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EDITORIAL

Does endoscopic ultrasound-guided biliary drainage really have clinical impact?

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Abstract

The well established, gold standard method for treatment of obstructive jaundice involves biliary drainage under endoscopic retrograde cholangiopancreatography (ERCP) performed by pancreatobiliary endoscopists. Recently, interventions using endoscopic ultrasound (EUS) have been developed not only for obtaining cytological and histological diagnosis, but also for biliary drainage as alternative method. EUS-guided biliary drainage (EUS-BD) was first reported by Giovannini *et al*. EUS-BD broadly includes EUS-guided rendezvous technique, EUS-guided choledochoduodenostomy, and EUS-guided hepaticogastrostomy. More recently, EUS-guided antegrade stenting and EUS-guided gallbladder drainage have also been reported. many case reports, series, and retrospective studies on EUS-BD have been reported. However, because prospective studies and comparisons between the different biliary drainage methods have not been reported, the technical success, functional success, adverse events, and stent patency with long-term follow up of EUS-BD are still unclear. Therefore, prospective, randomized controlled studies addressing these issues are needed. Despite this, EUS-BD undoubtedly is clinically useful as an alternative biliary drainage method. EUS-BD has the potential to be a first-line biliary drainage method instead of ERCP if results of clinical trials are favorable and the technique is simplified.

Key words: Endoscopic ultrasound; Endoscopic ultrasound-guided biliary drainage; Endoscopic ultrasound-guided hepaticogastrostomy; Endoscopic ultrasound-guided choledochoduodenostomy; Endoscopic ultrasound-guided antegrade stenting; Endoscopic ultrasound-guided gallbladder drainage

Core tip: To date, many case reports, series, and retrospective studies on EUS-guided biliary drainage (EUS-BD) have been reported. However, because prospective studies and comparisons between the different biliary drainage methods have not been reported, the technical success, functional success, adverse events, and stent patency with long-term follow up of EUS-BD are still unclear. Therefore, prospective, randomized controlled studies addressing these issues are needed. Despite this, EUS-BD undoubtedly is clinically useful. EUS-BD has the potential to be a first-line biliary drainage method instead of endoscopic retrograde cholangiopancreatography if results of clinical trials are favorable and the technique is simplified.

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INTRODUCTION

The well established, gold standard method for treatment of obstructive jaundice involves biliary drainage under endoscopic retrograde cholangiopancreatography (ERCP) performed by pancreatobiliary endoscopists[1-3]. Percutaneous transhepatic cholangiography (PTC) has also been established as an alternative method for biliary drainage[4,5]. However, PTC is associated with several complications, such as cholangitis, bile leakage, and pneumothorax. Moreover, the frequency of major complications, leading to prolonged hospital stay and permanent adverse sequelae, is 4.6%-25%, and that of procedure-related deaths is 0%-5.6%[4,5]. Cosmetic issues due to external drainage also compromise the patient's quality of life. Moreover, a large amount of ascites is a contraindication for PTC. Recently, interventions using endoscopic ultrasound (EUS) have been developed not only for obtaining cytological and histological diagnosis (EUS-guided fine needle aspiration), but also for biliary drainage. EUS-guided biliary drainage (EUS-BD) was first reported by Giovannini *et al*[6]. EUS-BD broadly includes EUS-guided rendezvous technique (EUS-RV)[7,8], EUS-guided choledochoduodenostomy (EUS-CDS)[9,10], and EUS-guided hepaticogastrostomy (EUS-HGS)[11,12]. Recently, EUS-guided antegrade stenting (EUS-AS)[13,14] and EUS-guided gallbladder drainage (EUS-GBD)[15,16] have also been reported.

Technical evaluation of EUS-BD

EUS-RV is mainly indicated for failed ERCP. This technique involves puncture of the intrahepatic or common bile duct using a 19G needle, following which a guidewire is advanced toward the duodenum through the site of stenosis or the ampulla of Vater. However, the technical success rate of this procedure is not very high (70%-100%)[17]. In addition, this technique is not indicated for cases of duodenal obstruction that are caused by tumor invasion, or those with altered anatomy, such as following the Roux-en-Y procedure. To enhance the technical success rate, the puncture needle and the guidewire should be stiff or include some additional technical features.

EUS-CDS is also normally indicated for failed ERCP. Performance of EUS-CDS requires puncture of the extrahepatic bile duct; therefore, this technique is indicated in cases of duodenal obstruction that do not involve the duodenal bulb. The extrahepatic bile duct is punctured by a 19G needle, the guidewire is inserted, and the fistula is dilated using a needle knife, dilator, or balloon dilator. Finally, a fully covered metallic stent is usually placed from the common bile duct to the duodenum. High technical and clinical success rates of this procedure have been reported. The biggest advantage of this method is that it is not associated with acute pancreatitis. EUS-CDS, thus, has the potential to be the biliary drainage method of choice instead of ERCP, although this needs to be confirmed by a randomized controlled trial comparing ERCP and EUS-CDS.

EUS-HGS has the widest indications among the different EUS-BD procedures. It can be performed in patients with altered anatomy, duodenal obstruction, and hepatic hilar obstruction[18]. In this procedure, the intrahepatic bile duct (segment 3) is punctured using a 19G needle, and the guidewire is advanced. Various devices are then used to dilate the fistula. Park *et al*[19] reported the predictors of adverse events with EUS-BD. In their study, post-procedure adverse events developed after EUS-BD in 11 patients (20%). Multivariate analysis demonstrated that use of a needle knife was the single most important risk factor for post-procedure adverse events after EUS-BD (OR = 12.4; P = 0.01). Hence, balloon or dilator catheters may be suitable for dilation of the fistula. In addition, metallic stents should also be used to avoid bile leakage. However, this technique is associated with the risk of fatal adverse events, such as stent migration[20]. If its adverse events can be minimized by various efforts, EUS-HGS may become the EUS-BD technique of choice because of its wide indications.

EUS-AS may also be a promising drainage method. After the intrahepatic bile duct is punctured using a 19G needle, the guidewire is advanced through the site of obstruction. Thereafter, a stent deliverer is inserted and the stent is placed in a trans- or supra-papillary position. In this technique, compared with EUS-HGS, stent migration does not occur, indicating that it seems to be a safe technique. However, re-intervention following stent occlusion, if required, can be challenging. If occlusion of the EUS-AS stent was to occur, we would need to do either of the following: puncture the intrahepatic bile duct and perform EUS-HGS, or place another stent inside the occluded stent. However, the intrahepatic bile duct may not always be dilated enough to allow for puncturing[21]. For this reason, EUS-AS should only be performed in selected patients, such as those with a limited prognosis.

EUS-GBD is probably the most easily performed of all the EUS-BD procedures, because the gallbladder presents a large target for puncture. The gallbladder can be visualized from the antrum or duodenal bulb. After it is punctured using a 19G needle, the guidewire is inserted. Then, the fistula

is dilated using a dilation or balloon catheter, while a pig tail type plastic stent is usually placed (sometimes combined with a metallic stent) to prevent stent migration. This technique is indicated in patients whose cystic duct is intact. If the cystic duct is invaded by tumor, stent dysfunction can occur. Although EUS-CDS or EUS-HGS is usually performed in cases requiring re-intervention, the patient's condition may not be suitable for re-intervention because of tumor progression. In such cases, when performance of EUS-CDS or EUS-HGS is challenging, EUS-GBD may be performed.

To date, there are no reports of randomized controlled studies comparing ERCP with EUS-BD. However, recently, a retrospective study comparing PTC and EUS-BD has been reported[22]. In this paper, of the 73 patients with failed ERCP complicated by distal malignant biliary obstruction who were included, EUS-BD was performed in 22 patients and PTC in 51 patients. Although the technical success rate of PTC was higher than that of EUS-BD, the adverse event rate and total cost were also higher than those of EUS-BD. Interestingly, EUS-BD is associated with a decreased adverse event rate and is significantly less costly due to the need for fewer re-interventions. However, these results should be further evaluated in a prospective clinical trial.

Techniques to minimize adverse events following EUS-BD

According to recent literature reviews of EUS-BD, the adverse event rates of these procedures are still high[17]. Reportedly, several techniques and devices have been introduced to reduce the adverse event rates. In EUS-CDS or EUS-GBD, novel metallic stents have been used to prevent stent migration. Itoi *et al*[23] reported the technique of EUS-GBD using AXIOS stent (Xlumena Inc., Mountain View, CA, United States). This stent is a fully covered, 10 mm diameter, 10 mm long braided stent with bilateral 20 mm diameter anchor flanges. Perez-Miranda *et al*[24] reported using this novel stent for EUS-CDS. This unique stent design may be effective in preventing stent migration. In addition, Teoh *et al*[25] described a simplified method of EUS-GBD using a novel cautery-tipped stent delivery system. However, since use of these novel stents or methods has only been reported as case reports, additional case studies and trials are required for further development of EUS-CDS and EUS-GBD as safe, simple, and effective biliary drainage methods. Likewise, several methods for improving the results of EUS-HGS have also been reported. The clinical impact of EUS-HGS combined with EUS-AS[21] and a novel method of stent placement of EUS-HGS[26] have been previously reported. Recently, Song *et al*[27] performed 10 EUS-HGS cases using a novel hybrid metallic stent that has proximal and distal antimigration flaps at both ends of the covered portion. Paik *et al*[28] described a simplified and modified technique of EUS-HGS, which resulted in a shorter procedural time ($P < 0.001$) and less frequent early adverse events ($P = 0.02$) compared with the conventional technique. Yet, although various techniques have been reviewed[29], the best techniques and devices still need to be determined by a prospective study.

CONCLUSION

In conclusion, to date, many case reports, series, and retrospective studies on EUS-BD have been reported. However, because prospective studies and comparisons between the different biliary drainage methods have not been reported, the technical success, functional success, adverse events, and stent patency with long-term follow up of EUS-BD are still unclear. Therefore, prospective, randomized controlled studies addressing these issues are needed. Despite this, EUS-BD undoubtedly is clinically useful as an alternative biliary drainage method. EUS-BD has the potential to be a first-line biliary drainage method instead of ERCP if results of clinical trials are favorable and the technique is simplified.

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REVIEW

Management of obstructed defecation

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Abstract

The management of obstructed defecation syndrome (ODS) is mainly conservative and mainly consists of fiber diet, bulking laxatives, rectal irrigation or hydrocolontherapy, biofeedback, transanal electrostimulation, yoga and psychotherapy. According to our experience, nearly 20% of the patients need surgical treatment. If we consider ODS an "iceberg syndrome", with "emerging rocks", rectocele and rectal internal mucosal prolapse, that may benefit from surgery, at least two out of ten patients also has "underwater rocks" or occult disorders, such as anismus, rectal hyposensation and anxiety/depression, which mostly require conservative treatment. Rectal prolapse excision or obliterative suture, rectocele and/or enterocele repair, retrograde Malone's enema and partial myotomy of the puborectalis muscle are effective in selected cases. Laparoscopic ventral sacral colporectopexy may be an effective surgical option. Stapled transanal rectal resection may lead to severe complications. The Transtar procedure seems to be safer, when dealing with recto-rectal intussusception. A multidisciplinary approach to ODS provides the best results.

Key words: Constipation; Obstructed defecation; Pelvic floor rehabilitation; Rectopexy; Rectal prolaxectomy

Core tip: Obstructed defecation mainly effect women and may be due both to functional and organic disorders. Some of them, *i.e.*, rectocele, are more evident and easy to detect. Two out of ten patients present with occult causes, more difficult to diagnose, which may be looked at as "underwater rocks" of an iceberg. Most patients may be treated conservatively, with fiber diet, laxatives, rectal irrigation, pelvic floor rehabilitation and psychotherapy; and a minority requires surgery, including rectocele repair, prolapse excision, rectopexy and, more rarely, transanal rectal resection. Due to its complex etiology and psychological involvement, obstructed defecation needs a multidisciplinary approach.

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SYMPTOMS AND DIAGNOSIS

Obstructed defecation syndrome (ODS) is a type of constipation characterized by fragmented stools,

need for straining at defecation, sense of incomplete evacuation, tenesmus, urgency, pelvic heaviness and self-digitation[1,2]. Most patients are females. The aim of self-digitation is two-fold: (1) by compressing the rectocele pouch through the vagina, the patient makes the anorectum straight and facilitates the evacuation of the stool; and (2) by pushing on the perineum, the transverse muscles of the perineum are stimulated, and this elicits a reflex rectal contraction aimed at evacuating the feces[3]. Unfortunately, self-digitation may cause anorectal ulcerations followed by bleeding and discomfort and anal fibrosis leading to a stricture. The diagnosis of ODS is based on a careful evaluation of the patient's clinical history. The severity of symptoms may be objectively evaluated using a validated score[4].

Transanal-vaginal ultrasound (US)[5], defecography[6], anorectal manometry and the balloon expulsion test[7], entero defecography, dynamic perineal US and magnetic resonance imaging defecography[8,9], pudendal nerve motor latency[10] and psychological evaluation[11] may be useful for the assessment of ODS.

ODS AS AN "ICEBERG SYNDROME"

ODS has been also defined an "iceberg syndrome", as the two most frequent lesions, *i.e.,* rectocele and rectal internal mucosal prolapse, present in more than 90% of patients with ODS, are easily detectable and may be considered "emerging rocks", whereas the "surgical ship" is likely to "sink" due to the "underwater rocks", *i.e.,* the occult lesions. At least two occult lesions were present in all patients with ODS in a prospective study conducted by our group[12].

They are more difficult to diagnose and may be either functional or organic. The former are: anxiety/depression, anismus or non-relaxing puborectalis muscle on straining, rectal hyposensation, pudendal neuropathy and spastic colon. The latter are: peritoneo- entero- and sigmoidocele, colpocele, cystocele, recto-rectal intussusception and solitary rectal ulcer. Therefore, rectocele and rectal prolapse, which are usually the target of surgery, are more effects than causes of symptoms[13].

An excessive straining is likely to be the "primum movens", causing tissue weakness and organ descent, and often is due to long-standing anxiety, muscle tension and consequent non-relaxing puborectalis muscle. The increased straining causes pudendal nerve stretch which may lead to a pudendal neuropathy which affects rectal sensation. The stool becomes small and hard and more difficult to evacuate, as they are less effective in stimulating the rectal wall, and then eliciting the peristaltic reflex aimed at inhibiting the rectal internal sphincter and facilitating the evacuation of the stool. It is clear that surgery has a secondary role in correcting the above-mentioned defects, as they are mainly psychological and/or muscular and/or neurological. Unfortunately, they would require long and complex treatment, *i.e.,* change of dietary regimen, psychological support, pelvic floor rehabilitation. Instead, both patients and surgeons prefer a faster solution of the problem, *i.e.,* a straightforward operation. This explains why most, if not all surgical procedures tend to fail in the long term[14-17].

As reported by the Wexner group, many women with rectocele suffer from ODS, but only in a minority of them are the symptoms due to rectocele[18].

Vermeulen *et al*[19], when reporting the high failure rate after surgery for rectocele, states that "to restore anatomy does not mean to restore function".

Therefore, caution is needed prior to recommending surgery in a patient with ODS.

CONSERVATIVE TREATMENT

Fiber diet, plenty of water and bulking laxatives are the most used frequently conservative treatments of ODS[20]. Chocolate and other foods which increase stool viscosity thus making more difficult stool expulsion "in one shot" should be avoided[21]. Hydrocolontherapy or lavage, consisting of retrograde large bowel irrigation with warm water through a tube gently inserted into the anorectum, also has a positive role in the treatment of ODS and there is no risk of side effects[22,23]. Several authors are in favour of rectal irrigation, which is reported to be effective in nearly half of the patients with intestinal dysfunction[24,25]. Nevertheless it is well known that the abuse of self- administered enemas may cause anorectal fibrosis and stricture, due to repeated microtrauma. Biofeedback is indicated in case of anismus[26,27] and rectal hyposensation[28].

Anismus may be also cured with yoga exercises[29] and botulinum toxin A, (50 units injected into the puborectalis muscle), with a short-term cure rate of about 50% and minor or rare side effects, such as transient anal incontinence and hypotension[30]. Transanal electrostimulation, which may be carried out as a home procedure using small probe inserted into the anus and connected with a portable electrostimulator, may be effective in both pudendal neuropathy and rectal hyposensation[31].

Rectocele and recto-rectal intussusceptions, despite being organic lesions, may be successfully treated with pelvic floor rehabilitation, provided that they are not long-standing. When these lesions become larger and more significant, they become causes of the ODS symptoms and require surgery[32,33].

Psychological counselling is helpful in patients with either depression or anxiety or both, whose psychosomatic condition may be also diagnosed

with reactive graphic tests such as the "draw-the-family" test[34,35]. It should be noted that one-third of the females complaining of ODS and proctalgia report episodes of sexual trauma during childhood or adolescence[36].

For the patients who are not willing to undergo formal psychotherapy, simple pelvic floor and abdominal muscle relaxation exercises taught by a psychologist may be useful to improve evacuation.

Recently a new procedure has been proposed by our group for patient with anismus and altered psychological pattern, combining guided imagery and relaxation techniques with ultrasound-guided biofeedback, namely psycho-echo-biofeedback, with is successful in half of the cases at two year[37].

Unfortunately, in many papers on the surgical treatment of ODS, at least half of the above- mentioned therapies are not listed among the conservative measures carried out before surgery is proposed[38,39], which shows that there is a tendency towards surgical overtreatment, *i.e.,* more than half of patients with ODS undergo stapled transanal rectal resection (STARR), compared with the lower operative rate of 14% according to other series[40,41].

SURGICAL TREATMENT: MANUAL TECHNIQUES

Basically, the options at disposal of the surgeon who deals with ODS are as follows: (1) to perform a kind of "surgical" irrigation; (2) to perform either a resection or a plication or a pexy in case of internal mucosal prolapse; (3) to reinforce the rectovaginal septum and/or, again, resect the redundant mucosa, in case of significant rectocele; and (4) to perform miotomy in case ODS is due to a muscular disorder.

Malone and others have reported surgical procedures which are modifications of the appendicostomy performed to treat children with chronic constipation[42,43].

