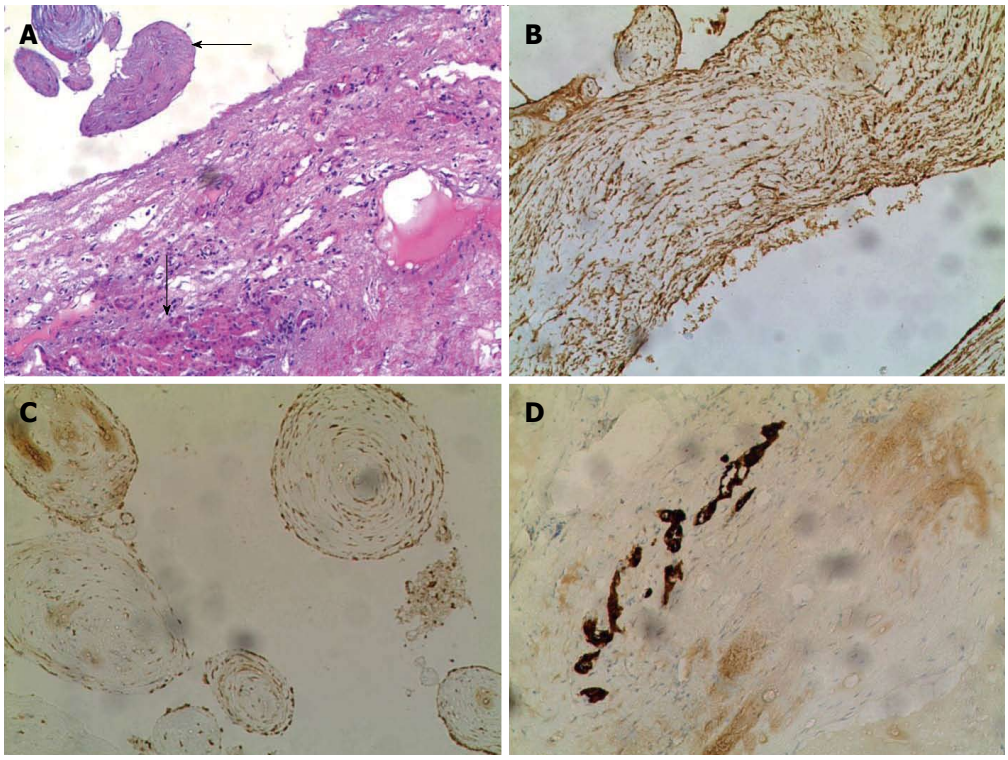

Figure 4 Clear boundary between liver parenchyma and proliferating connective tissue. A: The mass consisted of loose connective tissue full of myxoid matrix forming visible cysts (upper arrow). Small amounts of remaining liver tissue, with a lack of lobular architecture, were located in peripheral areas (lower arrow) (HE, original magnification × 100); Myxoid stroma with spindle cells showing smooth muscle differentiation were confirmed by positive staining for vimentin (B) and smooth muscle actin (C) (original magnification × 100); Benign dilated bile ducts were confirmed by positive staining for cytokeratin 7 (D) (original magnification × 100).

on the age of the patient. Most pediatric patients present with painless abdominal enlargement, normally appreciated by their parents[5]. However, in adult patients (age range: 19-87 years; females 62%, mean age 39 years; males 40%, mean age 60 years (Table 1), clinical features included hepatomegaly, and diffuse abdominal pain or pain in the right hypochondrium or left upper quadrant[12-14]. In severe cases, there may be compression of the diaphragm and lungs causing respiratory difficulties[4]. In the present case, the patient suffered from progressive abdominal distention and respiratory distress caused by the expanding multiple cystic masses distributed throughout the liver.

Concerning the localization and structure of the tumor, pediatric and adult populations have different characteristics. MHLs are more common in the left liver lobe in children. In adults, 17 cases (38%) were localized to the left lobe, 22 (49%) to the right lobe, and in six (13%) extended into both lobes (Table 1). All six cases of MHLs involving both lobes occurred in females. Among 45 cases of MHLs, 30 (67%) presented with cystic lesion, 12 (26%) with solid lesions, and three (7%) with both types. Of 30 cases of cystic MHLs, 21 (70%) were reported in females and only nine (30%) in males (Table 1).

MHLs are difficult to diagnose by laboratory tests or other investigations because of its non-specificity. Liver function tests and AFP values for MHLs are usually within normal limits[15]. Additionally, all imaging methods, including ultrasonography, CT and magnetic resonance imaging (MRI), provide nonspecific findings. The differential diagnosis of a cystic MHL includes simple liver cysts, hydatid cysts, biliary cystadenocarcinoma, and cystic metastases. If a lesion consists of a solid mass, the differential diagnosis includes focal nodular hyperplasia, hepatic adenoma, cavernous hemangioma, angiomyolipoma and hepatocellular carcinoma. In the present case, the initial abdominal enhanced CT scan revealed multiple liver cysts, which could easily have been misdiagnosed as a polycystic liver.

The diagnosis of MHL often relies on histological examination of tissue obtained by biopsy or by tumor resection; however, the histological appearance of the stromal component of an MHL can be variable. Hematoxylin and eosin (HE) staining, as well as immunohistochemical studies, have indicated MHLs as having spindle cells positive for vimentin and smooth muscle actin and negative for CD31, CD34 and S100 proteins, while the ducts stain positive for cytokeratin 7 and negative for cytokeratin 20[13,16].

MHLs have premalignant potential, particularly in adult patients[17]. The potential malignant evolution of a subset of MHLs into embryonal sarcoma or angiosarcoma supports the necessity for complete surgical excision both in children and adults[4,18]. Incomplete resection or marsupialization must be avoided because of the possibility of recurrence[19-21].