Either the appendix or a segment of terminal ileum are sutured to the underskin of the umbilicus or the right iliac fossa with a flap valve mechanism, aimed at preventing the external leakage of fecal matter.

Cosmesis is good because the surgical wound is small. The patient has to irrigate his/her intestine with water through a syringe, thus carrying out an anterograde enema and facilitating defecation. The limitation of the procedure is the relatively frequent occurrence of a sliding of the bowel which affects the valve mechanism and requires a reoperation.

Rectal prolapsectomy may be carried out via a transanal route in case of significant, *i.e.,* 2nd or 3rd degree rectal internal mucosal prolapse or recto-anal intussusceptions[44].

In case of high rectocele and large mucosal prolapse, the whole anterior aspect of the rectal mucosa is excised transanally, followed by a "concertina"- like plication of the denuded rectal muscle[45].

An alternative minimally invasive procedure, which is indicated when dealing with smaller prolapse and rectocele, consists of an over-running suture on the anterior midline starting from the dentate line up to the apex of the rectocele and then reversal, going back to the dentate line. The consequent plication of the rectal layers forms a kind of barrier and reinforces the weakened recto-vaginal septum obliterating both the prolapse and the rectocele[46].

In case of mucosal prolapse, another minimally invasive procedure has been proposed by El Sibai and Shafik[47]. It consists of cauterization and plication of the prolapse. This and other transanal, transperineal and abdominal operations have been reported by Pescatori and Zbar[48].

Resection rectopexy, the internal Delorme procedure or circumferential rectal mucosectomy with rectal muscle plication, sacral rectopexy and ventral laparoscopic rectopexy[49-53] have been used with satisfactory short-term outcomes when dealing with ODS due to recto-rectal intussusception, but the long-term outcomes are less encouraging, as nearly half of the patients have a recurrence of ODS symptoms at 4 years[14].

Ventral mesh rectopexy can also be carried out robotically[54].

Instead, nearly 90% of the patients are cured at 4 years after a combined transanal-transperineal and abdominal approach, *i.e.,* positioning of mesh at the pelvic outlet, for rectocele-internal rectal prolapse and enterocele. The reason for the high success rate is that the other occult concomitant functional diseases, such as anismus, are cured with psycho-echo-biofeedback. This novel procedure, which involves a psychologist, consists of breathing exercises accompanied by hypnotic words, while the patient strains and is encouraged to carefully watch the contraction-relaxation of the puborectalis muscle on the screen of the transanal or, better yet, the transvaginal US machine[37].

Recently, a surgical operation has been reported as effective in patients whose ODS is due to animus.

It consists of a transperineal bilateral partial myotomy of the puborectalis muscle, aimed at favouring its relaxation on straining. According to its inventor, the operation is more effective than biofeedback conditioning and botulinum toxin A injection[55,56].

We developed a modified Farid procedure using a semiclosed transperineal approach, with neither sepsis nor incontinence postoperatively and an 80% cure rate at one year in a small series (unpublished data).

Rectocele repair may be carried out using other

procedures than the above mentioned transanal approaches, such as transperineal or transvaginal interposition of a biological mesh of porcine collagen[57] anterior levatorplasty, which may cause dyspareunia in sexually active females[58] or the novel transvaginal Schwandner repair[59]. It may be also repaired using a laparoscopic approach, as reported by Vermeulen *et al*[19]. Sacral neuromodulation with an implanted pace-maker, which was first proposed by Pescatori *et al*[60-62] in constipated patients is helpful in case of slow transit constipation, but may be also effective for ODS according to Falletto *et al*[63], provided that patients are carefully selected.

The advantage is that the procedure is minimally invasive as it may be performed under local anesthesia. The disadvantage is the high cost of the pace-maker, around 15000 euros.

SURGICAL TREATMENT: STAPLING TECHNIQUES

The first stapled procedure for rectal mucosal prolapse causing ODS was reported by Pescatori *et al*[64], using a circular stapler in a small series with good short-term results and no relevant complications. It has been replicated by Zacharakis *et al*[65] with encouraging results.

The first study on STARR, was published by Boccasanta *et al*[66], who reported good results in around 90% patients in the short term, but painful defecation at one year in 20%. Post-STARR chronic proctalgia, which may be severe and affect patients' quality of life, is likely to be due to the fibrosis around retained staples, which triggers the nerve spindles on the levator ani and puborectalis muscles[67].

Removal of retained staples reduces pain only in a minor proportion of patients[68].

According to the invited comment of Robin Phillips to the above-mentioned paper by Boccasanta *et al*[66], "to resect the rectum for constipation is like resecting a lung for asthma".

Other complications have been reported following STARR, such as severe rectal bleeding, fecal urgency and anal incontinence, recto-vaginal fistulae, retrorectal hematoma, pelvic sepsis requiring a diverting stoma, and deaths[69-73]. A comprehensive review of post-STARR complications has been reported by Pescatori and Gagliardi[74].

Unfortunately, the potential risks of the STARR procedure are underestimated, also in part due to a strong media campaign, as shown by the definition of STARR as a "sutureless operation" given by newspapers[75].

Ira Kodner, the past-president of the American Society of Colon and Rectal Surgeons stated that those who report misleading messages should be excluded from the surgical community[76].

As far as clinical outcome is concerned, 55% of the patients still had at least 3 symptoms of ODS at 18 mo after STARR in a retrospective multicentric study of the Italian Society of Colo-Rectal surgery and 19% of the cases required a reintervention, either because of postoperative complications or recurrence of symptoms[77]. Reinterventions after STARR are likely to fail in patients with an altered mental pattern[78].

Some studies report lower complication rates and better clinical results[79,80].

A high recurrence rate in the long term has been reported by Hussein[15].

Fecal urgency and anal incontinence after STARR are most likely to be due to the reduced size of the rectal reservoir[81,82]. The more recent Transtar procedure, carried out with the Contour device (Ethicon Endosurgery, Cincinnati, Ohio, United States), seems to achieve better results, *e.g.*, in terms of chronic postoperative proctalgia[83]. Nevertheless, life-threatening retro-rectal hematoma has also been reported following Transtar[84]. A recent European multicentre study reported a relief of symptoms in most patient on the short term without a significant improving of St. Mark's score quality of life, neither major complication nor post operative mortality was reported[85].

Both clinical and functional results seem satisfactory in a large multicentre series reported by Jayne *et al*[86] but the follow-up is short and incomplete. However, this relatively novel procedure carried both satisfactory and unsuccessful outcomes as reported by several authors, and merits further evaluations prior to be considered a fully safe and effective operation[86-97]. It has to be performed by colorectal surgeons specifically trained with novel instruments, such as the Contour devices[98-100].

CONCLUSION

The outcome of surgery alone for ODS may be good in the short term, but it worsens over time, probably due to the fact that both the diagnosis and the management of the "occult" lesion(s) causing symptoms are neglected. The psychosomatic component of ODS should be recognized and managed as it affects two-thirds of the patients. Several conservative treatments are available and should be attempted prior to surgical management of ODS. The holistic approach is important, *i.e.,* psyche and soma should be considered a unique entity. The key to successful treatment of ODS appears to be a multidisciplinary approach.

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REVIEW

Markers of acute rejection and graft acceptance in liver transplantation

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Abstract

The evaluation of the immunosuppression state in liver transplanted patients is crucial for a correct post-transplant management and a major step towards the personalisation of the immunosuppressive therapy. However, current immunological monitoring after liver transplantation relies mainly on clinical judgment and on immunosuppressive drug levels, without a proper assessment of the real suppression of the immunological system. Various markers have been studied in an attempt to identify a specific indicator of graft rejection and graft acceptance after liver transplantation. Considering acute rejection, the most studied markers are pro-inflammatory and immunoregulatory cytokines and other proteins related to inflammation. However there is considerable overlap with other conditions, and only few of them have been validated. Standard liver tests cannot be used as markers of graft rejection due to their low sensitivity and specificity and the weak correlation with the severity of histopathological findings. Several studies have been performed to identify biomarkers of tolerance in liver transplanted patients. Most of them are based on the analysis of peripheral blood samples and on the use of transcriptional profiling techniques. Amongst these, NK cell-related molecules seem to be the most valid marker of graft acceptance, whereas the role $CD4^+CD25^+Foxp3^+$ T cells has still to be properly defined.

Key words: Liver transplantation; Acute cellular rejection; Tolerance; Biomarkers

Core tip: This review explores the available data in the literature concerning potential markers of acute cellular rejection and graft acceptance after liver transplantation, as well as their impact on decision-making for clinicians.

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INTRODUCTION

During the past 25 years liver transplantation has become the standard therapy for acute and chronic liver failure. Nowadays, with a 5-year patient survival rate of 73%[1], long-term outcome of patients is becoming the main concern for clinicians, who have to deal with the side effects of immunosuppressant drugs in the long-term.

Current immunological monitoring after liver transplantation relies mainly on clinical judgment and on measurement of immunosuppressive drug levels, without a proper assessment of the real suppression of the immunological system. Therefore, the evaluation of the immunosuppression state in liver transplanted patients is crucial for a correct post-transplant management and constitutes a major step towards the personalisation of immunosuppressive therapy.

The ideal diagnostic biomarker should be highly sensitive and specific, non-invasive, and rapidly available[2]. Despite the elevated interest in the evaluation of potential biomarkers of acute cellular rejection (ACR) and graft acceptance, and in the development of specific immune monitoring assays, only few of them are used routinely in clinical practice.

The aim of this review was to explore the available data in the literature concerning potential markers of ACR or graft acceptance after liver transplantation, as well as their impact on decision-making for clinicians.

MARKERS OF ACUTE CELLULAR REJECTION

Various markers have been studied in an attempt to identify a specific indicator of graft rejection after liver transplantation. However, the use of these markers has been hampered by the fact that there is considerable overlap with other conditions, and currently only a few of them have been validated[3].

Liver enzymes

The suspicion of ACR is usually driven by the rise of liver enzymes after transplantation. However, several reports have clearly shown that elevated standard liver tests have a low sensitivity and specificity for ACR and show a weak correlation with the severity of histopathological findings[4,5]. Moreover, liver enzymes do not allow for ACR to be differentiated from others complications. A study based on 70 post-transplant liver biopsies demonstrated that there is no single chemical parameter nor a combination of parameters that can statistically or clinically distinguish patients with ACR from those with other causes of graft dysfunction[6]. More recently, Rodriguez-Peralvarez *et al*[7] showed that patients with moderate/severe ACR are characterized by higher bilirubin levels and cholestasis parameters, with lower aspartate aminotransferase (AST), AST/alanine aminotransferase (ALT) ratio than those with mild or no ACR. However, the combination of these serum parameters in the logistic regression analysis had only a sensitivity of 73% and a specificity of 52.9%. ALT value was not related to the presence or grading of ACR, and although ALP values were related to ACR, this enzyme cannot be used as a marker of ACR nor its severity, due to the myriad of disorders in which it is elevated.

Cytokines

After liver transplantation, the characteristics of the inflammatory environment in which T cell recognition of the alloantigen takes place determines the lineage commitment of these cells. Thus, depending on the cytokines that are present when antigen activation occurs, naïve $CD4^+$ helper T cells may acquire cytopathic and/or immunoregulatory phenotypes[8,9].

Based on this immunological background, the first potential biomarkers studied to predict ACR were cytokines. Products of activated T lymphocytes, such as IL-2 or soluble components of its receptor (sIL-2R), have been particularly well studied.

Boleslawski *et al*[10] evaluated the intracellular IL-2 quantification in $CD3^+CD8^+$ cells in 21 liver transplant recipients for 6 mo after liver transplantation, showing that intracellular IL-2 expression in $CD8^+$ T cells before transplantation was closely related to the development of ACR. These results were later confirmed by Akoglu *et al*[11], who demonstrated that patients experiencing ACR showed a significantly higher intracellular percentage of $IL\text{-}2^+$ in $CD8^+$ T cells compared to stable liver transplant recipients. They also showed a good correlation between the percentage of $CD8^+IL\text{-}2^+$ cells and Banff score (Spearman's rho = 0.81; $P = 0.027$) (Table 1).

When the expression of IL-2 and IL-2 receptor was evaluated in liver grafts of patients with and without ACR, IL-2 and IL-2 mRNA were absent, with minimal expression of IL-2 receptor in patients experiencing ACR, whereas IL-4 and IL-4 mRNA were highly expressed during ACR, being absent in stable liver transplant recipients[12] (Table 1).

In a recent study, Millán *et al*[13] evaluated the intracellular expression and soluble production of IFN-γ and IL-2 in 47 liver transplanted patients. A pre-transplant cut-off value of 55.8% for the percentage of $CD8^+IFN\text{-}\gamma^+$ identified patients at high risk of ACR (sensitivity = 75% and specificity = 82%). In the first week after transplantation, patients with a percentage of inhibition for soluble IFN-γ, a percentage of $CD8^+IFN\text{-}\gamma^+$ and a percentage of $CD8^+IL2^+$ lower than 40%, developed ACR.

Regarding TNF-α, it has been shown that pre-

Table 1 Marker of acute cellular rejection after liver transplantation

Biomarker	Sample size	Ref.
Citokines		
IL-2	66	Akoglu *et al*[11]
	21	Boleslawski *et al*[10]
IL-4	20	Conti *et al*[12]
IL-6	169	Kita *et al*[17]
IL-15	35	Conti *et al*[18]
IL-18	Rat model	Fábrega *et al*[19]
IL-23	50	Fábrega *et al*[20]
IFN-γ	47	Millán *et al*[13]
TNF-α	50	Imagawa *et al*[15]
Other markers related to inflammation		
CD28	55	Minguela *et al*[22]
	237	Minguela *et al*[23]
	52	Boleslawski *et al*[24]
CD38	52	Boleslawski *et al*[24]
CD25	55	He *et al*[25]
ICAM-1	NA	Adams *et al*[26]
	12	Romero *et al*[27]
Bile markers		
Bikle acid concentrations	41	Janssen *et al*[30]
IL-6	51	Umeshita *et al*[31]
IL-8	45	Warlé *et al*[32]
Alanine Aminopeptidase N	9	Kim *et al*[33]
Ascites markers		
IL-2 receptor	30	Ganschow *et al*[34]
IL-6	30	Ganschow *et al*[34]
IL-1 receptor antagonist	30	Ganschow *et al*[34]

IL: Interleukin; IFN: Interferon; TNF: Tumor necrosis factor; ICAM-1: Intercellular adhesion molecule 1.

transplant "*in vitro*" production of this molecule was significantly increased in patients with post-transplant ACR (n = 9) compared with those who did not develop ACR (n = 12)[14]. When plasma levels of TNF-α were measured in 50 adult patients following liver transplantation, its concentration was significantly higher in patients experiencing ACR than in those with a stable clinical course (941 ± 83 pg/mL *vs* 240 ± 6 pg/mL, P = 0.0001)[15] (Table 1).

An important role of IL-18 in liver allograft rejection has been postulated in a recent study using a rat model of liver transplantation, which showed that specific suppression of IL-18 was associated with significantly decreased serum alanine amino-transferase levels and less histologic hepatic injury early after transplantation[16] (Table 1).

In another study, serum levels of IL-6 were evaluated in 20 liver transplanted patients with no infections, and it was demonstrated that levels of this cytokine were significantly higher 0-4 d before histological diagnosis of ACR compared to those of patients without ACR (131 ± 78 pg/mL *vs* 40 ± 21 pg/mL, P < 0.01). IL-6 elevation due to ACR appeared to be distinguishable from increases caused by infection, being serum IL-6 levels unrelentingly elevated during bacterial infection (> 1000 pg/mL). However, there was no correlation between IL-6 elevation of and histological grade of ACR[17] (Table 1).

Plasma levels and "*in situ*" expression of IL-15 are enhanced during ACR compared with patients without ACR (5.2 ± 1.3 pg/mL *vs* 0.6 ± 0.4 pg/mL, P = 0.02), with this expression being particularly evident when patients with steroid-resistant ACR were considered (6.9 ± 1.1 pg/mL)[18] (Table 1).

The role of IL-9, IL-23 and IL-17 in liver transplantation remains to be clarified. As far as IL-9 is concerned, when serum levels were determined in 50 liver transplanted patients (15 patients with ACR episodes, and 35 patients without ACR) on day 1 and 7 after liver transplantation and on the day of liver biopsy, no difference was found between patients with and without ACR[19]. Similarly, the serum concentrations of IL-23 and IL-17 were not different early in the post-transplantation period. However, a significant increase in serum IL-23 levels in the ACR group was seen at the time of liver biopsy[19,20]. These data were confirmed by a latter prospective study[21] showing that the levels of circulating $CD4^+IL\text{-}17^+$ T cells were higher in patients with ACR than those with no ACR (2.56% ± 0.43% *vs* 1.79% ± 0.44%, P < 0.001). Moreover, the frequency of $CD4^+IL\text{-}17^+$ cells in peripheral blood was correlated with the histological severity of ACR (r = 0.79, P = 0.0002) (Table 1).

In conclusion, pro-inflammatory and immuno-regulatory cytokines have been the most studied markers to predict ACR. Despite most of them showed an increased expression during ACR, many of these cytokines cannot differentiate between ACR and infections, making their utility limited in clinical practice.

Other markers related to inflammation

One of the first studies evaluating the expression of CD28 after liver transplantation demonstrated that patients experiencing ACR showed a clear increase with respect to patients without ACR, and to healthy controls. Significant differences in the total-$CD28^+$ lymphocytes between the ACR and non-ACR groups were reached on days 7 to 9 (P < 0.01) and 10 to 13 (P < 0.05) after transplantation[22]. The same group, in a subsequent study, showed that ACR and virus re-infection could be distinguished from each other because CD28 was up-regulated on $CD4^+$ lymphocytes only in recipients with ACR, irrespective of their HBV/HCV infection status[23] (Table 1).

The expression of CD28 and CD38 was also analysed on $CD3^+$, $CD4^+$ and $CD8^+$ cells in 52 liver transplanted patients in another study. The mean frequencies of CD28 and CD38-expressing T cells were significantly higher in patients with ACR (P = 0.01 and P = 0.001, respectively). Moreover, at multivariate analysis, only CD28 and CD38

frequencies at day 14 were independently associated with ACR (HR = 1.27, P = 0.04 and HR = 1.11, P = 0.01 respectively)[24] (Table 1).