Table 1 Cases of adult mesenchymal hamartomas of the liver reported in the literature

No.	Ref.	Year	Sex	Age (yr)	Clinical manifestation	Size (cm)	Gross appearance (cystic or solid)	Liver lobe(s) affected	Surgical treatment
1	Yamamura *et al*[26]	1976	F	22	NA	NA	Cystic	Both	NA
2	Grases *et al*[27]	1979	F	19	Abdominal pain, jaundice, hepatomegaly	24 × 19 × 8	Cystic	Left	Left hepatic lobectomy
3	Li *et al*[28]	1983	F	21	Asymptomatic	17 × 10	Cystic	Right	Hemihepatectomy
4	Kawata *et al*[29]	1984	F	43	NA	22 × 15 × 10	Solid	Left	NA
5	Ishizuka *et al*[30]	1985	M	59	NA	30 × 28 × 12	Cystic	Right	NA
6	Kawakami *et al*[31]	1986	M	67	NA	NA	Cystic	Right	NA
7	Jennings *et al*[32]	1987	F	32	Asymptomatic	14 × 11	Cystic	Left	Left hepatic lobectomy
8	Kato *et al*[33]	1988	M	66	Asymptomatic	NA	Solid	Left	Left hepatic lobectomy
9	Gutierrez *et al*[34]	1988	F	30	NA	18	Both	Both	Non-resectable
10	Gramlich *et al*[35]	1988	F	28	Abdominal distention, hepatomegaly	30 × 20 × 14	Solid	Right	Right hepatic trisegmentectomy
11	Alanen *et al*[36]	1989	F	20	Asymptomatic	6 × 8	Cystic	Left	Left hepatic lobectomy
12	Ito *et al*[37]	1989	F	43	NA	16 × 16 × 7.7	Cystic	Both	NA
13	Urabe *et al*[38]	1990	F	39	Asymptomatic	1.2	Solid	Left	Left hepatic lobectomy
14	Drachenb *et al*[39]	1991	F	69	Asymptomatic	26 × 20 × 11.5	Cystic	Left	NA
15	Wada *et al*[40]	1992	M	62	Asymptomatic	6 × 6 × 4.5	Solid	Left	Hepatectomy
16	Chau *et al*[41]	1994	M	53	Abdominal pain	20 × 14 × 10	Cystic	Right	NA
17	Megremis *et al*[42]	1994	F	56	Abdominal pain	7.5	Cystic	Both	NA
18	Yamamoto *et al*[43]	1994	M	52	Abdominal discomfort, weight loss	6 × 4 × 3.5	Cystic	Left	Lateral segmentectomy
19	Chung *et al*[44]	1999	F	57	Abdominal discomfort, weight loss	6 × 4 × 3.5	Solid	Right	Right hepatectomy
20	Papastratis *et al*[45]	2000	F	21	Abdominal pain, abdominal mass	17 × 10	Cystic	Right	Right hepatectomy
21	Cook *et al*[13]	2002	F	46	Abdominal pain	6 × 4 × 5	Cystic	Right	Right hepatectomy
22	Cook *et al*[13]	2002	F	66	Cough and shortness of breath	5 × 4 × 2	Cystic	Right	Right hepatectomy
23	Cook *et al*[13]	2002	F	63	Abdominal pain	11 × 16 × 24	Solid	Left	Left hepatic lobectomy
24	Mao *et al*[46]	2002	M	44	Abdominal discomfort	2 × 2	Solid	Left	Hepatectomy
25	Mao *et al*[46]	2002	F	43	Asymptomatic	3 × 4 × 4	Cystic	Right	Right hepatectomy
26	Mao *et al*[46]	2002	M	76	Abdominal pain	4 × 5 × 4	Cystic	Right	Right hepatectomy
27	Brkic *et al*[47]	2003	M	38	Abdominal pain	8 × 5	Solid	Right	Right hepatectomy
28	Kim *et al*[48]	2003	M	NA	Asymptomatic	5	Both	Right	NA
29	Yesim *et al*[12]	2005	F	54	NA	2.5 × 2.5 × 1.5	Cystic	Left	Total cystectomy
30	Yesim *et al*[12]	2005	F	51	NA	6 × 7 × 8	Cystic	Right	Unroofing procedure
31	Kim *et al*[49]	2006	F	40	Asymptomatic	5 × 5	Cystic	Right	Right hepatectomy
32	Ayadi-Kaddour *et al*[50]	2006	F	21	NA	11 × 5	Cystic	Left	NA
33	Hernández *et al*[25]	2006	M	51	NA	19 × 13	Solid	Right	Liver transplantation (4th reported[1])
34	Chang *et al*[51]	2006	M	79	Asymptomatic	2 × 2	NA	Right	NA
35	Chang *et al*[51]	2006	F	39	Asymptomatic	5 × 5	Cystic	NA	NA
36	Li *et al*[17]	2007	F	33	Abdominal distention	16	Both	Both	NA
37	Mori *et al*[52]	2008	F	36	Abdominal distention	20 × 15 × 10	Cystic	Right	Right hemihepatectomy
38	Giunippero *et al*[53]	2009	M	87	Abdominal distention	20 × 20	Cystic	Right	Hemihepatectomy
39	Nakajo *et al*[54]	2009	M	38	Asymptomatic	5 × 5	Solid	Right	Right hepatectomy
40	Klaassen *et al*[5]	2010	F	53	NA	9 × 9 × 7.5	Cystic	Right	Hepatectomy
41	Kulkarni *et al*[55]	2010	F	20	Abdominal mass, abdominal pain	14 × 11	cystic	Right	Mass resection
42	Tucker *et al*[56]	2012	W	74	Abdominal distention, abdominal pain	18 × 15 × 13	cystic	Left	Left hepatectomy
43	Liu *et al*[57]	2013	M	42	Asymptomatic	1.5 × 1.0 × 1.0	solid	Left	Hepatectomy
44	Lakić *et al*[58]	2014	M	44	Asymptomatic	2.9 × 3.1 × 3.5	NA	Left	Hepatectomy
45	Sharma *et al*[59]	2014	M	81	Abdominal distention	21.8 × 12.3 × 18.6	cystic	Left	Hepatectomy

46	Current case	2014	F	34	abdominal discomfort, dyspnea	41 × 32 × 31	cystic	Both	Liver transplantation (5th reported[1])

[1]The first three cases of liver transplantation for mesenchymal hamartomas of the liver were reported in pediatric patients. NA: Not available.

Laparoscopic liver resection for MHLs has been reported with successful outcomes[22].

Very rarely an MHL is non-resectable, even in an experienced center, and liver transplantation may have to be considered. Tepetes *et al*[23] reported two children who underwent liver transplantation following partial resections for MHLs. One died from intraoperative bleeding and the other survived. Bejarano *et al*[24] described a neonate with a recurrent MHL (after resection) who underwent successful liver transplantation. Hernández *et al*[25] reported the first case of an MHL in an adult that was treated by liver transplantation.

In conclusion, giant MHLs in adults are extremely rare. Clinical features, laboratory results and radiographic imaging are often nonspecific and inconclusive. Surgical excision of the whole lesion is imperative for both definitive diagnosis and cure. Liver transplantation should be considered as an option in the treatment of non-resectable MHLs.

COMMENTS

Case characteristics

A 34-year-old female with a history of progressive abdominal distention and respiratory distress.

Clinical diagnosis

Physical examination revealed a grossly distended abdomen without evidence of ascites, a firm and massively enlarged liver extending below the umbilicus, and tenderness in the upper quadrant.

Differential diagnosis

Polycystic liver, hydatid cyst, biliary cystadenocarcinoma and cystic metastases.

Laboratory diagnosis

Laboratory test results were unremarkable and non-diagnostic.

Imaging diagnosis

Abdominal computed tomography scan showed multiple liver cysts, with the diameter of the largest cyst being 16 cm × 14 cm. The liver hilar structures were not clearly displayed. The adjacent organs were compressed and displaced.

Pathological diagnosis

Histological examination showed dilated bile ducts and extensive connective tissue in the liver mass, while immunohistochemical staining showed positivity for vimentin, smooth muscle actin and cytokeratin 7.

Treatment

The patient underwent orthotopic liver transplantation.

Related reports

Mesenchymal hamartoma of the liver is a rare disease in adults and only 45 patients with this condition have been reported; the references are cited.

Term explanation

Mesenchymal hamartoma of the liver is a rare and potentially premalignant lesion that presents as a solid/cystic neoplasm. The pathogenesis remains incompletely understood; however, these lesions have generally been considered to represent a developmental abnormality in bile duct plate formation.

Experiences and lessons

This case report represents a successful application of liver transplantation for adult giant mesenchymal hamartomas of the liver, which could not be treated by conventional partial hepatectomy. We recommend that liver transplantation should be considered as an option in the treatment of non-resectable benign hepatic tumors.

Peer-review

This paper is a case report of a 34-year-old woman with liver transplantation for a giant mesenchymal hamartoma of the liver. Mesenchymal hamartoma of liver is a rare disease in adults and only 31 patients have been reported to date worldwide.

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CASE REPORT

Idiopathic neonatal pneumoperitoneum with favorable outcome: A case report and review

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Author contributions: Xu C designed the research; Ji Y and Sun XY collected and analyzed the data; He TZ wrote the paper; and Liu M critically revised the paper.
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Informed consent: All study participants or their legal guardian provided informed written consent prior to study enrollment.
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Abstract

Neonatal pneumoperitoneum is a surgical emergency indicative of gastrointestinal perforation that requires immediate treatment to prevent death. There have been non-surgical conditions secondary to neonatal pneumoperitoneum (*e.g.*, mechanical ventilation, pulmonary diseases and pneumatosis cystoides intestinalis) that neonates were able to overcome without the need for abdominal exploration. Idiopathic pneumoperitoneum, although similar to perforation of the alimentary tract and the previously mentioned non-surgical conditions, is a more rare and benign condition that does not yet have a definite cause. Hence, inexperienced surgeons may have a difficult time providing the right treatment for idiopathic pneumoperitoneum. We report a case of a neonate with a massive pneumoperitoneum who obtained a favorable outcome without surgical intervention. Nonetheless, the cause of pneumoperitoneum remains unclear. We hypothesize that the right sized perforation (range: 2 mm to 4 mm in diameter) at the anterior wall of the stomach is needed for pneumoperitoneum to occur. As the baby cries (aerophagia), the air in the stomach accumulates until it can enter the intraperitoneal cavity through the leak compressed by gastric peristalsis, hence forming a large pneumoperitoneum. Small amounts of gastric juice are able to penetrate the gastric wall; therefore, no signs or symptoms of peritonitis occur. The gastric leak self-seals, preventing further passage of the air, allowing the intraperitoneal free gas to dissipate gradually. This case demonstrated that laparotomy can be avoided in neonates with idiopathic pneumoperitoneum if a timely diagnosis is established.

Key words: Intestinal perforation; Pneumoperitoneum; Newborn; Therapeutics

Core tip: Neonatal pneumoperitoneum is often deemed an emergency requiring prompt abdominal exploration

to increase the chance of survival. Supporting therapy management has been successful in treating idiopathic neonatal pneumoperitoneum with excellent outcomes. This report describes a rare case of idiopathic neonatal pneumoperitoneum without a definite cause. A favorable prognosis was achieved without laparotomy. Therefore, a conservative treatment is feasible if prompt diagnosis is ascertained.

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INTRODUCTION

Neonatal pneumoperitoneum (NP) is a rare disease often requiring an acute surgical emergency intervention to maximize survival. NP is categorized into two types: surgical and nonsurgical pneumoperitoneum. Idiopathic pneumoperitoneum is classified as nonsurgical and usually has a more favorable prognosis. However, patients with idiopathic pneumoperitoneum who underwent surgery often had negative outcomes. The ability to differentiate between idiopathic pneumoperitoneum from a highly lethal perforation of air-containing viscus may reduce surgical intervention and increase the survival rate in neonatal patients. Future research is still necessary to understand the source of the free gas in the abdomen, as well as the underlying mechanism of pneumoperitoneum. Herein, we describe a rare case of NP without an established cause.