CD25 expression may also constitute a biological marker of immune activation in transplant recipients. Circulating $CD4^+CD25^{high}FoxP3^+$ T cells were significantly lower in patients with ACR compared with patients not experiencing ACR (2.23% ± 0.54% *vs* 2.99% ± 0.86%, P = 0.01) in a prospective analysis of 55 patients who underwent liver transplantation. Longitudinal analysis revealed circulating $CD4^+CD25^{high}FoxP3^+$ T cells of patients in the rejection group to be significantly lower during ACR than during quiescence (2.23% ± 0.54% *vs* 3.68% ± 0.70%, P = 0.0001). Furthermore, the frequency of circulating $CD4^+CD25^{high}FoxP3^+$ T cells negatively correlated with Rejection Activity Index (r = 0.80, P = 0.01)[25] (Table 1).

During graft rejection, adhesion molecules play a crucial role in infiltration, activation, and binding of effector cells to target tissues. The expression of intercellular adhesion molecule 1 (ICAM-1), for instance, has been studied on liver tissue after transplantation. It has been shown that ICAM-1 expression on bile ducts, endothelium, and perivenular hepatocytes (structures affected by the rejection process) is greater in patients with ACR than in patients with no ACR. Moreover, it was demonstrated that in patients with a resolving episode of rejection ICAM-1 expression was greatly reduced after high-dose corticosteroid treatment[26]. The effect of steroid therapy on ICAM-1 expression in liver biopsies of patients with post-transplant ACR was confirmed in the study by Romero *et al*[27]. After steroid treatment, the intensity of ICAM-1 expression decreased significantly in sinusoids (1.5 ± 0.67 *vs* 2.41 ± 0.66, $P < 0.05$) and in perivenular hepatocytes (0.25 ± 0.86 *vs* 0.83 ± 0.57, $P < 0.05$) compared to the pre-treatment liver biopsy samples (Table 1).

Lastly, graft eosinophilia has been identified as an independently associated feature of ACR in liver transplantation[28]. In one study, the absence of peripheral eosinophilia predicted the absence of moderate/severe ACR, however it could not be used to predict or to assess the response to corticosteroids for the treatment of acute rejection[29]. In a more recent study, based on 690 consecutive first liver transplant patients and using protocol liver biopsies, peripheral eosinophil count was strongly associated with moderate-severe ACR (OR = 2.15; P = 0.007), although the area under ROC curve (AUROC) was 0.58. These investigators also found that the delta in eosinophil count between the biopsies performed before and after ACR treatment was the only independent predictor of histological improvement (OR = 3.12; P = 0.001)[7] (Table 1).

In conclusion, the expression of CD28 and CD38 on T cells at specific interval time form liver transplantation, seems to be a reliable marker of ACR, being able to differentiate between ACR and infection. Promising results have been found when eosinophil count was evaluated, especially because it is strongly associated with moderate-severe ACR, which often require steroid treatment.

Bile and ascites markers

Contrasting data are available on the role of bile and ascites markers as potential tools for predicting which patients will develop ACR after liver transplantation.

In a study on 41 patients who underwent liver transplantation, the investigators performed serum bile acid concentration measurements and correlated these with findings at liver biopsy. In patients with ACR, bile acid concentrations were statistically significantly increased 3 d prior to liver biopsy (from a mean of 37 ± 31 μmol/L to 118 ± 46 μmol/L; P = 0.001). Moreover, successful antirejection treatment correlated with a significant decrease of serum bile acid as early as 1 d after initiation of therapy (P = 0.008)[30] (Table 1).

Patients with ACR showed a significant increase of bile IL-6 compared with patients who had uneventful postoperative courses (1090 ± 990 pg/mL *vs* 18 ± 3 pg/mL, $P < 0.05$) in a study performed on 51 liver transplant recipients[31]. In a prospective study on 45 patients who underwent liver transplantation, biliary IL-8 levels were also demonstrated to be significantly increased at the onset of ACR (11.62 ± 4.25 pg/mL, $P < 0.001$) compared with patients with an uneventful course and those with infectious complications[32] (Table 1).

Lastly, in a more recent study, alanine aminopeptidase N (APN/CD13) enzyme activity in bile samples collected within 3 d before post-transplant liver biopsy was significantly higher in patients with ACR (584 ± 434 U/g protein) than in those free of ACR (301 ± 271 U/g protein) (P = 0.004)[33] (Table 1).

In another study, the value of cytokine quantification in drained ascites was evaluated in 30 children in the first 2 wk after liver transplantation. There were no significant elevations of IL-2 receptor and IL-6 in serum and ascites between patients with and without ACR. However, the concentration in ascites of the IL-1 receptor antagonist increased 48 h before ACR (P = 0.01 *vs* no ACR). The IL-1 receptor antagonist concentration in ascites was up to 11-fold higher than in serum during ACR (15.43 *vs* 1.38 ng/mL)[34] (Table 1).

In conclusion, despite encouraging results, bile and ascites markers have a controversial use in diagnosing ACR. The main limit of these diagnostic approaches is that they often requires invasive procedures such as the position of a T tube (which is no longer used in most of the liver transplant centres) or performing a paracentisis. Therefore

this aspect is of great relevance in clinical practice because, liver biopsy, which is the gold standard for ACR diagnosis, is an invasive procedures and the clinical attention is posed mainly to non-invasive markers.

Future markers

To date, the Cylex ImmuKnow assay, which quantifies the amount of adenosine triphosphate produced by $CD4^+$ T cells after *in vitro* stimulation by a non-donor-specific mitogen (phytohemagglutinin-L), is the only commercially available test to evaluate the immune status in transplanted patients.

A recent systematic literature review evaluated the use of ImmuKnow in liver transplant recipients. The study identified five studies analysing ImmuKnow performance for infection and 5 studies analysing ImmuKnow performance for ACR. Considering the ability to predict ACR, the pooled sensitivity, specificity, positive likelihood ratio, diagnostic odds ratio, and AUROC curve for this analysis were 65.6% (95%CI: 55.0%-75.1%), 80.4% (95%CI: 76.4%-83.9%), 3.4 (95%CI: 2.4-4.7), 8.8 (95%CI: 3.1-24.8), and 0.835 ± 0.060 respectively, while the respective values in the setting of infection were 83.8% (95%CI: 78.5%-88.3%), 75.3% (95%CI: 70.9%-79.4%), 3.3 (95%CI: 2.8-4.0), 14.6 (95%CI: 9.6-22.3), and 0.824 ± 0.034, respectively. Notably, heterogeneity was low for infection studies and high for ACR studies[35]. Based on these data, it appears that this assay could be more useful in order to assess over-immunosuppression rather than under-immunosuppression[36].

Due to the high number of proteins involved in the ACR process, proteomic analysis could have a crucial role in identifying a potential biomarker of ACR. However, despite several studies have been performed, the results are not conclusive and these techniques have to progress from the research bench to the clinical routine[37].

In conclusion, future markers of ACR such as ImmuKnow and proteomic analysis, have been evaluated, but well designed, prospective studied are needed in order to better understand their clinical applicability.

MARKERS OF GRAFT ACCEPTANCE

To date, liver biopsy is the gold standard to assess the graft status after liver transplantation, but it is an invasive procedure and is not suitable for monitoring the graft on a daily basis. Moreover, it does not provide any useful information for predicting future development of tolerance[38]. Therefore, biomarkers of graft acceptance could be crucial in order to select patients eligible for enrolment in immunosuppressive drug weaning or withdrawal protocols. Thus, several studies have been performed to identify biomarkers of tolerance in liver transplanted patients; most of them are based on the analysis of peripheral blood samples and on the use of transcriptional profiling techniques[39].

Non-specific genomic analysis

In the first study using microarray gene expression profiling, Martínez-Llordella *et al*[40] found that genes encoding for γδT-cell, for NK receptors, and for proteins involved in cell proliferation arrest were up-regulated in tolerant liver transplanted patients (n = 16) compared to immunosuppression-dependent patients (n = 16) or healthy individuals (n = 10). A second study by the same group, using a larger cohort of patients, confirmed these results. Again, NK cell and $\gamma\delta TCR^+$ T cell transcripts were predominantly expressed in tolerant liver transplanted patients[41].

In a more recent study, transcriptional profiles from 300 samples were examined by microarrays and RT-PCR measurements of blood specimens from paediatric and adult liver transplant recipients and of normal tissues. Tolerance-specific genes were validated in independent samples across two different transplant programs and validated by RT-PCR. A minimal set of 13 unique genes, highly expressed in NK cells (P = 0.03), were significantly expressed in both paediatric and adult liver transplanted tolerant patients, and the performance of this gene set analysis, tested in independent samples, yielded a 100% sensitivity and 83% specificity[42].

Lastly, Bohne *et al*[43] recently reported the results of a multicentre prospective study evaluating 75 liver transplant recipients from whom cryopreserved liver tissue samples had been obtained before the initiation of drug minimization and were available for transcriptional analyses. Amongst these, 33 recipients successfully discontinued all immunosuppressive drugs, while 42 rejected their allografts. Before initiation of drug withdrawal, operationally tolerant and non-tolerant recipients differed in the intragraft expression of genes involved in the regulation of iron homeostasis. Moreover, operationally tolerant patients exhibited higher serum levels of hepcidin and ferritin and increased hepatocyte iron deposition compared to non-tolerant ones.

Peripheral blood immunophenotyping

An increase Vδ1/Vδ2 γδT-cells ratio has been found in operationally tolerant liver transplanted patients (n = 12, ratio = 1.5) when compared with liver transplanted patients on immunosuppression (n = 19, ratio = 0.8; $P < 0.01$) and with age-matched healthy controls (n = 24, ratio = 0.3; $P < 0.05$)[44]. The increase in the number of circulating $V\delta1^+$ T cells in tolerant patients has also been confirmed

in a later study by Martínez-Llordella *et al*[40] who demonstrated that Vδ1$^+$ subtype is the predominant γδT-cell subpopulation in tolerant recipients.

Based on this, altered distribution of the Vδ1 and Vδ2 γδT cells in operationally tolerant liver transplant recipients, γδT cells subset quantification was proposed as a biomarker of immunologic risk in liver transplantation. However, a recent study showed that alterations in the γδT cell compartment are not restricted to tolerant liver recipients, and that most immunosuppressed liver recipients display an enlarged peripheral blood γδT cell pool mainly resulting from an expansion of Vδ1 T cells exhibiting an oligoclonal repertoire and different phenotypic and cytokine production traits than Vδ2 T cells. The authors proposed that persistent viral infection might be the cause of these alterations[45].

Several studies have shown that the numbers of circulating CD4$^+$CD25$^+$ T-cells is increased in operationally tolerant patients after liver transplantation[40,44,46,47]. When peripheral blood mononuclear cell populations were analysed in 12 liver transplant recipients with stable graft function for more than 2 years, the percentage of CD4$^+$CD25^{high+} cells was significantly higher in tolerant patients (n = 12, 2.3% ± 0.6%), compared with patients who were still on immunosuppression (n = 19, 0.9% ± 0.7%; P < 0.01), and with age-matched volunteers (n = 24, 1.8% ± 0.6%; P < 0.05)[44]. This data were confirmed by Pons *et al*[46] who found an increased frequency of CD4$^+$CD25^{high+} cells when immunosuppressive therapy was withdrawn in tolerant patients (n = 5). The most interesting data of this study was that relative mRNA FoxP3 expression increased 3.5-fold before the complete withdrawal of immunosuppression in tolerant patients, and this increase continued when the immunosuppressive therapy was stopped. Conversely, patients who suffered ACR (n = 7) did not exhibit an increase in CD4$^+$CD25^{high+} cells or FoxP3 expression.

When the expression of Foxp3 mRNA and the presence of CD4, CD8, and Foxp3 cells were quantified in liver biopsies from tolerant living-donor liver transplanted patients, it was found that Foxp3 mRNA expression was higher in tolerant patients (n = 28), compared with patients on immunosuppression (n = 29; P = 0.07), but was equivalent to patients who experienced chronic rejection (n = 7; P < 0.01). The number of Foxp3 cells was significantly increased in tolerant patients, compared with patients on immunosuppression (P < 0.05), although the number of CD4 or CD8 cells did not differ between the two groups[48].

CONCLUSION

The evaluation of the real suppression of the immune system after liver transplantation would allow transplant clinicians to modulate the immunosuppressive therapy according to patient needs, identifying, not only patients at risk of acute rejection, and infection, but also understanding if the immunological background would allow a progressive reduction of the immunosuppressive therapy, favouring graft acceptance.

Despite these considerations, the current immunological monitoring after liver transplantation relies mainly on clinical judgment and on immunosuppressive drug levels, without a proper assessment of the real suppression of the immunological system.

Therefore, it becomes crucial to identify potential biomarkers of immune activity, which can be used to tailor immunosuppression after liver transplantation.

In this manuscript, we reviewed available data on studies assessing the role of different biomarkers of ACR and graft acceptance after liver transplantation.

Considering biomarkers for ACR, pro-inflammatory and immunoregulatory cytokines are the most studied ones, showing an increased expression during ACR. However many of these cytokines cannot differentiate between ACR and infections, making their utility limited in clinical practice. The expression of other proteins related to inflammation, not only in the blood, but also in the bile and in the ascites has been evaluated, but the results are controversial. Moreover, the use of ascites markers is an invasive method and it needs the presence of ascites after liver transplantation, therefore it is not applicable on a daily basis.

When we evaluated available markers of graft acceptance after liver transplantation, we found that data are more encouraging compared to biomarkers of ACR. Patients undergoing immunosuppression withdrawal seem to present specific characteristics compared to non-tolerant patients. One of the most reliable blood marker, which could help clinicians to differentiate between tolerant and non-tolerant patients, are NK cells and their related transcripts. It has been clearly demonstrated that they are already present in the blood of tolerant liver transplanted patients before the withdrawal of immunosuppressive therapy. The role CD4+CD25+Foxp3+ T cells, which seem to have a immunoregulatory effect, is less clear due to the use of immunosuppressive drugs, which could alter their expression. Independently from the markers identified, there is a substantial difference between the expression of specific markers in the blood and their expression in the transplanted liver. This difference makes blood-related biomarkers less accurate in order to predict graft acceptance and forces clinician still to use liver biopsy to monitor patients undergoing immunosuppression withdrawal.

Lastly, it is becoming evident that a single biomarker cannot be able to reflect all the alterations of the immune system associated with organ

transplantation. Therefore a panel of different biomarkers will be needed to properly evaluate the immunological suppression and to modify immunosuppressive treatment according to patient needs. Once a panel of markers is identified, it should undergo validation in large multicentre studies in order to prove its real clinical utility.

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REVIEW

Preventing post-endoscopic retrograde cholangiopancreatography pancreatitis: What can be done?

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Abstract

Post-endoscopic retrograde cholangiopancreatography pancreatitis (PEP) is the most common complication of endoscopic retrograde cholangiopancreatography. The incidence of post-endoscopic retrograde cholangiopancreatography (ERCP) pancreatitis varies substantially and is reported around 1%-10%, although there are some reports with an incidence of around 30%. Usually, PEP is a mild or moderate pancreatitis, but in some instances it can be severe and fatal. Generally, it is defined as the onset of new pancreatic-type abdominal pain severe enough to require hospital admission or prolonged hospital stay with levels of serum amylase two to three times greater than normal, occurring 24 h after ERCP. Several methods have been adopted for preventing pancreatitis, such as pharmacological or endoscopic approaches. Regarding medical prevention, only non-steroidal anti-inflammatory drugs, namely diclofenac sodium and indomethacin, are recommended, but there are some other drugs which have some potential benefits in reducing the incidence of post-ERCP pancreatitis. Endoscopic preventive measures include cannulation (wire guided) and pancreatic stenting, while the adoption of the early pre-cut technique is still arguable. This review will attempt to present and discuss different ways of preventing post-ERCP pancreatitis.

Key words: Endoscopic retrograde cholangiopancreatography; Post-endoscopic retrograde cholangiopancreatography pancreatitis; Sphincterotomy

Core tip: Endoscopic retrograde cholangiopancreatography (ERCP) is a widely used procedure for diagnosing and treating diseases of the pancreatobiliary tree. Post-ERCP pancreatitis is the most frequent complication. Prophylactic measures of post-endoscopic pancreatitis include pharmacological and mechanical ERCP related approaches. Prevention is suboptimal and still not widely accepted.

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is a widely used procedure for diagnosing and treating diseases of the pancreatobiliary tree, with over 500000 ERCP procedures performed annually in the United States alone[1]. Most common complications of ERCP are hemorrhage, pancreatitis, cholangitis and perforation, with pancreatitis after ERCP, or post-endoscopic pancreatitis (PEP) being the most frequent complication. The incidence of post-ERCP pancreatitis varies substantially and is reported to be around 1%-10%, although there are some reports with an incidence of around 30%[2,3]. Usually, it is a mild or moderate pancreatitis, but in some instances it can be severe and fatal[2]. According to a consensus from 1991, PEP is the presence of new pancreatic-type abdominal pain severe enough to require hospital admission or prolonged hospital stay with levels of serum amylase two to three times greater than normal, occurring 24 h after ERCP[4,5]. Although PEP is mostly a mild complication of ERCP, it causes prolonged hospitalization, anatomical complications, and further procedures (endoscopies, laparoscopies, open surgery, *etc.*). It can cause the deterioration of the patient's health, as well as a huge financial burden to hospitals. Therefore, preventing PEP could benefit both patients and hospitals. Attempts at preventing PEP have been carried out using pharmacological prophylaxis, technical measures or proper patient selection.

Prophylactic measures of PEP include pharmacological and mechanical ERCP-related approaches. Mechanical solutions for PEP prevention have been found in prophylactic stenting of the pancreatic duct in high risk patients and early pre-cut cannulation. As a current gold standard, placement of a pancreatic stent is recommended. Nevertheless, endoscopists are looking for a pharmacological solution which will be safe, cheap, easily administered just before the procedure and applicable to all types of patients requiring ERCP.

In this review, we attempt to present and discuss different ways of preventing post-ERCP pancreatitis by reviewing the literature that describes various factors of PEP prevention and the possible utilization of endoscopic techniques and drugs in preventing PEP or lowering its incidence and severity.

PHARMACOLOGICAL PREVENTION

Many pharmacological agents have been considered in the prevention of PEP, although their effectiveness remains debatable. These include allopurinol, gabexate mesylate, octreotide, somatostatin, antibiotics, non-steroidal anti-inflammatory drugs (NSAIDs) and many others. The current literature reveals that basically all of the suggested pharmacological agents have either disappointing or inconclusive results so far, with the exception of NSAIDs[2,3,6-9].

Nitrates

Some potential in the prevention of PEP has been observed for glyceryl nitrate (GTN). Due to his dilatatore properties, it is believed that its usage could relax biliary and pancreatic sphincters, thus alleviating cannulation of the common bile duct (CBD). GTN can reduce the pressure of the sphincter of Oddi[10]. If used during and after ERCP, GTN can relax pancreatobiliary sphincters, facilitating CBD cannulation and reducing the chances of obstruction of the pancreatic outflow. GTN is also cost effective and easily administered. A meta-analysis was conducted exploring the aforementioned effects of GTN. More precisely, Chen *et al*[11] investigated the effect of prophylactic administration of GTN on the incidence of PEP, and the success of cannulation of the CBD duct. They conducted the analysis on a total number of 1841 patients. Out of the total number, 150 patients developed PEP; 55 were given GTN and 95 were given a placebo. They found a statistically significant difference in risk for acquiring PEP between the group who received GTN and the placebo group. They also analyzed the route of administration of GTN, and found that there were 10/128 patients (7.8%) who developed PEP after sublingual administration of GTN in comparison to 26/132 patients in the placebo group. On the other hand, transdermal application of GTN was less successful, and PEP developed in 32/626 (5.1%) patients, whereas 50/640 (7.8%) patients in the placebo group acquired PEP. They concluded that sublingual administration of GTN had a better success rate in prevention of PEP. The second aim of their analysis was to determine if GTN contributes to a more successful cannulation. They found five and seven articles with 900 and 1294 patients, respectively, in which they did not find any significant differences, meaning that prophylactic administration of GTN has no effect on facilitating bile duct cannulation. Wehrmann *et al*[12] also concluded that there was no difference between a group which received GTN and a group which received placebo in time needed for successful cannulation and the number of cannulation attempts. They concluded that topical administration of GTN

does not alleviate cannulation of the bile duct during ERCP. The only adverse effects worth mentioning were hypotension and headache, both easily treated with intravenous administration of crystalloids. Chen *et al*[11] concluded: (1) GTN administration can prevent PEP and reduce its incidence; (2) GTN does not facilitate cannulation of the CBD; and (3) GTN is effective, cheap and easily administered.

There are other conflicting findings regarding the efficacy of GTN in PEP prophylaxis. Kaffes *et al*[13] conducted a prospective, double-blind, placebo-controlled trial in which they evaluated the effect of GTN on the prevention of PEP and success rates of cannulation during ERCP. They included a total number of 318 patients divided into two groups-one on a GTN transdermal patch (155 patients) and the other receiving placebo. There was no notable distinction between the two groups considering the success of initial cannulation, deep cannulation, time needed to achieve successful cannulation, usage of the needle knife or guide wire and PEP. Following their results, they concluded that transdermal GTN had no effect on the prevention of PEP or improvements in cannulation success rates. On the other hand, Bai *et al*[14] performed a meta-analysis of randomized, double-blind, placebo controlled trials evaluating the prophylactic properties of GTN in PEP prevention. They analyzed eight studies with a total of 1920 patients and found that GTN treatment significantly lowered the incidence rate of PEP; incidence of PEP in the GTN group and placebo group was 5.9% and 9.8%, respectively. Also, patients who received GTN had a 39% less chance of acquiring PEP.

Another, similar meta-analysis was performed by Ding *et al*[15].They included 12 randomized, controlled trials with 2649 patients; 11 of those trials reported the occurrence of PEP and compared GTN's and placebo's effect on PEP prevention. The results showed an overall incidence of PEP of 8.8% with a PEP incidence of 7.1% and 10.5% among GTN and placebo patients, respectively. They also conducted a sub-group meta-analysis comparing transdermal and sublingual application of GTN with the results suggesting that sublingual administration of GTN had far more success in prevention of PEP. In conclusion, their results indicated that GTN administration is an effective prophylactic measure in the prevention of PEP. An interesting approach has been made by a group of Iranian authors. Sotoudehmanesh *et al*[16] conducted a randomized trial with a combination of sublingual nitrates and indomethacin *vs* indomethacin alone as a method of preventing PEP. They reported RR = 0.39, 95%CI: 0.18-0.96, P = 0.016, favoring the combination therapy. Drug-induced adverse events were equal among the study groups. They suggested that the aforementioned combination of drugs is more effective in reducing PEP incidence than indomethacin by itself. In conclusion, GTN is not recommended for routine use in PEP prophylaxis but GTN in combination with some other agent such as NSAIDs may further reduce PEP incidence. Further research is needed in order to confirm and support these findings.

Heparin

A group of Chinese authors[17] performed a review and a meta-analysis of clinical trials on the potential beneficial properties of low-dose heparin in the prevention of PEP. Heparin has proven beneficial effects in acute pancreatitis in animals. Low-molecular-weight heparin (LMWH) promotes the survival rate and decreases mortality in cases of severe acute pancreatitis. It also reduces the severity of pancreatitis related microcirculatory disorders in rats. In combination with insulin, heparin is beneficial in acute hyperlipidemic pancreatitis. However, there is conflicting data about its prophylactic effect. In their review Li *et al*[17] analyzed seven studies with a total number of 1438 patients. The incidence of PEP was 5.65% in the group which was given heparin and 7.91% in the control group. Severe PEP occurred in eight cases; 2/562 (0.35%) in the heparin group and 6/872 (0.69%) in the control group. Post-ERCP hemorrhage occurred in 23 patients; 8/562 (1.42%) in the heparin group, and 15/872 (1.72%) in the control group. These results showed no significant correlation between the use of heparin and reduction inPEP incidence. There was no connection between the use of heparin and post-ERCP hemorrhage; low doses did not worsen post-ERCP hemorrhage. They also compared low dose unfractioned heparin and low dose LMWH, finding no difference in the success of PEP reduction, reduction in the severity of PEP, or hemorrhage complications after ERCP. However, Rabenstein *et al*[18] produced results showing significant success in lowering PEP incidence in patients using heparin. They conducted an analysis on 815 patients that underwent ERCP and sphincterotomy. Heparin was given to 268 patients, while the rest of the patients, precisely 547 of them formed the control group. The incidence sof PEP were 3.4% and 7.9%, in the heparin group and the control group, respectively. Furthermore, heparin did not increase hemorrhagic complications. Based on their findings, they concluded that heparin administration correlated with a significantly lower incidence of PEP.

Ung *et al*[19] also conducted a randomized, double-blind, placebo-controlled trial over 89 patients. They were randomly given either 0.2 mL of 25000 IE of heparin or 0.2 mL of saline subcutaneously 4 h before and 4 and 18 h after ERCP. They found that patients which were given heparin had no elevations in levels of amylase, ALT and AST. They concluded

that heparin reduces the increase in amylase levels which is typical for PEP. Li *et al*[17] concluded that neither low dose unfractioned heparin nor LMWH had a significant impact on reducing PEP incidence or its prevention. Despite some promising results where the beneficial effects of heparin were emphasized, this is still not a recommended prevention method. In addition to GTN and heparin, there are several other potential chemoprophylactic agents considered to be beneficial in the prevention of PEP.

Somatostatin and protease inhibitors

Somatostatin is a drug considered to have a beneficial effect on PEP prevention. It inhibits the secretory functions of the pancreas. It can also restrain the motility of the sphincter of Oddi. This combined action can contribute to PEP prevention. The problem with somatostatin is that it has a short half-life and has to be continuously administered intravenously. Due to those disadvantages, octreotide, a somatostatin analogue is used. It has a half-life of 3 h, and can be administered subcutaneously. Arcidiacono *et al*[20] conducted a study on 151 patients who were randomly divided into two groups. Group A (75 patients) was given 0.1 mg of octreotide subcutaneously 120 and 30 min before and 4 h after endoscopy, while group B (76 patients) was given a placebo (1 mL of saline).

They measured serum amylase levels before octreotide administration and 4, 24 and 48 h after ERCP. Group B had a greater rise in serum amylase levels, but the statistically significant difference was measured only 48 h after ERCP. Both groups had five cases of pancreatitis and two cases of cholangitis. Overall, octreotide administration showed no advantages in the prevention of PEP. On the other hand, octreotide contributed to less severe cases of pancreatitis in the treated group, although the difference was not statistically significant. Further research should be conducted, especially on high risk patients.

In relation with somatostatin, a randomized, prospective, double blinded trial was conducted by Katsinelos *et al*[21] on a total number of 540 patients divided into group A and group B in order to see the potential benefits of administering a combination of somatostatin and diclofenac sodium in the prevention of PEP. Both groups had the same number of patients, patients in group A received 1.5 mg of somatostatin intravenously diluted in 500 mL of saline solution 30 min before and 6 h after ERCP. They also received a suppository of 100 mg diclofenac sodium 30-60 min prior to ERCP. Patients in group B received 500 mL of saline and placebo suppositories which were same in appearance as diclofenac sodium suppositories. Patients who had complications or adverse reactions during ERCP, such as hypotension, intolerance to somatostatin or placebo, drop in oxygen saturation, or inability to reach the papilla, were excluded from the study. Cannulation of the common bile duct was performed by a sphincterotome. If unsuccessful after 5 min, a guide wire was used. In cases where guide wire cannulation failed after 5 min, pre-cut papillotomy was performed. A sphincterotome with guide wire cannulation time over 5 min was deemed difficult cannulation. They defined PEP according to the consensus from 1991[4]. PEP was graded as "mild" if lasting 3 d, "moderate" if therapeutic measures were required for 4 to 10 d after ERCP and "severe" if complications lasted longer than 10 d or if death occurred. Also, PEP was severe if a CT scan showed the presence of tissue necrosis in > 30% of the pancreas or if it showed peripancreatic fluid.

The aims of this study were to determine whether the aforementioned combination of drugs could prevent PEP and affect the type of PEP and side effects caused by the same combination[21]. The advantage of this study was that both groups were comparable in sex, age, indications for therapeutic ERCP and ERCP findings. Also, there was no significant difference in cannulation difficulty, pancreatic opacification, number of guide wires inserted, and use of pre-cut papillotomy.

After data analysis, the overall PEP incidence was 7.2%, occurring in 39 patients. Mild PEP occurred in 29 patients (5.6%), moderate in 8 (1.5%) and severe PEP in 2 patients (0.4%). They found a significant difference between the two groups in the rate of PEP: 4.7% in group A and 10.4% in group B. Moreover, the incidence of PEP in high risk patients was significantly lower in the group receiving the diclofenac and somatostatin combination than in the placebo group, *i.e.,* 5.8% and 12.3%, respectively. However, there was no significant distinction in low risk patients (group A 1.5% and group B 3.5%). Based on univariate and multivariate analyses, they found that a history of acute pancreatitis, pancreatic opacification of the first class branches and beyond, and the absence of pharmacoprophylaxis were all independent risk factors for PEP development. Several problems arose in this study. It was difficult to differentiate patients with high and low risk for PEP. There are patient-related factors such as suspected dysfunction of the sphincter of Oddi and previous acute pancreatitis which can be easily identified prior to the procedure. However, ERPC-related risk factors such as difficult cannulation, opacification of the pancreatic duct and pre-cut papillotomy can be identified only during and after ERCP. Logic therefore infers that an ideal pharmacoprophylactic agent has to include all patients undergoing ERCP. Further limitations to this study were the low number of pancreatic sphincterotomes, and only a few suspected sphincters of Oddi dysfunctions (SOD), which are

both known and confirmed risk factors for PEP. Furthermore, ERCP was performed by experienced endoscopists, which contributes to lower rates of PEP.

The protease inhibitors gabexate mesilate, ulinastatin and nafamostat mesilate have been registered for the treatment of acute pancreatitis. The rationale for their usage is a reduction in the pancreatic secretion of proteolytic enzymes. Gabexate mesilate has been shown to decrease the incidence of PEP[22,23], but this agent has to be infused continuously for as long as 13 h because of its short half-life; ulinastatin can be injected as a bolus. Furthermore, Masci *et al*[23] compared two infusion rates of gabexate mesilate; 13 h infusion and 6.5 h infusion. They found no difference in efficacy between the two infusion rates. In a recent meta-analysis by Yuhara *et al*[24], only nafamostat mesilate and NSAIDs showed the potential to reduce PEP, while the other two protease inhibitors were, gabexate mesilate and ulinastatin were shown not to be efficient in reduction of PEP incidence. Due to their high price and inconvenient route of administration, protease inhibitors cannot be recommended as a routine prophylactic measure. Positive results from Japanese trials should be replicated at other centers.

Non-steroidal anti-inflammatory drugs

In some previous studies, it has been pointed out that phospholipase A2 has a pivotal role in the initial inflammatory cascade in acute pancreatitis by regulating a variety of proinflammatory mediators, including arachidonic acid products and platelet-activating factors[25-27]. Murray *et al*[28] was the first one who described the potential of NSAIDs in preventing PEP. These results have been confirmed in several other trials[7,29-32]. Cheon *et al*[29] showed no difference between oral administration of diclofenac and placebo. They conducted a study on 207 patients, 72% of whom were high risk patients (suspected SOD or pancreatic therapy). This suggests that rectal administration of diclofenac has advantages over oral administration. Katsinelos *et al*[21] concluded that a combination of diclofenac and somatostatin significantly lowers the incidence of PEP, especially in high risk patients. Univariate and multivariate analyses confirmed that pre-procedure administration of the mentioned combination is associated with a significantly reduced risk of PEP. They also found no relevant adverse effects of these medications, especially no increases in bleeding after sphincterotomy.

There is more evidence supporting the administration of NSAIDs. Elmunzer *et al*[30] performed a meta-analysis of studies which investigated the efficacy of NSAIDs on the prophylaxis of PEP. They analyzed four studies by Murray *et al*[28], Khoshbaten *et al*[31], Sotoudehmanesh *et al*[16] and Montãno Loza *et al*[33]. First two studies compared rectal administration of 100 mg of diclofenac with placebo, while the latter two compared rectal administration of 100 mg of indomethacin with placebo. Sotoudehmanesh *et al*[32] conducted a trial on 442 patients who were given either indomethacin or placebo just before ERCP. Overall, the PEP incidence was 4.9%, which could be explained by the fact that only 10% of the patients in this trial had SOD. There was no significant difference in the PEP incidence between the placebo group and the indomethacin group, *i.e.,* 3.2% (7/221) and 6.8% (15/221), respectively. However, an additional analysis found that indomethacin had a beneficial effect in patients undergoing pancreatic duct injection. The same group conducted an interesting trial where they compared indomethacin plus sublingual nitrates *vs* indomethacin alone. They reported a further reduction in PEP incidence in the combined group (indomethacin plus nitrates), *i.e.,* RR = 0.39 and 95%CI: 0.18-0.86, which may be of particular interest in high risk patients[16]. None of those patients developed moderate or severe pancreatitis, unlike the seven patients in the placebo group who had developed both modalities. Montãno Loza *et al*[33] conducted the same test with indomethacin and placebo. Their findings were different, and suggested a statistically significant difference in PEP incidence; 5.3% in the indomethacin group and 16% in the placebo group. Murray *et al*[28] and Khohsbaten *et al*[31] conducted research as mentioned previously. They found that the incidence of PEP in the placebo group was higher, making the difference between the two groups statistically significant. Murray *et al*[28] reported a PEP incidence of 6.4% and 15.5% in the diclofenac and placebo groups, respectively, while Khoshbaten *et al*[31] reported a PEP incidence of 15% in the diclofenac group and 26% in the placebo group. No adverse effects were noted in this meta-analysis. Elmunzer *et al*[30] concluded that patients who received NSAIDs were 64% less likely to develop pancreatitis and 90% less likely to develop moderate to severe pancreatitis. Both diclofenac and indomethacin have been proven to be effective in preventing the development of moderate or severe PEP. All of the four studies that were included in this meta-analysis show a positive trend for prophylactic use of NSAIDs.

Furthermore, these studies showed that using NSAIDs is more cost effective. If an institution performs 750 ERCPs annually, and the incidence rate of PEP is 5%, we come to a number of 38 PEPs per year. United States Medicare provides financial support for PEP in the amount of 5700 USD per case, which when multiplied by the number of PEPs comes to 216600 USD per year. The cost of one dose of NSAIDs is between 1.25 and 2 USD. The annual cost of administering diclofenac before every

ERCP would be around 1500 USD, but it would reduce the number of PEPs to 13. Thus, a lower number of PEPs equals a smaller amount of money spent annually; we come to a figure of 74100 USD per PEP. Adding the cost of NSAIDs (1500 USD), the institution would spend 76500 USD, or 141000 USD less than if they were not using NSAIDs. Their meta-analysis supports the use of NSAIDs in PEP prophylaxis, giving an advantage to diclofenac.

Elmunzer *et al*[7] conducted an additional trial concerning the rectal application of NSAIDs. They performed a multicenter, randomized, placebo-controlled, double-blind clinical trial including 602 patients with a high risk of PEP development. A high risk for PEP was established based on previously validated patient-related and procedure-related risk factors. Out of the total number of patients, 493 (82%) had a suspicion of SOD. Patients were divided into two groups: one received a single dose of indomethacin rectally (295 patients) and the other received placebo (307 patients). PEP occurred in 27 patients (9.2%) in the indomethacin group and in 52 patients (16.9%) in the placebo group (P = 0.005). Furthermore, moderate/severe PEP was observed in 13 patients (4.4%) in the indomethacin group and in 27 patients (8.8%) in the placebo group (P = 0.03). They concluded that rectal administration of indomethacin notably reduced the incidence of PEP in patients who were at a high risk of PEP development. At the moment, it is absolutely clear that rectal administration of NSAIDs (diclofenac sodium and indomethacin) is the preferred method for reducing the incidence of PEP. Due to their good safety profile, low price and easy availability, NSAIDs are at this moment the best pharmacological prophylactic method. In the future, we are expecting the results from more randomized controlled trials regarding combination therapy (NSAIDs plus nitrates or antibiotics) and possible further reductions in the incidence of PEP.