CASE REPORT

A newborn female (8 d and 16 h old) was admitted to our department with an 8 d long tachypnea and abdominal distension lasting 4 d. Prenatal examination was uneventful. The patient's birth weight was 2950 g. The baby was delivered vaginally from a gravida 2, para 2 mother at the 37th week of gestation. The Apgar score was 8 and 10 at 1 and 5 min, respectively. Upon admission, the baby weighed 2500 g; abdominal distension was visible and the bowel sound was weak. Chest examination showed no abnormal findings. Laboratory evaluation noted a white blood cell count of 18420/mm^3 with 75.2% segmented neutrophils. The anteroposterior X-ray in the lateral position showed free gas in the right part of the abdomen surrounding the liver and intestine (Figure 1). These results differed from the ones obtained four days earlier when radiograph results showed no free gas under the diaphragm. The inflated stomach was decompressed using a gastric tube.

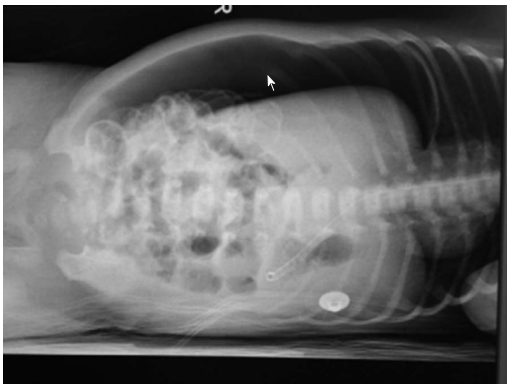

Figure 1 Neonate's radiograph on the 8th d after birth revealed massive free gas in the right part of the abdomen on the liver and intestine.

The initial diagnosis was neonatal gastrointestinal perforation. The recommended treatment plan included an immediate abdominal exploration. However, the patient's parents found the operational risk and expenses too high and refused the treatment. The newborn was released home where she was first fed with a little bit of water followed by breast milk on the third day. The neonate made a bowel movement on the first day after being released and her stool appeared to be normal. The parents took the baby to the hospital four days later; the radiograph indicated most of the free gas had dissolved. The baby appeared to be in good condition and was asymptomatic. A roentgenogram of the abdomen on the 22nd d after the initial admission showed intraperitoneal free air was absent. The 4 year follow-up (twice per year) check-ups, including clinical and radiological examination, were uneventful.

DISCUSSION

An X-ray taken in an upright position showing free gas under the diaphragm usually leads to the diagnosis of gastrointestinal perforation. Our patient was diagnosed with alimentary perforation, a controversial diagnosis. The large amount of free gas observed in our patient is very uncommon. Furthermore, the patient lacked symptoms associated with general peritonitis, such as fever and feeding intolerance. Therefore, a diagnosis of hollow viscus perforation could not be made.

Diesen *et al*[1] reported a case of neonatal intestinal perforation caused by necrotizing enterocolitis (NEC) which was spontaneously sealed by omentum, suggesting the possibility that NEC patients are able to heal without laparotomy. Other instances of nonsurgical pneumoperitoneum have been reported, such as pneumoperitoneum secondary to mechanical ventilation and tension pneumothorax[2]. More recently, the relationship between pneumoperitoneum and respiratory diseases has been widely debated[3-5]. Macklin[6,7] demonstrated in cats with ventilation assistance that at higher levels of intrathoracic pressure, alveoli could rupture, allowing air to enter the interstitial

Table 1 Details of idiopathic neonatal pneumoperitoneum in the literature

Ref.	Age at onset (d)	Birth weight (g)	Gestation (wk)	Delivery mode (V or C)	Apgar score (1, 5 min)	Clinical sign	Peritonitis (Yes or No)	WBC, N (109, %)	Exploration (Yes or No)	Findings (P or N)	Survival (Yes or No)
Vohra *et al*[18], 1992	2	1300	30	V	6, 7	AD, RD	N	3.6, 28	Y	N	Y
Khan *et al*[22], 2011	12	1700	38-42	V	U	AD	N	14.5, U	N	-	Y
	12	1900	34	V	U	AD	U	U	N	-	Y
Bedi *et al*[20], 1991	0-1	2400	U	C	U	AD, RD	N	U	Y	N	Y
Shah *et al*[17], 1992	5	2300	36	U	8, 10	AD	N	U	Y	N	Y
Steves *et al*[21], 1987	U	U	U	U	U	AD, HMD	N	U	Y	N	Y
Porter[19], 1956	2	2900	U	C	U	RD, EC	N	U	Y	N	Y

V: Vaginal delivery; C: Cesarean section; WBC: White blood cell count; P: Positive; N: Negative; U: Unmentioned; AD: Abdominal distention; RD: Respiratory distress; HMD: Hyaline membrane disease; EC: Extremity cyanosis.

tissues and the mediastinum along the perivascular sheaths of the lung, reaching the abdomen. Based on Macklin's theory, Eisen[8] concluded that the distribution of the gas in the patients experiencing ventilation must have been the same as that in experimental animals. Other communication pathways between the chest and abdomen include the periaortic and periesophageal space and congenital defect[9,10] or pleuroperitoneal fistula[11] that permit the air to pass through. Our patient did not experience positive end-expiratory pressure ventilation and there was no evidence of pneumothorax or pneumomediastinum. Therefore, we concluded that the alveoli in the thoracic cavity were not the source of the intraperitoneal free gas.

Under rare circumstances, NP can also be seen with pneumatosis cystoides intestinalis (PCI)[12]. Although there was no indication of the gas in the submucosal and subserosal spaces of our patient's intestine in the plain film, the PCI diagnosis cannot be excluded. Tiny free gas within the intestinal wall (linear sign) on plain film is difficult to identify because of the interruption of the overlapped organ or tissue image, especially when rupture of the gas-filled cyst occurs. A computed tomography scan allows for a defined PCI diagnosis; however, we were not able to obtain one for our patient. In most cases, PCI is seen in preterm neonates with NEC[13,14]. NEC is characterized by abdominal distension, gastrointestinal bleeding, abdominal tenderness (even sepsis and shock at advanced stage) and the presence of PCI on abdominal X-ray film[14,15]. Our patient was born at the 37th week of gestation (a full-term) with normal body weight and did not display typical symptoms or signs of NEC other than abdominal distention and tachypnea. Considering that the presence of PCI in neonates combined with massive pneumoperitoneum is extremely rare, we cautiously excluded the NEC diagnosis.