Antibiotics

Prophylactic use of antibiotics is recommended by the British Society of Gastroenterology during ERCP in patients who are expected to obtain full patency of the bile duct, patients with advanced hematologic cancer, history of liver transplantation, pancreatic pseudocyst and patients with severe neutropenia. Others recommend antibiotic prophylaxis before ERCP, especially in the presence of biliary obstruction. Antibiotics should decrease or prevent post-ERCP complications, such as cholangitis, cholecystitis, septicemia and pancreatitis. A meta-analysis was conducted by Brand *et al*[34] on nine randomized, controlled trials including 1573 patients. They showed the beneficial properties of antibiotic prophylaxis, but only in patients whose biliary obstruction persisted after ERCP. In patients whose biliary obstruction was resolved, antibiotics did not have much effect. The conclusion was drawn that, although antibiotics show beneficial properties in PEP prophylaxis, the presence or absence of biliary obstruction after ERCP is the determining factor in the efficacy of antibiotics and the incidence of post-ERCP infections.

Antibiotic prophylaxis of PEP is still to be proven and established and there are conflicting viewpoints on this matter. For instance, the American Society for Gastrointestinal Endoscopy recommends antibiotic prophylaxis for ERCP in patients with bile duct obstruction.

Research performed by Räty *et al*[35] suggests that antibiotic prophylaxis effectively decreases the risk of PEP development. They conducted a study on 321 patients, who were divided into two groups: a prophylaxis group and a control group. There were 161 patients in the prophylaxis group; all received 2 g of cephtazidime, and 160 patients in the control group who did not receive an antibiotic. Patients with allergy to cephalosporins, immunodeficiency, clinical jaundice or with any other condition requiring antibiotic usage were excluded. Also, pregnant patients did not participate. The diagnosis of acute pancreatitis was based on increased levels of serum amylase (> 900 IU/L), CRP level, leukocyte count, no increase in liver chemical values and clinical findings. Nine patients in the prophylaxis group (6%) and 15 patients in the control group (9%) had a notable increase in serum amylase levels (> 900 U/L) after ERCP, but only four out of nine patients in the prophylactic group developed clinical signs of pancreatitis, leukocytosis and pain. In comparison, all 15 patients from the control group with hyperamylasemia had pain, elevated CRP, leukocytosis and other signs of pancreatitis. Multivariate analysis showed that lack of antibiotic prophylaxis and sphincterotomy are independent risk factors for the development of PEP. They concluded that the application of antibiotics as chemoprophylaxis effectively decreases the chances of PEP development.

However, in the most extensive review and meta-analysis by Bai *et al*[36] on antibiotic prophylaxis of post-ERCP cholangitis, the authors included seven trials and 1389 patients which were divided into two groups: 705 patients in the control group and 684 in the treated group. Cholangitis occurred in 5.8% of control group patients and 3.4% of treated patients, with no statistical significance. In accordance with the ASGE recommendations for antibiotic prophylaxis, sensitivity analysis was performed targeting patients with suspicious biliary obstruction. It showed that the incidence of post-ERCP cholangitis was 2.8% in patients who received antibiotics and 5.4% in control group patients, suggesting that there is no protective effect of antibiotics. In their

summary, they agreed that antibiotics cannot be used as an effective means of post-ERCP cholangitis prevention.

Although their data showed no correlation between antibiotic prophylaxis and a reduced rate of post-ERCP cholangitis, we can assume that the same premise can be applied to the connection of antibiotic prophylaxis and PEP prevention, *i.e.,* antibiotic administration will not be effective in the prophylaxis of PEP. However, due to the lack of sufficient data on this topic, we believe that further research should be conducted in an attempt to show the potential benefit of antibiotics as chemoprophylactic agents.

Other pharmacological treatments

There are some other pharmacological agents thought to be potentially beneficial in PEP prophylaxis. For example, allopurinol has demonstrated beneficial properties in animal models. However, three trials with human subjects offer conflicting and inconclusive results[37,38]. In two trials, the authors showed benefits from the usage of allopurinol. Kastinelos *et al*[37] gave 600 mg of allopurinol *per os* to their patients 15 and 3 h before ERCP and saw significantly lower rates of PEP in comparison to the placebo group; 3.2% and 17.8%, respectively. Furthermore, patients with pancreatitis who received allopurinol had shorter duration of hospital stay than those who were in the placebo group. Martinez-Torres *et al*[38] gave 300 mg of allopurinol *per os* to 85 patients at same times as in the Katsinelos trial, while the other 85 patients received oral placebo. They observed significantly lower rates in PEP incidence, *i.e.,* 2.3% in comparison to 9.4% in the placebo group. However, Mosler *et al*[39] conducted a trial where they randomly administered allopurinol and placebo 4 h and 1 h prior to ERCP. PEP incidence was 12.96% and 12.14%, in allopurinol and placebo groups, respectively. They concluded that there is no efficacy of allopurinol prophylaxis of PEP.

A new possible treatment to the prevention of PEP is being used by German physicians who recently published a study protocol[40]. They designed a randomized, double-blind, placebo controlled study where they will test the effect of magnesium sulfate on the incidence and severity of PEP. They will include a total of 502 patients distributed into two groups. One group of patients will receive 4930 mg of magnesium sulfate 60 min before and 6 h after ERCP and the other group will receive placebo at the same time intervals. The incidence of PEP and hyperlipasemia, the degree of pain, analgesic usage and the length of hospitalization will be observed and analyzed. Their opinion is that, if successful, magnesium sulfate could become a routinely used a pharmacological prophylactic agent.

There are some alternative approaches with promising results such as aggressive hydration with Ringer's lactate[41,42]. Buxbaum *et al*[41] performed a study in which patients who were undergoing ERCP for the first time were randomly assigned to groups (2:1) that either received aggressive hydration with lactated Ringer's solution (3 mL/kg per hour during the procedure, a 20-mL/kg bolus after the procedure, and 3 mL/kg per hour continuously for 8 h post-ERCP) or standard hydration with Ringer's solution (1.5 mL/kg per hour during and for 8 h post-procedure). They concluded that aggressive intravenous hydration with lactated Ringer's solution reduces development of PEP. Since these are the results of a pilot study with only 62 patients, this benefit has to be shown in trials with an adequate sample size.

Although we have adequate pharmacological agents such as NSAIDs, which can significantly reduce the incidence of PEP, possible new approaches are very welcome. We are eager to see the results from adequately powered trials regarding aggressive hydration. If we get positive results, this may become the easiest preventive method.

Non-pharmacological approaches

Vila *et al*[43] presented an article reviewing the factors contributing to PEP and other post-ERCP complications, such as non-technical factors and technical factors. They also emphasized the role of pancreatic stenting and NSAIDs in PEP prophylaxis as the two methods with the most scientific evidence. Non-technical factors include placement of the pancreatic stent and administration of NSAIDs. Multiple studies have shown the benefits of placing a pancreatic stent.

Pancreatic stent placement

There are many reviews and analyses suggesting the beneficial impact of pancreatic stent placement. Singh *et al*[44] conducted a meta-analysis which included five studies and 481 patients. They showed that the incidence of PEP in the stented group was significantly lower (5.8%) in contrast to the no-stent group (15.5%).They drew a conclusion stating that temporary placement of a stent in the main pancreatic duct lowers risk for PEP. Additional meta-analysis of one more study by Andriulli *et al*[45] showed similar results. They conducted a meta-analysis of 6 controlled studies with an addition of 12 uncontrolled studies. Their results showed that the stented group had a PEP rate of 12% while the control group rate was 24.1%. They also showed a reduction in the number of cases of severe pancreatitis in stented patients. Choudhary *et al*[46] conducted a meta-analysis on eight randomized, controlled trials and 656 patients, and 10 non-randomized studies including 4904 patients. They observed the incidence of PEP, incidence of hyperamylasemia, incidence of mild, moderate and

severe pancreatitis, and possible adverse effects of stent placement. The results of the randomized, controlled studies showed a significant decrease in the PEP incidence in the stented group, *i.e.,* 4.6%. The incidence of PEP in the control group was 19.7%. Furthermore, fewer PEP cases were observed in patients with a stent < 3 cm, but with no statistical significance. No statistically significant difference was noted in using flanged and unflanged stents. Concerning hyperamylasemia, levels were significantly lower in the stented group. Analysis of the non-randomized trials also showed a statistically significant lower incidence of PEP in five trials. Moreover, pancreatic stenting led to fewer cases of severe pancreatitis. Although there is no doubt that pancreatic stents decrease the incidence of PEP, several questions remains unanswered, possibly vital questions which if answered could lower the PEP incidence even more. Who should get a pancreatic stent? What is the best time of placement - before or after therapy, *e.g.,* before sphincterotomy? How long do stents have to remain in place? For now, pancreatic stents are placed in high risk patients. Further research has to be done in order to provide answers to these questions. However, the European Society of Gastrointestinal Endoscopy recommends stent placement in high risk patients undergoing ERCP[2]. High risk patients are, according to a consensus, patients with SOD, young women, patients with previous pancreatitis, and patients with a high number of cannulations and injections of the pancreatic duct during ampullectomy or cannulation. The recommended size of the stent is 5-Fr. Furthermore, pancreatic stents should be placed taking endoscopists's rate of success into consideration, which has to be > 75%[2].

Mazaki *et al*[9] reviewed and conducted a meta-analysis on 8 studies including 680 patients. All studies included different kinds of high risk patients, such as SOD, difficult cannulation, precut sphincterotomy, biliary balloon dilatation of an intact papilla for stone extraction, ampullectomy or pancreatic brush cytology. Pancreatic stent placement had a success rate of 90% to 100% in five studies. Out of the total number of 680 patients, 336 received a pancreatic stent, while 344 were in the control group. Total number of PEPs was 82; 19 patients (6%) in the stent group, and 64 patients (19%) in the control group, which was statistically significant. They also showed that pancreatic stents were more efficient in high risk patients. This meta-analysis showed that pancreatic stent placement is a good and effective prophylaxis for PEP. Furthermore, it is consistent with previously performed meta-analyses[44,45].

Ito *et al*[47] conducted a study on 9192 ERCP procedures. Out of the total number of ERCPs, 414 patients were included in this study as they were high risk patients for the development of PEP. High risk criteria were: female gender, history of pancreatitis, SOD, difficult cannulation of the bile duct, pancreatic duct cytology/biopsy, precut sphincterotomy, pancreatic sphincterotomy and endoscopic ampullectomy. The size of stents used was 5-Fr, 3 cm long with a single pigtail. The goals of this study were to explore the frequency and severity of PEP, the frequency of hyperamylasemia and risk factors for PEP. The incidence of PEP was 9.9% and 90% of those were mild cases. Taking the high risk factors of these patients into consideration, the PEP incidence in this study was acceptable. The frequency of moderate to severe cases of PEP was 10%. In two important studies[48,49], the rates of moderate and severe pancreatitis were 47% and 25%, respectively. In conclusion, the results of this study suggested that pancreatic duct stenting decreases the incidence of PEP, and could possibly contribute to less severe cases of PEP, thereby easing the patient's recovery.

Zolotarevsky *et al*[50] conducted a trial regarding the optimal stent size for insertion into the pancreatic duct for PEP prevention. The current viewpoint on stent size goes in favor of 5-Fr stents. Zolotarevsky *et al*[50] compared 5-Fr and 3-Fr stents in order to see which one led to better results in pancreatic stenting. A large trial by Rashdan *et al*[51] was conducted on 2283 patients who underwent ERCP and had a 3-4-Fr, unflanged stent placed. Incidence of pancreatitis was 7.5% and 10.6% for 3-Fr and 4-Fr stents, respectively in comparison with rates of 9.8% and 14.6% for larger stents, 5-Fr and 6-Fr, respectively. They concluded that smaller sized stents were superior to larger ones in preventing PEP. However, Chahal *et al*[52] showed a completely different situation; 5-Fr stents correlated with higher rates of spontaneous stent passage and lower rates of PEP. In addition, this study showed that placing 3-Fr stents had more failed attempts. Failure in placing a stent can prolong the procedure and thus augment the chances of PEP development. A comparison of 5-Fr and 3-Fr stents done by Zolotarevsky *et al*[50] was performed on 234 patients by random assignment of those stents. Out of the total number of patients, 78 were at high risk for PEP. Pancreatic stent placement was successful in 77 patients. Spontaneous passage rates duringa two-week period were 68.4% and 75% for the 5-Fr and 3-Fr stents, respectively. Lack of stent passage at 2 wk was also nearly the same, *i.e.,* 10.5% and 10% for the 5-Fr and 3-Fr stents, respectively.

Another important aspect in comparison of these two stents and their efficacy was the number of wires needed for stent placement. One wire was sufficient in 22 cases of 5-Fr stent placement (59.4%), whereas 3-Fr stent placement with one wire occurred in only eight cases (20.5%); a

significant difference. The time required to place a stent was more frequently prolonged during the placement of 3-Fr stents. Furthermore, the placement of 5-Fr stents was deemed easier than placing a 3-Fr stent.

Eleven patients (14.1%) developed PEP, which manifested a sa mild or moderate form. There was no statistically significant difference in PEP incidence between the stent groups. In conclusion, an increased number of wires needed for successful stent placement, prolonged attempts for stent placement and a higher number of failed stenting attempts may be associated with a higher incidence of PEP. In this study, the results showed that the technical aspects of the 5-Fr stent render it favorable over the 3-Fr stent; its placement is easier, faster and requires fewer wires. These criteria alone should be enough to give the 5-Fr stent an advantage in choosing the better and more effective stent in pancreatic stent placement. A recent, excellent network meta-analysis has provided definite results regarding some of these dilemmas. Afghani *et al*[53] analyzed 6 randomized, controlled studies including 561 patients. The authors concluded that the 5-Fr stent is superior to the 3-Fr pancreatic stent for the prevention of PEP in high risk patients. Also, the performance of 5 Fr stents was not influenced by the design (flanged, straight, pigtail), suggesting that the diameter is more important for the prevention of PEP than the type of stent.

Despite the robust data which favor the usage of NSAIDs and pancreas stenting in the prevention of PEP, gastroenterologists still have some doubts. Dumonceau *et al*[54] completed a survey about prophylactic pancreatic stenting and NSAID administration. They distributed the survey to 467 medical doctors, but collected only 141 completed ones. The majority of respondents (61.7%) worked in a community hospital where the ERCP volume was $\leqslant$ 500 per year. Diagnostic ERCP was used in < 20% of cases by the majority of respondents (83%). The majority of the respondents did not attempt prophylactic pancreatic stenting in the presence of procedure-related risks for PEP, such as prolonged or difficult cannulation, previous PEP or needle pre-cut. Only in the case of ampullectomy did the majority of respondents (54.5%) attempt pancreatic stenting. They attempted the procedure in more than 50% of cases. However, pancreatic stenting was attempted more frequently when patient-related risk factors were present. Thirty respondents (21.3%) did not attempt pancreatic stenting at all. Those who attempted pancreatic stenting used mainly 5-Fr stents (64.5%). Fourteen of them used either 3-Fr or 7-Fr stents. The majority of respondents, namely 118 of them (83.7%) did not use NSAIDs for PEP prevention; 88.1% of those 118 respondents cited lack of evidence as the main reason.

This survey showed a huge gap between the scientific evidence supporting prophylactic pancreatic stenting and its actual application in practice. The reasons for this are a lack of experience and the difficulty of the procedure itself (pancreatic stenting has the highest degree of difficulty). Many of the respondents have little or no confidence in using NSAIDs due to the lack of supporting evidence. Further investigations into PEP prophylaxis and better and more frequent ERCP education could provide a more stable ground for the implementation of techniques and increasing knowledge of the prevention of PEP.

Cannulation

Other technical factors include techniques used in duct cannulation, sphincterotomy and ampullectomy. Guide wire cannulation is one of these factors, and there are many variations. Guide-wire hovering is a variation of direct cannulation where the guide wire hovers a few millimeters to a couple of centimeters through the catheter or sphincterotome. It is useful in pancreatic cannulation when access through the minor papilla is needed.

The guide wire technique has advantages in comparison to contrast cannulation. For example, Cennamo *et al*[55] conducted a meta-analysis of five randomized controlled studies with 1762 patients who showed that guide wire cannulation improves the cannulation rate from 74.9% to 85.3% and, more importantly, reduces the incidence of PEP from 8.6% to 1.6%. Subsequently, guide wire cannulation is considered to be the standard for cannulation. Another variation is pancreatic stenting after guide wire placement. Fogel *et al*[56] reported a significant difference in the incidence of PEP between pancreatic stent placement followed by needle-knife sphincterotomy and double wire cannulation. Placing a stent led to a PEP incidence of 10.7%, while the double wire technique had a rate of 28.3%. Madacsy *et al*[57] also showed the benefits of stent placement. There were no cases of PEP in stented patients, while the PEP incidence in patients who underwent needle-knife with guide wire cannulation was 43%.

It is well known that pre-cut sphincterotomy increases the rate of PEP. It is still not well-defined regarding what is the best approach: to persistently attempt to cannulate or an early (five to ten minutes) switch to pre-cut. A meta-analysis by Cennamo *et al*[58] analyzed six trials by comparing the rates of cannulation and the incidence of PEP in early pre-cut cannulation and persistent cannulation with a late pre-cut. The analyzed data showed no difference in the success rate, *i.e.,* 90.2% and 89.6%, respectively. However, the incidence of PEP differed significantly. PEP occurred in 2.48% in early pre-cut, while its rate was 5.34% in late pre-cut. Another meta-analysis by Gong *et al*[59] also suggests that early pre-cut is more beneficial in PEP prophylaxis. Debate is still ongoing because two recent meta-

analyses have not provided a consistent answer[60,61]. While one group suggests that the rates of PEP are similar in the pre-cut sphincterotomy group and in the persistent attempt group (OR = 0.58, 95%CI: 0.32-1.05)[61], in the other meta-analysis, the authors claimed to have concluded that the early pre-cut technique decreases the trend of PEP incidence[60]. According to the recent literature, we may conclude that we have an obvious trend towards a reduction in PEP incidence by adopting the early pre-cut approach, but further data are needed.

While it is obvious that the wire-guide cannulation technique and pancreatic duct stenting significantly reduce PEP incidence, we are still lacking data regarding the early pre-cut technique. Endoscopists have dilemmas about continuing with attempts to cannulate and possibly further traumatizing the papilla, which can hamper cannulation later on, or switch to the needle knife early but possibly increase the risk of PEP, bleeding or perforation. With unequivocally positive results regarding early pre-cut, our decision would be easier.

CONCLUSION

In summarizing the prophylactic measures against PEP, we can conclude that only two methods of prophylaxis are currently recommended: pancreatic stent placement and NSAID administration, preferably with diclofenac.

Pancreatic stent placement is a recommended and effective method for preventing PEP today. Much is known of its beneficial properties, the type of stent needed, the duration of stent placement and so on. It is a method which has been proven to be effective. NSAIDs are cheap, can be easily given to patients and have little or negligible adverse effects, making diclofenac and other NSAIDs an attractive approach in PEP prevention, but there is still resistance to its usage due to the lack of reliable supporting evidence and/or the lack of information.

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Hyperhomocysteinemia as a potential contributor of colorectal cancer development in inflammatory bowel diseases: A review

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Abstract

Homocysteine is an amino acid generated metabolically by the S-adenosylmethionine-dependent transmethylation pathway. In addition to being a well-known independent risk factor for coronary heart disease, is also a risk factor for cancer. Patients suffering from inflammatory bowel diseases (IBD) including ulcerative colitis and Crohn's disease are at increased risk of developing colorectal cancer in comparison to healthy individuals. Furthermore, the risk of hyperhomocysteinaemia is significantly higher in IBD patients when compared with controls. In the present article, we review the mechanisms in which hyperhomocysteinemia may contribute to increased risk of colorectal cancer in IBD patients.

Key words: Hyperhomocysteinemia; Colorectal cancer; Inflammatory bowel disease

Core tip: There is growing evidence suggesting hyperhomocysteinemia to be associated with increased colorectal cancer risk. Taking this into account that hyperhomocysteinemia and its related contributors are prevalent among patients with inflammatory bowel disease, we suggest performing well designed epidemiological, experimental, and clinical trial studies to investigate such association in these patients.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic relapsing-remitting immune disorder of unknown

etiology that afflicts millions of individuals throughout the world with debilitating symptoms, which impair performance and quality of life[1]. IBD is precipitated by a complex interaction of environmental, genetic, and immunoregulatory factors. Higher rates of IBD are seen in northern, industrialized countries[2]. Recurrent inflammation with ulceration and tissue restitution confers an increased risk of colorectal cancer in both ulcerative colitis (UC) and Crohn's disease (CD)[3]. Although colorectal cancer occurs in a minority of IBD patients (1%), it carries a high mortality and accounts for 20% of IBD-related mortality[4].

Homocysteine is a sulfur-containing amino acid derived from the metabolism of methionine via methyl group metabolism[5]. There is little doubt that hyperhomocysteinemia plays a role in the development of cardiovascular disease. This is not only supported by human population studies identifying it as an independent risk factor, but strong evidence resides in animal models, as well[5]. More recently, a relationship between hyperhomocysteinemia and increased risk of different cancers has been indicated[6-11]. In the present article, we review the association between hyperhomocysteinemia and increased risk of colorectal cancer in IBD and the possible mechanisms.

INFLAMMATORY BOWEL DISEASE

IBD, including UC and CD, is characterized by chronic inflammation of the gastrointestinal tract in genetically susceptible individuals that are exposed to environmental risk factors[12]. CD may affect all parts of the gastrointestinal tract, from mouth to anus, but most commonly involves the distal part of the small intestine or ileum, and colon. UC results in colonic inflammation that can affect the rectum only (proctitis) or can cause continuous disease from the rectum proximally, to involve part of or the entire colon. Clinical symptoms include diarrhea, abdominal pain, gastrointestinal bleeding, and weight loss[13]. IBD has become one of the most common chronic inflammatory conditions worldwide[14]. The incidence and prevalence of IBD are increasing with time and in different regions around the world, indicating its emergence as a global disease[12]. In Canada, there are approximately 280000 patients with medically diagnosed IBD, which accounts for 0.8% of the population[15]. Although IBD has long been considered a disease that affects predominantly Western populations, recent data have shown significantly higher rates in Asians and time trend studies have shown an increase in its incidence across Asia[16]. IBD is mostly prevalent in young adults and currently is not curable, with patients usually requiring lifelong medication and may undergo devastating surgeries[17].

COLORECTAL CANCER IN INFLAMMATORY BOWEL DISEASE

The development of colorectal cancer is a serious long-term complication of chronic inflammation[13]. Colorectal cancer still accounts for 10%-15% of deaths in patients with IBD. Herrinton *et al*[18] demonstrated a 60% greater relative risk of colorectal cancer among individuals with CD and UC compared with an age- and gender-matched cohort of patients without IBD. IBD-associated colorectal cancer affects patients at a younger age than sporadic colorectal cancer. The prognosis for sporadic colorectal cancer and IBD associated colorectal cancer is similar, with a 5-year survival of approximately 50%. The increased risk of colorectal cancer in association with IBD is thought to be due to genetic and acquired factors[19]. The relationship between inflammation and cancer has been well established in the gastrointestinal system. The role of toll-like receptors and tumour necrosis factor-α (TNF-α) in the activation of nuclear factor κB, which induces transcription of genes involved in tumorigenesis, including COX-2 have been indicated in colitis-associated cancer. Defective signaling via p53 may be an early event in the progression of colitis-induced dysplasia to cancer. Without p53-induced apoptosis, aberrant cells are not eliminated and cancer may develop[20].

RISK FACTORS OF COLORECTAL CANCER DEVELOPMENT IN INFLAMMATORY BOWEL DISEASE

The extent and duration of colonic disease, the coexistence of primary sclerosing cholangitis, and a family history of sporadic colorectal cancer have been confirmed as risk factors of colorectal cancer in IBD patients. The risk of UC-associated colorectal cancer starts to increase after 7 years of extensive colonic disease[21]. In a meta-analysis of 41 studies the cumulative incidence of IBD associated colorectal cancer in patients with UC was 2% at 10 years, 8% at 20 years, and 18% after 30 years of disease[22]. The extent of mucosal inflammation has also been correlated with the risk of developing colorectal cancer. While patients with extensive disease (pancolitis and left-sided colitis) have an increased risk of developing colorectal cancer, patients with only proctitis or proctosigmoiditis do not[21]. There is conflicting evidence as to whether younger age at diagnosis of IBD is an independent risk factor for colorectal cancer in IBD. This evidence is not easy to evaluate, as children tend to have more extensive and severe colitis than those diagnosed as adults, and younger people have the potential for longer

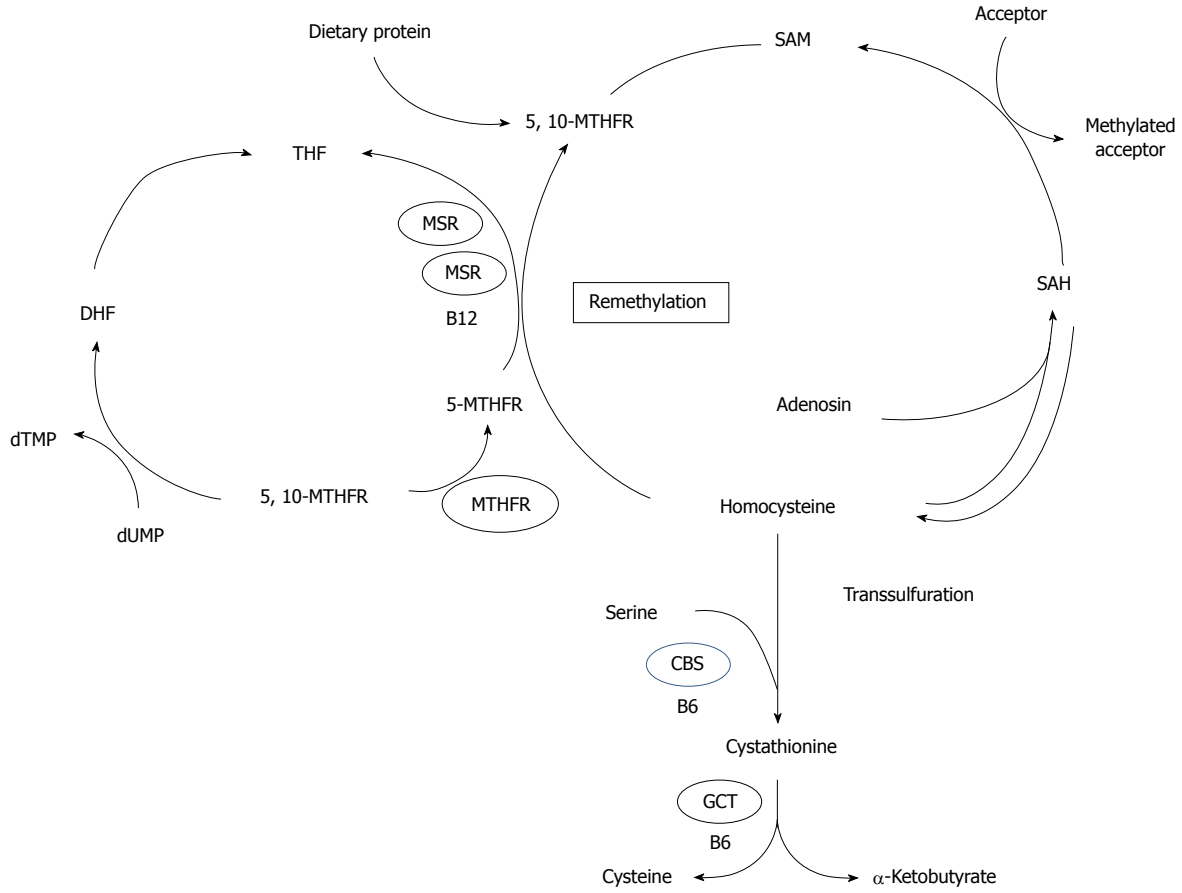

Figure 1 Metabolism of homocysteine[7]. dUMP: Desoxyuridine monophosphate; dTMP: Desoxytimidine monophosphste; THF: Tetrahydrofolate; DHF: Dihydrofolate; 5-MTHF: 5-methyltetrahydrofolate; 5,10-MTHF: 5,10-methyltetrahydrofolate; 5,10 MTHFR: 5,10- methyltetrahydrofolate reductase; MS: Metionin synthase; MSR: Metionin synthase reductase; B12: Vitamin B12; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; CBS: Cystathionine β-synthase; GCT: γ-cystathionase; B6: Vitamin B6.

colitis duration, which is itself a risk factor[19]. IBD patients with a first-degree relative with colorectal cancer have twice the risk of developing colorectal cancer than those who do not. Moreover, if a first-degree relative suffered from colorectal cancer before the age of 50 years, the risk of developing colorectal cancer in IBD patients increases nine-fold[21]. Some genetic polymorphisms have been proposed to be associated with the risk of colorectal cancer in UC patients[23]. So far, there has not been any specific biomarker useful to identify the high-risk patients for progression to colorectal cancer in IBD patients[21].

HOMOCYSTEINE METABOLISM AND PATHOGENESIS OF HYPERHOMOCYSTEINEMIA

Homocysteine is a non-protein-forming, sulfur amino acid whose metabolism is at the intersection of two metabolic pathways[24]: remethylation and transsulfuration (Figure 1). In remethylation, homocysteine acquires a methyl group from N-5-methyl-tetrahydrofolate (MTHF) or from betaine to form methionine. The reaction with MTHF occurs in all tissues and is vitamin B12-dependent, while the reaction with betaine is confined mainly to the liver and is vitamin B12-independent. ATP then activates a considerable proportion of methionine to form S-adenosylmethionine (SAM). SAM serves primarily as a universal methyl donor to a variety of acceptors including guanidinoacetate, nucleic acids, neurotransmitters, phospholipids, and hormones. S-adenosylhomocysteine (SAH), the by-product of these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer. In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5'-phosphate (PLP)-containing enzyme, cystathionine β-synthase (CBS). Cystathionine is hydrolyzed by a second PLP-containing enzyme, gamma-cystathionase, to form cysteine and alpha-ketobutyrate. Excess cysteine is oxidized to taurine and inorganic sulfates or excreted in the urine. Thus, in addition to the synthesis of cysteine, this transsulfuration pathway effectively catabolizes excess homocysteine which is not re-

quired for methyltransfer, and delivers sulfate for the synthesis of heparin, heparan sulfate, dermatan sulfate, and chondroitin sulfate. It is important to note that since homocysteine is not a normal dietary constituent, the only source of homocysteine is methionine[25].

Two enzymes and three vitamins play a key role in the regulation of circulating homocysteine levels. Of the enzymes, cystathionine-β-synthase controls the breakdown of homocysteine to cystathionine in the transsulfuration pathway, while methylene tetrahydrofolate reductase (MTHFR) is involved in the remethylation pathway, in which homocysteine is converted back to methionine. Folic acid, vitamin B6 and vitamin B12 are essential cofactors in homocysteine metabolism and a lack of them due to a deficient diet or disease can produce elevated plasma homocysteine[26]. In addition, a genetic defect in one of the enzymes of homocysteine metabolism can lead to metabolic disruption and potentially to hyperhomocysteinemia[24]. Of the gene defects, the most common is the C-to-T substitution at nucleotide 677 in the coding region of the gene for MTHFR, the so-called thermolabile variant. There is an elevated homocysteine concentration and a decreased plasma folate concentration in the homozygous mutant genotype of C677TMTHFR gene[26].

Depending on its severity, hyperhomocysteinemia is classified into several categories: (1) severe hyperhomocysteinemia which is characterized by high homocysteine levels at all times, caused by deficiencies in CBS, MTHFR, or enzymes of vitamin B12 metabolism; (2) mild hyperhomocysteinemia during fasting which is characterized by moderately high homocysteine levels under fasting conditions and reflects impaired homocysteine methylation (folate, vitamin B12, or moderate enzyme defects (*e.g.*, thermolabile MTHFR); and (3) mild hyperhomocysteinemia during post-methionine load that is defined as abnormal increase in homocysteine after methionine load which reflects impaired homocysteine transsulfuration (heterozygous CBS defects, vitamin B6 deficiency)[25].

It should be noted that in addition to the above mentioned key enzymes and vitamins, a variety of other factors affect the regulation and function of these enzymes, including diet, age, physiological state, and hormonal imbalance. Moreover, and in addition to the MTHFR C677T polymorphisms, the majority of these enzymes exhibit polymorphic forms that certainly have the potential to influence homocysteine balance for specific individuals, as has been discussed[27].

ROLE OF HYPERHOMOCYSTEINEMIA IN CANCER DEVELOPMENT

Many malignant cells are methionine dependent, resulting from the inability of malignant cells to convert homocysteine to methionine[28]. Elevated total homocysteine could be an early marker of carcinogenesis and a sensitive marker for detecting recurrence. The change of serum levels of homocysteine paralleled that of different tumor markers in cases of ovarian, breast, pancreatic and colon cancer suggesting that serum total homocysteine level, like tumor markers, reflected the tumor cell activity or the rapid proliferation rate of tumor cells. In addition, hyperhomocysteinemia caused by the proliferation of tumor cells was also demonstrated from the study of cell tissue cultures[28].

Several biochemical changes indicate that elevated homocysteine in blood creates a risk for cancer, and it is likely that hyperhomocysteinemia is a risk factor for carcinogenesis. Hyperhomocysteinemia is frequently associated with folate deficiency. In fact, homocysteine has become a sensitive marker for the deficiency of folate and the majority of the cancer risk derived from hyperhomocysteinemia is likely to be related to folate status. Polymorphism of MTHFR may reduce the production of its product, 5-MTHF, and increase the risk for cancer. 5-MTHF is the major form of folate in serum that provides the methyl group for DNA methylation. Reduction of 5-MTHF results in global genomic hypomethylation[28], which is an early and consistent event in carcinogenesis. Global hypomethylation of the coding and noncoding regions and demethylation of repetitive DNA sequences may contribute to the development of cancer through the following mechanisms: chromosomal instability, increased mutations, reactivation of intragenomic parasitic sequences that could be transcribed and moved to other sites, where they could disrupt normal cellular genes mitotic recombination leading to loss of heterozygosity and promotion of rearrangements, aneuploidy, loss of imprinting, and up-regulation of proto-oncogeneses[29]. Hyperhomocysteinemia has been shown to be a potential oxidative stress indicator via its impact on folate status. The overproduction of oxygen free radicals generated from the oxidation of homocysteine causes of endothelial injury and DNA damage. As reduced free homocysteine contains a free sulfhydryl group, free radicals including hydrogen peroxide can be generated upon oxidation of homocysteine, forming a disulfide linkage with free sulfhydryl group of albumin, cysteine or homocysteine. Actually, the plasma level of reduced free homocysteine affects and enhances oxidative stress. The endogenous attack on DNA by hydrogen peroxide and oxygen free radicals may cause gene mutations such as P53 and ras gene, and eventually lead to carcinogenesis[28,30,31]. However, a recent case-control study by Chiang *et al*[32] indicated that that increased homocysteine concentration is strongly associated with the risk of colorectal cancer

independently of oxidative stress indicators and antioxidant capacities.

Another mechanism by which homocysteine might predispose to cancer is the activation of proinflammatory genes due to region-specific hypomethylation. Results of *in vitro* and *in vivo* experiments have suggested that homocysteine might provoke intestinal mucosal injury by modulating TNF-α-mediated cytotoxicity. Indeed, plasma homocysteine has been regarded as a determinant of TNF-α in pathological conditions characterized by low-grade inflammation and targeting the TNF pathway can significantly reduce homocysteine, suggesting a role for this cytokine in homocysteine metabolism[33]. Finding out the biological mechanisms in which hyperhomocysteinemia plays its carcinogenic effects requires further investigations including well-designed experimental studies.