In the literature, idiopathic pneumoperitoneum has been reported as a condition with no demonstrable risk factor for the development of intraperitoneal free gas production[16]. The results of the literature review on the cases of idiopathic pneumoperitoneum are listed in Table 1. Of seven cases, five had a negative laparotomy[17-21] while the rest[22] survived with watchful therapy, thus avoiding the laparotomy. Retrospectively, there would not have been positive findings like intestinal perforation or general peritonitis if abdominal exploration was undertaken on our patient; therefore, the diagnosis of idiopathic pneumoperitoneum was appropriate considering our patient did not have typical symptoms of either general peritonitis or NEC.

The underlying cause and mechanism of pneumoperitoneum remain unclear. The current consensus is that gastric tissue ischemia, secondary to hypoxia, is responsible for the etiology of spontaneous neonatal gastric perforation. In contrast, the theory that the gastric leak is a mechanical disruption is becoming more acceptable[23]. It has been postulated that a competent anti-reflux mechanism at the gastroesophageal junction and a proximal gastric obstruction, caused by an angulation at the hiatus due to sudden gastric distention, are the main factors for gastric leak formation.

We propose a novel hypothesis based on the literature review and our clinical experience. In neonatal patients, a single perforation on the anterior wall, frequently close to the greater curvature[23], is formed due to ischemia[24] or mechanical disruption[23] of the gastric wall. The stomach acts like a partially-filled bag. The diameter of the leak is neither too big nor too small, ranging from 2 mm to 4 mm at the empirical base. At the lower range, the leak is more prone to self-sealing. In contrast, large perforations permit the free gas to pass through into the intraperitoneal cavity. As a result, large amounts of air are swallowed into the stomach when newborns cry (also called aerophagia), thus gradually accumulating pressure inside the stomach. A single-direction valve mechanism is essential for pneumoperitoneum production until the pressure inside the stomach and peritoneal cavity equalizes. Considering newborns spend most of their time in a supine position, the gastric juices at the bottom of the stomach have a low chance of entering the peritoneal cavity from the anterior wall of the stomach during gastric peristalsis. The strong gastric muscle generates

enough power for continuous compression of the air in the stomach into the peritoneal cavity, forming a large scale pneumoperitoneum. The thickness of the gastric wall plays an important role in preventing the gastric juice from penetrating the gastric wall. The leak disappears with the development of a self-sealed mechanism once there is no air being compressed through the leak when the inner and outer pressures of the stomach wall equalize. Air in the peritoneal cavity gradually gets absorbed by the peritoneum and viscera.

In conclusion, when there are no abdominal physical signs indicative of peritonitis and the neonatal patient has a normal body temperature and white cell count, conservative management is preferred. Special attention should be paid to neonates because negative laparotomies would jeopardize their already precarious conditions.

ACKNOWLEDGMENTS

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COMMENTS

Case characteristics

A newborn female presented with tachypnea and abdominal distension.

Clinical diagnosis

The Apgar score of the newborn was 8 and 10 at 1 and 5 min, respectively, and inspection revealed obvious abdominal distension; auscultation revealed weak bowel sounds.

Differential diagnosis

Neonatal pneumoperitoneum (including idiopathic and secondary ones), alimentary tract perforation, necrotizing enterocolitis presenting signs of pneumatosis cystoides intestinalis.

Laboratory diagnosis

White blood cell count of 18420/mm^3 with 75.2% segmented neutrophils.

Imaging diagnosis

The lateral position anteroposterior X-ray showed a significant amount of free gas in the right part of the abdomen on the liver and intestine.

Pathological diagnosis

There was no specimen for pathological diagnosis.

Treatment

The patient received supporting therapy such as fluid resuscitation.

Related reports

Rare cases reported in the literature mentioned that idiopathic neonatal pneumoperitoneum can be cured with conservative methods; the patients who underwent an operation had negative outcomes.

Term explanation

Idiopathic neonatal pneumoperitoneum, also termed benign, non-surgical and spontaneous, is a rare entity where free gas enters the abdominal cavity through an unknown passage without any leading risk factors and is often mistaken for hollow viscus perforation.

Experiences and lessons

The case report presents a neonate with a massive pneumoperitoneum who obtained a favorable outcome without any surgical intervention; differential diagnoses included idiopathic neonatal pneumoperitoneum, gastrointestinal perforation and another non-surgical condition presenting as pneumoperitoneum.

Peer-review

In this article, the authors reported a rare case of neonatal pneumoperitoneum without a definite cause and explained the interesting phenomenon by proposing a hypothesis based on the literature review. This case is clinically relevant and illustrated that laparotomy could be avoided in neonates if a timely diagnosis of benign pneumoperitoneum is made.

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CASE REPORT

Reversible sinusoidal obstruction syndrome associated with tacrolimus following liver transplantation

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Author contributions: Shen T treated the patient and drafted the paper; Feng XW performed pathological analysis; Geng L did radiological analysis; Zheng SS designed the study; and all authors have read and approved the final version to be published.

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Ethics approval: The study was reviewed and approved by the Zhejiang University Institutional Review Board.

Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.

Conflict-of-interest: The authors declare that there is no conflict of interest in this study.

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Abstract

Sinusoidal obstruction syndrome (SOS), previously known as hepatic veno-occlusive disease, is a rare disorder in solid organ transplant patients, and is an uncommon complication after liver transplantation. Severe SOS with hepatic failure causes considerable mortality. Tacrolimus has been reported to be an offending agent, which potentially plays a role in the pathophysiological process of SOS. SOS due to tacrolimus has been reported in lung and pancreatic transplantations, but has never been described in a liver transplant recipient. Herein, we present a case of SOS after liver transplantation, which was possibly related to tacrolimus. A 27-year-old man developed typical symptoms of SOS with painful hepatomegaly, ascites and jaundice after liver transplantation, which regressed following withdrawal of tacrolimus. By excluding other possible predisposing factors, we concluded that tacrolimus was the most likely cause of SOS.