HOMOCYSTEINE STATUS IN INFLAMMATORY BOWEL DISEASE

In 1996, Lambert *et al*[34]. were the first who reported elevated homocysteine levels in patients suffering from CD in comparison with healthy controls Since then, several studies reported the higher prevalence of hyperhomocysteinemia in IBD patients in comparison with healthy subjects. Recently, Oussalah *et al*[35] performed a meta-analysis of 28 studies that had evaluated plasma homocysteine level and/or hyperhomocysteinemia risk in IBD patients. They found that the mean plasma homocysteine level was significantly higher in IBD patients when compared with controls and the mean plasma homocysteine level did not differ between UC and CD. In addition, they reported that the risk of hyperhomocysteinemia was significantly higher in IBD patients when compared with controls (OR = 4.65; 95%CI: 3.04-7.09). Hyperhomocysteinemia in IBD patients has been mainly attributed to low folate[36-38], vitamin B12[36-38], and vitamin B6 status[39]. A meta-analysis on genetic variants of homocysteine metabolism pathway in IBD did not find a relationship between MTHFR C677T polymorphism and IBD risk[40]. It should be noted that the impact of MTHFR C677T polymorphism on IBD risk according to plasma folate concentration was not assessed in this study. However, in another meta-analysis, Oussalah *et al*[35] found that MTHFR 677TT genotype was associated with higher IBD risk in patients with low plasma folate status. As mentioned before, this genotype is accompanied by elevated homocysteine concentration[26]. Furthermore, the hyperhomocysteinemia in IBD patients is suggested to be associated with advanced age, male sex, vitamin B12 deficiency or lower vitamin B12 serum levels, multivitamin therapy, and disease severity[41].

HYPERHOMOCYSTEINEMIA IN THE PATHOGENESIS OF COLORECTAL CANCER

It has been shown that homocysteine enhances growth of colon cancer cells in culture and the growth-promoting effect of homocysteine is reversed by folate[42]. In 1999, Kato *et al*[43] published the first epidemiological study showing the relationship between biological markers for folate status and colorectal cancer risk among women. Since 1999, different studies that have investigated the potential role of homocysteine status in the pathogenesis of colorectal cancer reported controversial results.

In a case-control study, total homocysteine levels were significantly higher in cancer patients (18 cases of breast cancer and 29 cases of colorectal cancer) compared to controls[44]. Univariate analysis demonstrated that total homocysteine levels significantly correlated with both interleukin-6 and TNF-α both in breast and colorectal cancer patients. In addition, TNF-α was independently associated with total homocysteine in patients with breast or colorectal cancer suggesting that cancer-related inflammation may be associated with elevated total homocysteine levels. The authors concluded that homocysteine-induced damage related to cell adhesion molecules, cytokines and chemokines might therefore contribute to the biology of cancer[44]. Battistelli *et al*[26] reported that non-metastatic colorectal cancer patients, who were eligible for curative surgery, had statistically higher levels of homocysteine than healthy individuals did. They also found that the increase of plasma homocysteine observed in the C/C and C/T genotype of C677TMTHFR gene carriers in the cancer group might be related to the methionine-dependent proliferation rate of colorectal cancer cells and might act as a permissive factor for thrombosis in the context of cancer thrombophilia. The homocysteine increase observed in T/T genotype carriers in both groups, on the other hand, was probably dependent on the enzymatic deficit associated with the homocysteine conversion to methionine and/or the depletion of folate. However, it should be mentioned that conflicting data exists on the relationship between different C677TMTHFR polymorphisms and risk of colorectal cancer development. For instance, while The TT genotype of MTHFR was found to associated with an increased risk of CRC in older populations, possibly due to age related disturbances in folate metabolism[45], the C677T was reported to have a protective effect on colorectal cancer development in a population with low allelic variability and an optimal intake of folic acid[46]. A recent meta-analysis of 70 published studies concluded that the MTHFR 677TT allele was

associated with a decreased risk of colorectal cancer in comparison to CT + CC polymorphisms (OR = 0.86; 95%CI: 0.76-0.96)[47].

The mean plasma homocysteine level in 226 cases of colorectal cancer and 437 matched referents from the population-based Northern Sweden Health and Disease Study did not differ significantly and plasma homocysteine concentrations were not significantly associated with colorectal cancer risk[48]. Although high homocysteine concentration was reported to be inversely correlated with colorectal tumorigenesis in patients suffering from end-stage renal disease[49], the association between increasing plasma total homocysteine levels and colorectal cancer was reported in three other case-control studies[50-52]. Kim *et al*[53] performed an observational study on 30 persons with colorectal polyps and found that the mean concentration of serum homocysteine was 22% higher in patients with adenomatous polyps than in those with hyperplastic polyps. It should be noted that hyperplastic polyps are generally regarded as not having malignant potential. A recent study among 422 Korean patients with colorectal adenoma and 617 controls indicated a higher plasma homocysteine concentration to be significantly correlated with increased risk of adenoma among women[54]. In a nested case-control study within the Norwegian JANUS cohort, total homocysteine was associated with increased risk of colorectal cancer[55]. Odds ratio (OR) for the upper *vs* lower tertile was 1.32 (95%CI: 1.04-1.68; *P*-value for trend = 0.02). In addition, no interaction between MTHFR polymorphisms and total homocysteine was detected. However, in a case-control study nested within the Multiethnic Cohort study in United States, investigators analyzed prospectively collected blood samples from 224 incident colorectal cancer cases and 411 matched controls and reported no association between plasma homocysteine levels and risk of colorectal cancer[56]. Similarly, in another nested case control study from the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study cohort in Finland, serum homocysteine was unrelated to risk of colon or rectal cancer[57]. In a recent nested case-control study, Miller *et al*[58] demonstrated that high plasma homocysteine was associated with increased risk of colorectal cancer among a large sample (*n* = 988/group) of United States postmenopausal women. In this study, multivariate-adjusted OR (95%CI) for colorectal cancer was 1.46 (1.05, 2.04) for the highest quartile of homocysteine compared with the lowest quartile. In another recent case-control study in Taiwan, high serum homocysteine level was significantly associated with increased odds of colorectal before and after adjustment for different potential confounders including oxidative stress indicators and antioxidant capacities[32].

One of the most promising molecular markers of outcome that have been investigated in colorectal cancer is microsatellite instability (MSI). Approximately 15% of colorectal cancers are characterized by MSI, reflecting inactivation of the mismatch repair genes. The remaining 85% of colorectal cancers develop from the chromosomal-instability (microsatellite-stable) pathway. In comparison to patients with microsatellite stable tumors, those with tumors having a high degree of MSI (MSI-H) have a significantly better prognosis[59]. A strong association between sporadic MSI-H and plasma homocysteine has been indicated in Danish patients with colorectal cancer[57]. In addition, the authors indicated that systemic folate did not reflect the level of folate in tumor tissue and systemic homocysteine but not systemic folate found to be a biomarker for MSI-H[60]. Hyperhomocysteinemia has also been suggested as the missing link between type 2 diabetes mellitus and colorectal cancer risk[61].

The relationship between homocysteine status and colorectal cancer has been investigated in clinical trials, as well. Martínez *et al*[62] assessed the relation of plasma folate and homocysteine and colorectal adenoma recurrence separately in two studies. The first involved an intervention of a cereal supplement that contained folic acid, wheat bran fiber (WBF), and the second was conducted primarily during postfortification of the food supply using ursodeoxycholic acid (UDCA). It is worthy to note that UDCA may prevent colonic neoplastic transformation by countering the tumor-promoting effects of secondary bile acids, such as deoxycholic acid (DCA). UDCA exerts cytoprotective effects and has been shown to antagonize DCA-induced cell death of transformed colonocytes[63]. In these trials, among non-multivitamin users, individuals in the highest *vs* the lowest quartile of homocysteine had higher odds of adenoma recurrence, in both the WBF (OR = 2.25) and UDCA (OR = 1.93) populations. Using the data from WBF trial, Martínez *et al*[64] found that relative to subjects in the highest quartile of plasma homocysteine, those in the lowest quartile had an OR of adenoma recurrence of 0.69 (*P*-value for trend = 0.02) after adjustment for confounding factors. They reported a significant dose response between plasma homocysteine and adenoma recurrence. Using the data from 627 participants from the control arm of Polyp Prevention Trial, a large 4-year multicenter randomized, controlled trial in United States the authors found that high homocysteine concentrations were positively associated with two times increased likelihood of any and multiple adenoma recurrence[65]. Also, there was a suggestive positive association between high homocysteine concentrations and high-risk adenoma recurrence[65]. In the analysis of subjects, participating in a randomized clinical trial of folate and/or aspirin for the prevention of colorectal

adenomas there was no association between baseline plasma total homocysteine and adenoma recurrence risk in either the placebo or the folic acid supplementation groups[66]. The lack of association between plasma total homocysteine and recurrence risk was similar for all adenoma end-points. In this study, baseline plasma total homocysteine was associated with the number of adenomas at the baseline examination, but this association was attenuated and no longer statistically significant after controlling for potential confounders, including plasma total folate and other B vitamins. About half the subjects in the study were recruited after voluntary folate fortification of the United States food supply began in 1996, and the first 3-year observation period overlapped a time of gradually increasing folic acid availability in United States and Canadian diets, with consequently decreasing total homocysteine levels. The authors discussed that it was possible that their negative results were due to the progressively lower plasma total homocysteine, which might have fallen to levels below a threshold for an association with adenoma risk. They concluded that their data would suggest one of two possibilities: there is no independent association between plasma total homocysteine and adenoma recurrence risk or that any association between plasma total homocysteine and adenoma recurrence may be limited to plasma total homocysteine levels higher than their study population who were largely folic acid-fortified[66].

ROLE OF HYPERHOMOCYSTEINEMIA IN THE DEVELOPMENT OF COLORECTAL CANCER IN INFLAMMATORY BOWEL DISEASE

Although the role of folate deficiency in the increased risk of colorectal cancer in IBD patients has been indicated in different studies[67-69], to date only one study investigated the relationship between homocysteine status and colorectal cancer in IBD patients. Phelip *et al*[29] performed a cross-sectional study to analyze the factors (especially hyperhomocysteinemia and folate deficiency) associated with the development of dysplasia-associated lesions or masses, or colorectal carcinoma in 114 IBD patients. In univariate analysis, the risk of oncogenesis in the IBD patients was significantly associated with low level of folate, and hyperhomocysteinemia. In multivariate analysis, neither hyperhomocysteinemia nor folate deficiency were associated with increased risk of colorectal cancer. However, when hyperhomocysteinemia was associated with folate deficiency, there was a significant increased risk of carcinogenesis (OR = 16.9, 95%CI: 2.3-126.7). Although, patients with hyperhomocysteinemia without folate deficiency had 2.5 times as many carcinogenic lesions as patients with normal homocysteinemia, the association was not statistically significant (P = 0.08). They concluded that hyperhomocysteinemia was significantly associated with oncogenesis when there was concomitant folate deficiency and in the subgroup of patients with low folate and no hyperhomocysteinemia, no increased risk of oncogenesis or preoncogenesis was shown.

CONCLUSION

Overall, studies investigating the relationship between hyperhomocysteinemia and risk of colorectal cancer have shown a tendency toward increased risk of colorectal cancer in association with elevated homocysteine levels. Although, most studies have also demonstrated that the effect of hyperhomocysteinemia on carcinogenesis is associated with low folate status and other vitamin B deficiencies mainly due to the underlying metabolic pathways that cause hyperhomocysteinemia, there is some evidence from well-designed studies showing independent effects of hyperhomocysteinemia on colorectal cancer development. In addition, there is some evidence suggesting that hyperhomocysteinemia may be a risk factor for cancer development in IBD. There should be further well designed prospective studies to investigate if hyperhomocysteinemia is associated with increased colorectal cancer risk in IBD patients. Currently, the primary strategy for managing colorectal risk in IBD is to conduct high quality colonoscopy screening at regular intervals in at risk individuals[70]. With the finding that hyperhomocysteinemia is associated with increased risk of colorectal cancer, it is highly suggested to include IBD patients with elevated levels of homocysteine as an "at risk" group of patients to perform regular colonoscopic screening, and in addition, to provide hyperhomocysteinemia lowering therapy using B vitamins (*e.g.*, folic acid, B6 and B12).

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Benign esophageal lesions: Endoscopic and pathologic features

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Abstract

Benign esophageal lesions have a wide spectrum of clinical and pathologic features. Understanding the endoscopic and pathologic features of esophageal lesions is essential for their detection, differential diagnosis, and management. The purpose of this review is to provide updated features that may help physicians to appropriately manage these esophageal lesions. The endoscopic features of 2997 patients are reviewed. In epithelial lesions, the frequency of occurrence was in the following order: glycogenic acanthosis, heterotopic gastric mucosa, squamous papilloma, hyperplastic polyp, ectopic sebaceous gland and xanthoma. In subepithelial lesions, the order was as follows: hemangioma, leiomyoma, dysphagia aortica and granular cell tumor. Most benign esophageal lesions can be diagnosed according to their endoscopic appearance and findings on routine biopsy, and submucosal lesions, by endoscopic resection. Management is generally based upon the confidence of diagnosis and whether the lesion causes symptoms. We suggest endoscopic resection of all granular cell tumors and squamous papillomas because, while rare, these lesions have malignant potential. Dysphagia aortica should be considered in the differential diagnosis of dysphagia in the elderly.

Key words: Benign tumor; Esophagus; Epithelial lesions; Subepithelial lesions; Endoscopy

Core tip: Benign esophageal tumors have a lower detection rate due to the fact that most patients are asymptomatic. The majority of these benign lesions are asymptomatic, and diagnoses are often made incidentally during investigations for other symptoms. Although biopsy or excision is required for a definitive

diagnosis, understanding the endoscopic appearances provides essential help for differential diagnosis. In epithelial lesions, the frequency of occurrence was in the following order: glycogenic acanthosis, heterotopic gastric mucosa, squamous papilloma, hyperplastic polyp, ectopic sebaceous gland and xanthoma. In subepithelial lesions, the order was as follows: hemangioma, leiomyoma, dysphagia aortica and granular cell tumor.

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INTRODUCTION

Esophageal benign lesions have a diverse spectrum of etiologies in terms of clinical course and underlying pathologic features. Benign esophageal tumors, while uncommon compared with esophageal carcinoma, can sometimes cause dysphagia but often have insignificant clinical outcomes. Endoscopic findings are essential for detection, diagnosis, staging, and treatment planning. Benign esophageal tumors are rare, with a prevalence $\leqslant$ 0.5%[1], while benign tumors represent 20% of esophageal neoplasms on autopsy[2]. Since many of these tumors are small and asymptomatic, few benign esophageal lesions attract clinical attention. Benign esophageal lesions could be detected more often with the widespread use of endoscopes, radiologic imaging[3], and increased awareness of the disease. Esophageal lesions can be classified in two different ways; histologically depending on the involved layer into epithelial or subepithelial lesions and endoscopically depending on endoscopic features such as flat, raised, or cystic lesions.

In this article, we review the endoscopic and pathological features of esophageal benign lesions. In all, 149 benign esophageal lesions in 2997 endoscopic examinations are retrospectively reviewed. We removed the esophageal epithelial lesions by biopsy or resected the subepithelial lesions by endoscopic mucosal resection or endoscopic submucosal dissection for histological analysis. In this article, we divide these lesions into epithelial or subepithelial lesions based on the final histological findings. The Institutional Review Board at Mackay Memorial Hospital approved this project.

EPITHELIAL LESIONS

Heterotopic gastric mucosa

In all, 21 patients had histologically proven heterotopic gastric mucosa (HGM) (12 men, 9 women; mean age: 48 years, range: 25-69 years), which appears salmon-colored under conventional esophagogastroduodenoscopy (EGD) and is recognized as flat or slightly elevated (Figure 1A), while the iodine reaction is negative. Narrow band imaging facilitates mucosal surface evaluation by adjusting the reflected light to enhance the contrast between the esophageal mucosa and the HGM and may improve the diagnostic rate of HGM (Figure 1B). It is easily overlooked because it is typically located just below the upper esophageal sphincter.

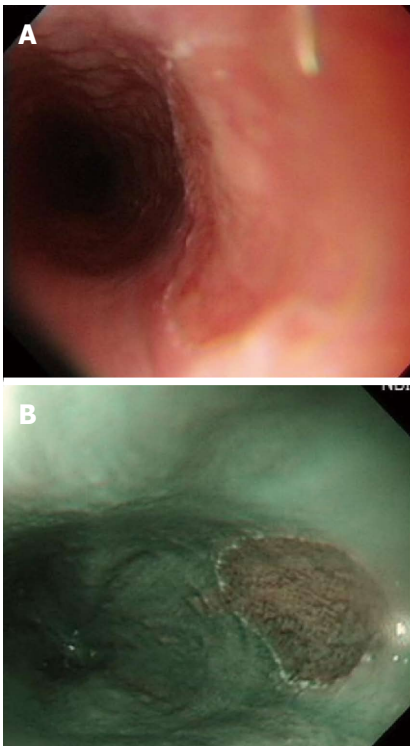

Figure 1 Heterotopic gastric mucosa. A: Heterotopic gastric mucosa appears salmon-colored under conventional endoscopy and is recognized as flat or slightly elevated; B: Narrow band imaging facilitates mucosal surface evaluation of heterotopic gastric mucosa by adjusting reflected light to enhance the contrast between the esophageal mucosa and the gastric mucosa and may improve the diagnosis of heterotopic gastric mucosa.

The prevalence of HGM is 0.1%-10%[4-6], and most patients have no symptoms[5,6]. Some patients present with pharyngeal globus sensation, heartburn, acid regurgitation, or dysphagia because HGM produces mucin and acid. Two types of HGM have been recognized: one with foveolar epithelium and fundic glands and the other with only foveolar epithelium[7]. The foveolar epithelium produces neutral mucins. The number of reported cases with neoplastic changes or malignant transformation is very low and these cases are considered extremely rare[8]. Management is generally required only if symptoms or complications develop.