Key words: Liver transplantation; Sinusoidal obstruction syndrome; Veno-occlusive disease; Tacrolimus; Predisposing factor

Core tip: We describe a rare case of sinusoidal obstruction syndrome following liver transplantation, which was possibly related to tacrolimus. We believe that this condition is uncommon and has rarely been reported in liver transplant recipients.

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INTRODUCTION

Sinusoidal obstruction syndrome (SOS), previously

known as hepatic veno-occlusive disease, is a rare disorder with the unique etiopathogenesis of toxic injury to hepatic sinusoids, which induces progressive fibrotic obliteration of centrilobular veins. Painful hepatomegaly, ascites and jaundice are typical symptoms of SOS[1-3]. In general, SOS is a difficult condition, in which 16%-50% of patients are likely to develop irreversible illness, and have a fatal outcome due to hepatic failure. Severe SOS causes mortality in approximately 84%-90% of the patients[2,4].

SOS can occur in post-transplant patients, and the majority of research has been carried out in post-hematopoietic stem cell transplantation (HSCT) patients related to preconditioning treatment[1,2,4]. A limited number of cases of SOS have been reported after renal, lung, pancreatic and liver transplantations[5-8]. In liver transplantation, SOS is unusual, and azathioprine therapy or acute rejection is considered the most common etiology[8-10]. Tacrolimus may be another possible and rare pathogenic agent as it has potential cytotoxicity to endothelial cells and precipitates their dysregulation[11]. To the best of our knowledge, SOS due to tacrolimus has been reported in lung and pancreatic transplantations, but has never been described after liver transplantation[6,7]. Herein, we present a case of SOS following liver transplantation, who achieved complete clinical remission after discontinuation of tacrolimus.

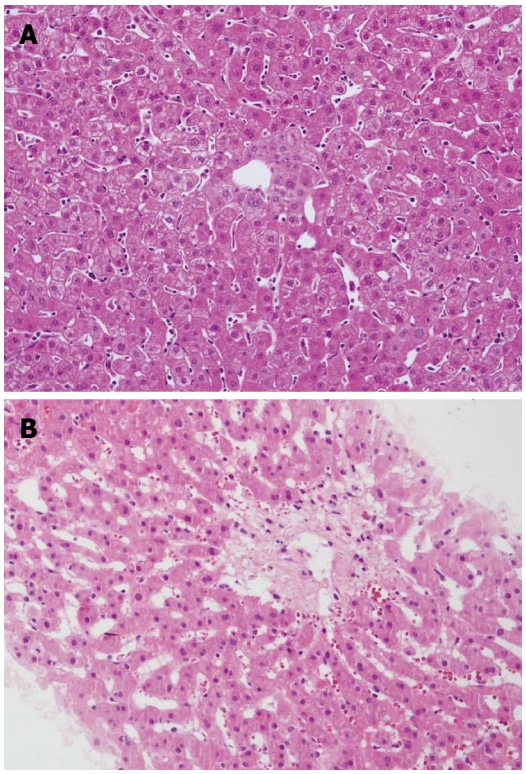

Figure 1 Biopsy of the donated graft at the time of transplantation was normal (HE, × 200) (A) and liver biopsy showed sinusoidal congestion and fibrosis of centrilobular veins at 88 d after transplantation (HE, × 200) (B).

CASE REPORT

A 27-year-old man underwent an ABO-identical liver transplantation for acute hepatic failure due to hepatitis B. The graft was obtained from a donor after cardiac death with a warm ischemia time of 5 min and cold ischemia time of 8 h. No specific pathology was observed on biopsy of the donated graft at the time of transplantation (Figure 1A). Operation time was 6 h with satisfactory reconstruction of vessels and biliary duct. Early post-operative recovery period was uneventful. Piperacillin-tazobactam, fluconazole and ganciclovir were administered as prophylaxis against infection. Entecavir and hepatitis B immunoglobulin were administered as prophylaxis against hepatitis B virus recurrence. A routine immunosuppressive regimen consisting of tapering prednisone, tacrolimus and mycophenolate mofetil was applied. Remission of hepatic function and coagulation was achieved one week after transplantation. The patient was discharged on normal graft function with excellent flow in the hepatic veins, portal vein and hepatic artery on day 20 (Figure 2A and B).

The patient remained stable on tacrolimus (trough level 7-10 ng/mL), mycophenolate mofetil and entecavir for two months, but was hospitalized on day 80 due to anorexia, abdominal pain and polypnea. No natural remedies before the current admission were used. Physical examination revealed palpable liver 3 cm below the ribcage and positive shifting dullness. Serological tests, including liver function, renal function, routine blood examination, coagulation and tacrolimus concentration (trough level of tacrolimus was 8.9 ng/mL), were normal. Serological markers of viral infection such as hepatitis A, B, C, D and E, cytomegalovirus and Epstein-Barr virus were negative. Ultrasonography demonstrated ascites and enlarged liver with regular blood flow. Computed tomography (CT) showed enlarged liver with patchy enhancement, thin hepatic veins and massive ascites (Figure 2C and D). Initial medical treatment with a diuretic was ineffective. The patient's condition continued to deteriorate, accompanied by weight gain of 3 kg above his baseline, obvious abdominal distention and high bilirubin (57 μmol/L on day 87). Faint yellow ascites and pleural effusion were drained to alleviate the symptoms. A percutaneous liver biopsy was obtained under ultrasound guidance after complete drainage of ascites on day 88, which showed the pathological findings of sinusoidal congestion and fibrosis of centrilobular veins (Figure 1B). Moreover, no evidence of acute rejection or viral hepatitis was found on pathology. All the findings supported the diagnosis of SOS, and excluded the possibility of chylous fistula, obstruction of outflow, hepatitis recurrence or rejection. He was started on a regimen of defibrotide for 2 wk, which was then stopped due to lack of efficacy, with increased bilirubin (76 μmol/L on day 104), rapidly expanding ascites and obvious weight gain. Tacrolimus

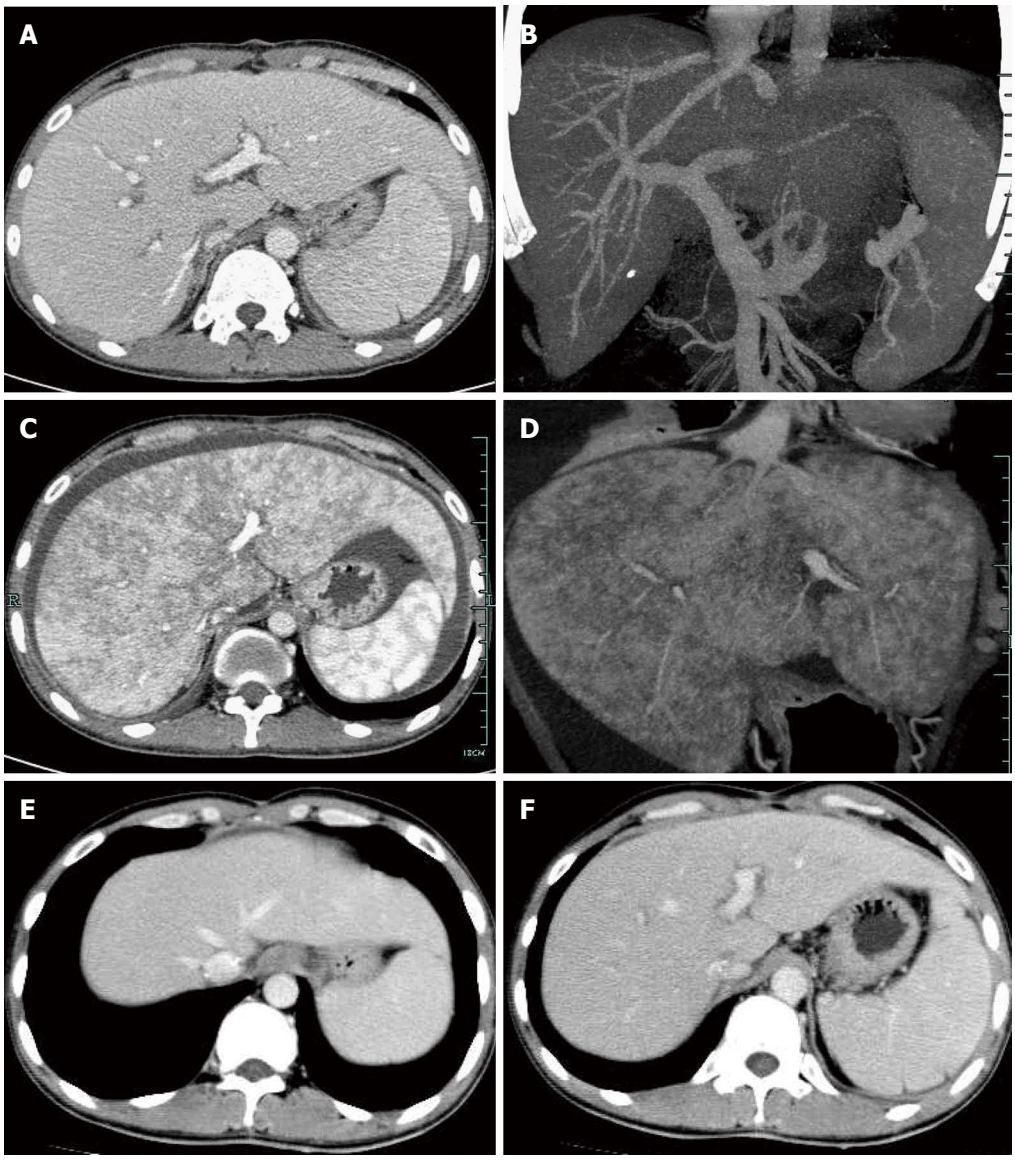

Figure 2 Computed tomography revealed excellent reconstructed blood flow in the hepatic veins, portal vein and hepatic artery at 14 d after transplantation (A, B), enlarged liver with patchy enhancement, obscure hepatic veins and massive ascites at 80 d (C, D), and normal radiologic presentations with resolved ascites and patchy enhancement, and recovered hepatic vein flow at 120 d (E, F).

concentration was controlled at 5-7 ng/mL and was subsequently discontinued as it was considered the only offending drug and was replaced by cyclosporine A at a concentration of 150 ng/mL. Complete resolution of clinical manifestations such as ascites and graft dysfunction was observed, with normalized radiologic presentations (Figure 2E and F) during the 2-wk period after tacrolimus discontinuation. The patient has remained asymptomatic on cyclosporine A, mycophenolate mofetil and entecavir for 6 mo. The clinical data including graft function, coagulation, immunosuppressant level and body weight are presented in Table 1.

DISCUSSION

Certain toxic drugs containing pyrrolizidine alkaloids, chemo-irradiation preconditioning regimens in stem cell transplantation and immunosuppressive therapy with azathioprine are common predisposing factors for SOS[4,5,12,13].

SOS is a rare finding after liver transplantation, but can be an infrequent cause of graft dysfunction. According to a review article, approximately 1.9% of cadaveric liver transplant recipients suffered from SOS after transplantation[10]. Another investigation of 1346 biopsy samples obtained after liver transplantation showed an SOS incidence of 2.3%[8]. Clinical manifestations such as body weight gain, ascites, hepatomegaly and jaundice are recognized as diagnostic clues for SOS[2]. It is important that common post-operative complications such as acute rejection, obstruction of outflow (anastomotic stenosis or twisting of the hepatic vein), biliary complications, and viral hepatitis should be excluded in post-liver transplant individuals. Unique pathologic findings characterized by

Table 1 Clinical characteristics during disease course

Days after LT	50	80	87	104	111	118
Clinical course		Current admission	Before defibrotide	After defibrotide/TAC withdrawal		Resolution
ALT (U/L)	17	35	39	43	26	23
TB (μmol/L)	16	20	57	76	42	13
Platelet (10^9/mL)	156	171	183	175	158	173
PT (s)	11.5	11.3	12.5	12.7	11.5	12.2
D-dimer (mg/L)	0.27	0.34	0.52	0.41	0.32	0.33
TAC (ng/mL)	7.9	8.9	7.2	5.6		
CSA (ng/mL)					126	165
Wt (kg)	64.5	66.0	67.9	69.5	67.5	64.8

LT: Liver transplantation; ALT: Alanine aminotransferase; TB: Total bilirubin; PT: Prothrombin time; TAC: Tacrolimus; CSA: Cyclosporine A; Wt: Body weight.

fibrosis and obliteration of hepatic centrilobular veins, hemorrhagic centrilobular necrosis, and sinusoidal congestion are suggestive of the diagnosis of SOS, which ultimately result in outflow obstruction, portal hypertension and hepatic injury[1]. Enlarged liver with patchy enhancement, ascites, usually accompanied by pleural effusion, and obscure main hepatic veins due to congestive liver, are the most typical presentations on CT or magnetic resonance imaging. In particular, the area of the liver where veno-occlusion occurs may show relatively lower enhancement and form a patchy enhancement sign, which is rare in other liver disorders. Therefore, it is the most valuable radiologic feature in diagnosing SOS, and the grade of patchy enhancement is also associated with clinical severity[12]. Although the mortality rate for SOS is unclear, the deteriorating manifestations of ascites and graft failure could be a threat to survival, making re-transplantation inevitable[8].

Azathioprine was implicated as the main predisposing factor for onset of SOS in post-liver transplant patients when it was widely used in the last century, due to its vascular hepatotoxicity[9]. In addition, an immunological reaction has been proven to participate in the pathophysiological process of SOS after liver transplantation, and it is suggested that SOS is part of the presentation of rejection with endothelial predilection[8,10]. Therefore, the disease could be reversed by intensive immunosuppressive treatment with corticosteroids or intervention for antibody-mediated rejection in liver transplant recipients[14]. However, the exact causative factor is still undetermined[8]. It should be emphasized that the determination of SOS etiology is important as withdrawal of the offending drug plays a key role in the treatment of SOS following liver transplantation.

Tacrolimus, one of the most widely used calcineurin inhibitors, is safe and efficient in the prophylaxis and treatment of acute rejection in organ transplantation. However, as tacrolimus has potential cytotoxicity to endothelial cells and precipitates their dysregulation, it is suspected to contribute to the onset of SOS in some cases, although the pathogenic mechanism remains to be elucidated[11].

According to the published literature, there have only been two cases of SOS described after lung and pancreatic transplantation, which were clinically proven to be induced by tacrolimus[6,7]. The present case is the first to show a definite association between tacrolimus and SOS after liver transplantation. Our patient developed refractory ascites which was the most obvious complaint other than slightly increased jaundice. This probably indicated that sinusoidal injury had a significant effect on hepatic outflow and contributed to portal hypertension, but had a mild influence on hepatic metabolism of bilirubin in this case. Although recommended in reported guidelines[2], defibrotide therapy for this patient lacked efficacy. At the onset of the disease, it was difficult to confirm the predisposing factor. Considering that the patient had never been exposed to azathioprine or other specific suspicious drugs, with the exception of tacrolimus, and there was no evidence of acute rejection, tacrolimus was considered the offending drug, and was therefore discontinued. Clinical and radiological regression was observed following withdrawal of tacrolimus.

In conclusion, we describe a patient with SOS following liver transplantation. The possible causative drug was tacrolimus as complete recovery was observed after its withdrawal. Transplant surgeons should be aware of this rare condition following liver transplantation, and tacrolimus should be considered as a possible causative agent.

COMMENTS

Case characteristics

A 27-year-old man presented with anorexia, abdominal pain, jaundice and body weight gain after liver transplantation.

Clinical diagnosis

Sinusoidal obstruction syndrome which led to ascites, hepatomegaly and graft dysfunction.

Differential diagnosis

Acute rejection, hepatitis B recurrence, anastomotic stenosis of the hepatic vein.

Laboratory diagnosis

Alanine aminotransferase (43 U/L), total bilirubin (76 μmol/L), tacrolimus (5.6 ng/mL), platelet count (175 × 10^9/mL), D-dimer (0.41 mg/L), hepatitis B surface antigen, hepatitis B virus - DNA, anti- hepatitis A virus IgM/IgG, hepatitis C virus

- RNA, hepatitis D virus - RNA, anti- hepatitis E virus IgM/IgG, cytomegalovirus pp65 and epstein-Barr virus - DNA were all negative.

Imaging diagnosis

Computed tomography showed enlarged liver with patchy enhancement, thin hepatic veins and massive ascites.

Pathological diagnosis

Biopsy of the graft revealed the pathological findings of sinusoidal congestion and fibrosis of centrilobular veins.

Treatment

Tacrolimus was discontinued as the only suspicious offending drug and replaced by cyclosporine A.

Related reports

Sinusoidal obstruction syndrome (SOS) due to tacrolimus has been reported in lung and pancreatic transplantations, but has never been described after liver transplantation.

Term explanation

SOS is an unusual clinical syndrome characterized by hepatomegaly, ascites, and jaundice, with the unique pathological findings of fibrosis and obliteration of hepatic centrilobular veins, hemorrhagic centrilobular necrosis, and sinusoidal congestion, due to injury of sinusoidal endothelial cells.

Experiences and lessons

Transplant surgeons should be aware of this rare condition following liver transplantation, and tacrolimus should be considered as a possible causative agent.

Peer-review

The authors report a case of patient with sinusoidal obstruction syndrome following liver transplantation due to hepatitis B-related acute hepatic failure. It is the first reported case of tacrolimus-related sinusoidal obstruction syndrome in liver transplant recipient. The paper is well structured and except some minor language issues well written.

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