Squamous cell papilloma

In all, 20 patients had histologically proven squamous cell papilloma (SCP) (3 men, 17 women; mean age:

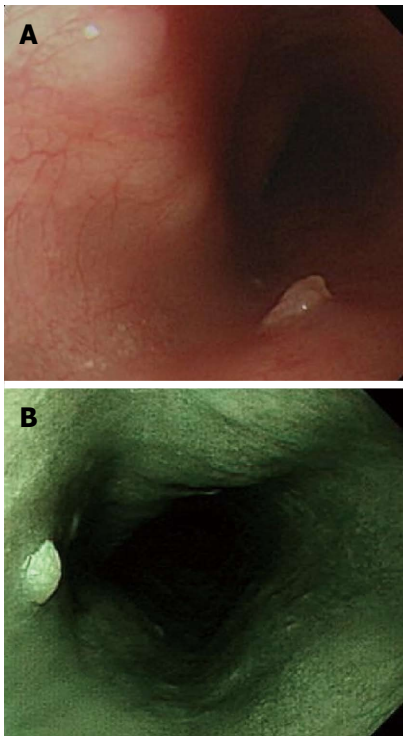

Figure 2 Squamous cell papilloma. A: Squamous cell papilloma is recognized as whitish-pink, wart-like exophytic projections on conventional endoscopy; B: Narrow band imaging facilitates mucosal surface evaluation of squamous cell papilloma and shows that microvessels in the lesion are not dilated.

49 years, range: 26-68 years), which is recognized as whitish-pink, wart-like exophytic projections on conventional endoscopy (Figure 2A) and is a rare benign esophageal lesion, with a prevalence of 0.01%-0.45%[9,10]. Narrow band imaging facilitates mucosal surface evaluation of squamous cell papilloma and shows that microvessels in the lesion are not dilated (Figure 2A). Most cases of SCP are solitary and asymptomatic[10,11]. The etiology of SCP is not fully understood, although there are two possible hypotheses: chronic mucosal irritation and infection with human papillomaviruses[12]. The hypotheses of inflammatory reactions such as gastroesophageal reflux disease were based on the high frequency of SCP occurring in the lower third of the esophagus. To our best knowledge, whether SCP is associated with human papillomavirus is controversial[13]. Papillomatosis in the proximal esophagus seems to favor involvement of the human papillomavirus[14].

In the majority of the cases, SCP of the esophagus can be removed using endoscopic biopsy forceps because most are only a few millimeters in size. Larger papillomas can be removed using endoscopic mucosectomy. We suggest removing these lesions endoscopically because, while rare, these lesions have malignant potential.

Hyperplastic polyp

In all, 18 patients had histologically proved hyperplasia polyp (HP) (12 men, 6 women; mean age: 36 years, range: 18-69 years). In our patients, HPs were most common in the region of the esophago cardiac junction (67%), followed by the distal esophagus (27%) and mid-esophagus (6%). HP appears as a polypoid lesion on edematous inflamed gastric folds at the gastroesophageal junction (Figure 3). HPs of the esophagus and esophagogastric junction region are characterized by the presence of mixed inflammatory infiltrates with plasma cells, eosinophils, fibroblasts, and inflamed stroma. HPs are associated with concurrent erosive esophagitis in the majority of cases, but other potential etiologies including medication-induced pill esophagitis, infection, and previous anastomosis or polypectomy have been reported[15]. These results suggest that the pathogenesis of HP is a mucosal regenerative response to surrounding mucosal injury[16].

Treatment for HP is similar to that for gastroesophageal reflux. In our experience, HP regresses after anti-acid therapy such as proton pump inhibitors. Careful clinical history and biopsy of the non-polypoid mucosa are essential for determining the clinicopathological context in which the HP developed. When HP is found with Barrett's esophagus, endoscopic mucosal resection is recommended[17].

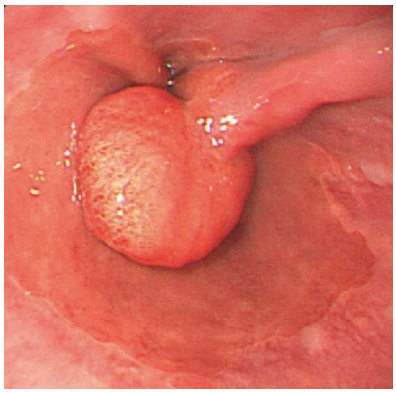

Figure 3 Hyperplasia polyp. Hyperplasia polyp occurs as a polypoid lesion and is located on edematous inflamed gastric folds at the gastroesophageal junction.

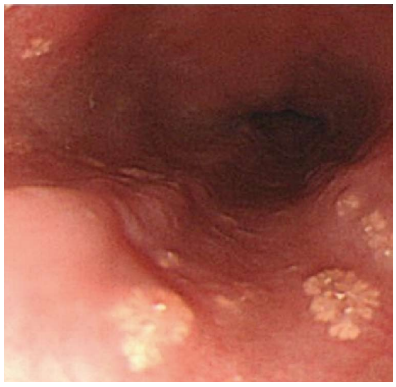

Figure 4 Xanthoma. Xanthomas are endoscopically recognized as elevated, granular, yellowish, fern-like lesions and scattered on a normal mucosal surface.

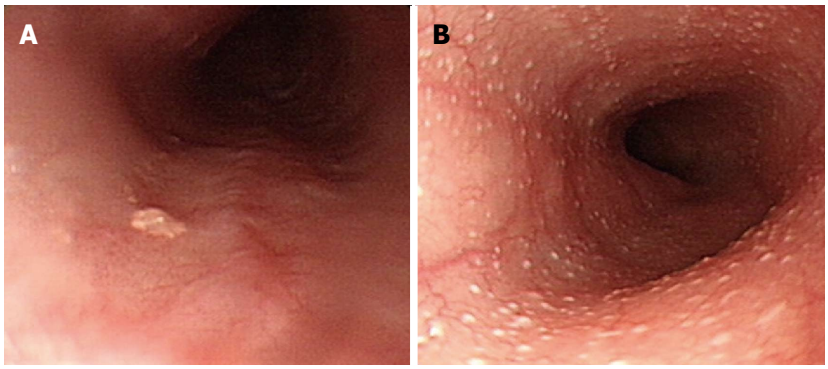

Figure 5 Ectopic sebaceous gland. A: The number of ectopic sebaceous gland is variable from single to B: more than one hundred yellowish plaques measuring 1 to 2 mm in the esophagus.

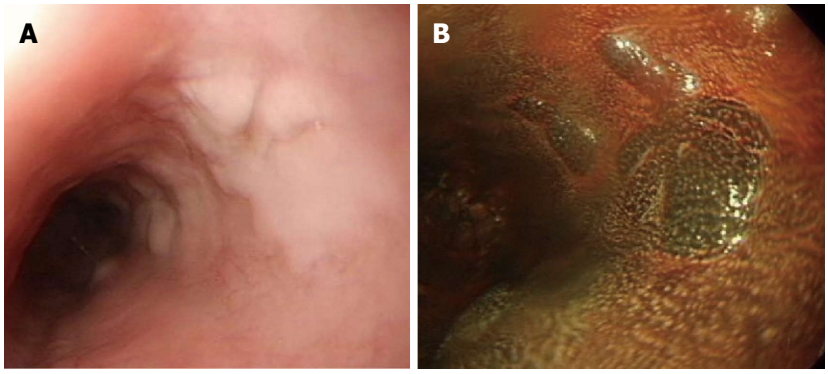

Figure 6 Glycogenic acanthosis. A: Esophagogastroduodenoscopy reveals multiple, uniformly sized, oval or round glycogenic acanthosis usually < 1 cm, involving otherwise normal esophageal mucosa; B: In chromoscopy with iodine spray, glycogenic acanthosis is recognized as slightly elevated iodine-positive, brownish areas.

Xanthoma

We reported on esophageal xanthomas localized in the lower esophagus in a 62-year-old man. EGD revealed some well-defined, fern-like, and yellowish lesions scattered over the middle and lower esophagus (Figure 4).

In terms of endoscopic findings, xanthoma is most commonly solitary, 2-10 mm across, and located in the lower esophagus, but cases of multifocal lesions have been reported[18,19]. Microscopically, they consist of fat accumulation in foamy histiocytes beneath the squamous epithelium[20]. One study showed the most common location of ectopic xanthoma in the gastrointestinal tract was the stomach (76%), followed by the esophagus (12%) and duodenum (12%)[21]. The etiology of esophageal xanthoma remains unknown, but one study theorized that these xanthomas were derived from focal mucosal damage, and that lipids derived from broken down cell membranes are captured by interstitial histiocytes[13]. This may explain why they occur less frequently in the esophagus than stomach because the esophageal mucosa can better tolerate mucosal injury[21].

Xanthoma must be distinguished on endoscopy from other yellowish lesions such as carcinoid tumor, granular cell tumors, and ectopic sebaceous glands (ESGs).

ESGs

In all, three patients (one man and two women; mean age: 52 years, range: 45-69 years) had histologically proven ESGs within the esophagus. EGD revealed variable numbers from single (Figure 5A) to > 100 (Figure 5B) yellowish plaques measuring 1-2 mm in diameter in the middle and lower esophagus.

ESGs have been found in various tissues, such as the lips and mouth, external genitalia, parotid glands, palms, soles, and various organs[22]. Typically, the mean age of affected patients is approximately 50 years, and the condition has equal gender distribution[23]. The numbers of ESGs in the esophagus varied from single to > 100, while their size was 1-20 mm (the majority were < 0.5 cm)[23,24]. The ESGs are most likely the result of a metaplastic process rather than a congenital anomaly because the ESGs are derived from endodermal tissue unlike the sebaceous glands, which are derived from ectodermal tissue[22,25].

The apparent low incidence of this condition may be because of the lack of obvious clinical signs and symptoms. Most cases have been discovered incidentally by endoscopy during a referral for a

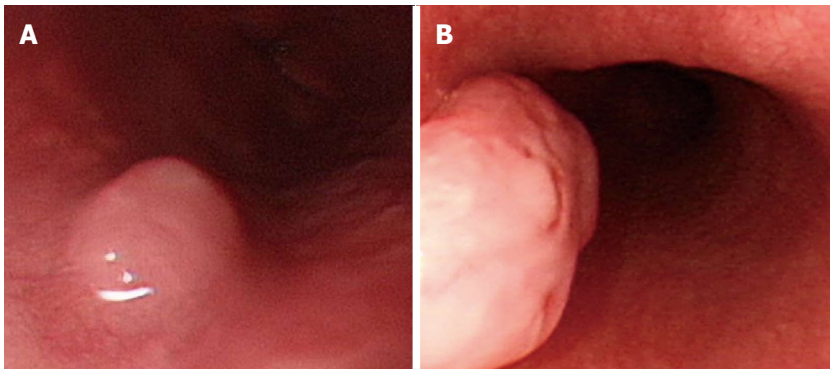

Figure 7 Leiomyoma. A: Leiomyoma commonly arises from the muscularis propria layer of the esophagus and presents as submucosal tumor; B: Leiomyoma arising from the muscularis mucosae can present as a polypoid intraluminal tumor.

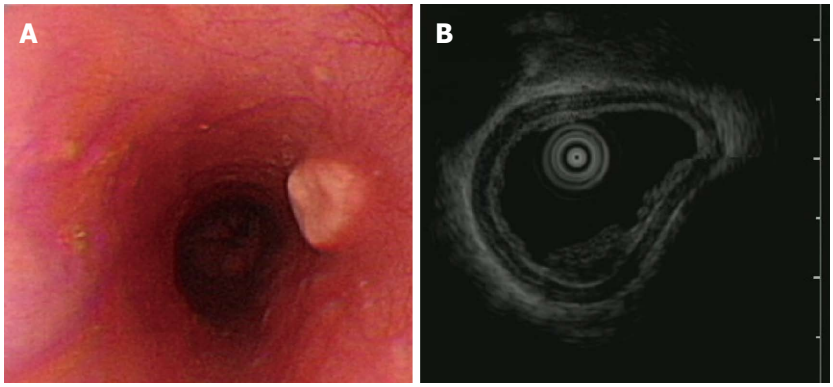

Figure 8 Granular cell tumor. A: Granular cell tumor is endoscopically recognized as a firm, yellowish subepithelial tumor covered with the normal mucosa in the esophagus; B: Endoscopic ultrasonography of granular cell tumor shows a homogenous hypo-echogenic tumor extending from the muscularis mucosa layer, and the musculais propria is not involved.

gastrointestinal tract examination. Most cases have no significant overall changes in ectopic sebaceous gland number, size, or shape during follow-up[26]. We suggest that ESGs do not need further treatment.

Glycogenic acanthosis

Glycogenic acanthosis was demonstrated as nodules involving otherwise normal esophageal mucosa in 66 patients (42 men and 24 women; mean age: 52 years, range: 45-79 years). Glycogenic acanthosis is defined as nodules involving otherwise normal esophageal mucosa. This is endoscopically recognized as slightly elevated areas on conventional endoscopy (Figure 6A) and slightly elevated iodine-positive areas on chromoscopy (Figure 6B). These lesions most commonly appear as multiple, uniformly sized, and oval or round elevations < 1 cm in diameter[27]. Biopsies after periodic acid-Schiff staining demonstrate that the nodules that represent glycogenic acanthosis are combinations of cellular hyperplasia and increased cellular glycogen[27]. Although glycogenic acanthosis was thought to be related to gastroesophageal reflux, antireflux therapy improved symptoms but failed to eradicate glycogenic acanthosis lesions[27,28].

SUBEPITHELIAL LESIONS

Leiomyoma

Leiomyoma was observed in three patients (3 men; mean age: 37 year, range: 25-43 years). Leiomyomas were located at the middle esophagus in 1 case and at the distal esophagus in 2 cases. The mean size of leiomyoma is 1.26 ± 0.4 cm. It commonly arises from the muscularis propria layer of the esophagus and presents as a submucosal tumor (Figure 7A). In rare circumstances, those cases arising from the muscularis mucosae can present as polypoid intraluminal tumors (Figure 7B).

Leiomyomas are the most common benign esophageal neoplasm, accounting for roughly two-thirds of all benign tumors of this organ[29]. Because they arise from smooth muscle cells, they are located mainly in the middle and distal thirds of the esophagus but are uncommon in the upper third of the esophagus, where the muscular layer is predominately skeletal in origin.

Most patients are asymptomatic, but dysphagia and pain may develop depending on lesion size and encroachment degree into the esophageal lumen. Treatment options include endoscopic enucleation[30],

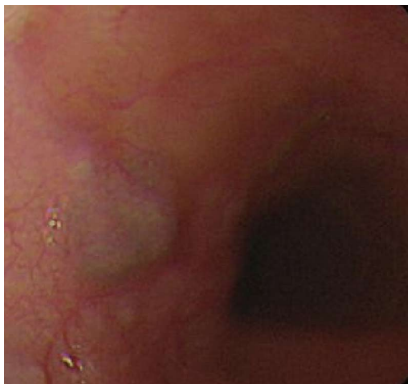

Figure 9 Hemangioma. On endoscopy, esophageal hemangioma appears cystic and bluish-red and can be pressed with biopsy forceps.

submucosal tunneling endoscopic resection[31], and surgical enucleation[32] or observation. Esophageal leiomyomas have a benign clinical course and typically do not recur after surgery.

Granular cell tumor

Granular cell tumor was observed in one 39-year-old health woman who presented with acid regurgitation and discomfort sensation when swallowing. EGD revealed one 12 mm × 15 mm firm, slightly elevated, whitish-to-yellow, and smooth nodular tumor covered by an intact epithelium (Figure 8A). Endoscopic ultrasonography showed one homogenous hypo-echogenic tumor extending from the muscularis mucosa layer (Figure 8B). The muscularis propria was not involved. Endoscopic submucosal dissection was performed.

Granular cell tumors are the secondary common cause of non-epithelial tumors in the esophagus. Most of the patients with esophageal granular cell tumor are asymptomatic[33], but some complain of mild dysphagia or retrosternal discomfort. Although most granular cell tumors have a clinically indolent course, it is estimated that 1%-3% are malignant with a 5-year survival rate < 35%[34]. Microscopically, granular cell tumors are composed of nests of ovoid or polygonal cells separated by collagen bundles. The tumor has malignant potential in the presence of necrosis, increased mitotic count, vesicular nuclei with large nucleoi, and high Ki67 index[35].

Resection is the main treatment for granular cell tumors. Endoscopic mucosal resection and endoscopic submucosal dissection were introduced and were considered the therapy of choice for tumors within the subepithelial layer or submucosa separated from the muscularis propria.

Hemangioma (venous bleb)

Esophageal hemangiomas were observed in 13 patients (4 men, 9 women; mean age: 67 years, range: 45-82 years). On endoscopy, they appear

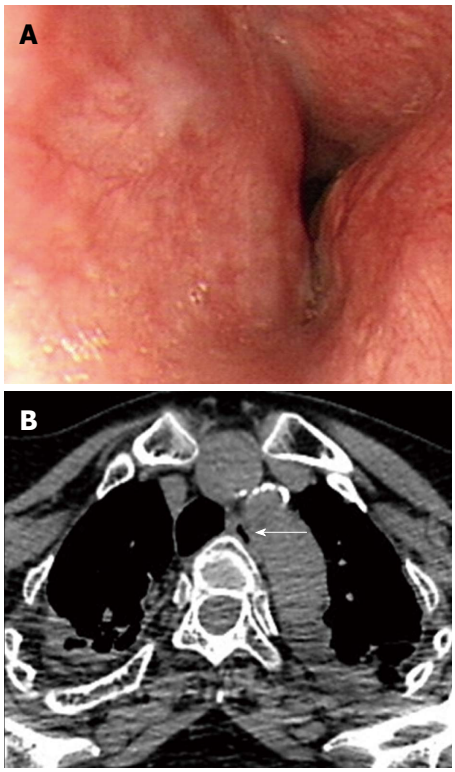

Figure 10 Dysphagia aortica. A: Esophagogastroduodenoscopy reveals a pulsatile extrinsic compression at about 25 cm from the incisor; B: The chest computed tomography showed aortic arch and descending aorta tortuosity with compression into adjacent esophagus. The arrow indicates the esophagus.

cystic and bluish-red and can be pressed with biopsy forceps (Figure 9). The prevalence of esophageal hemangiomas in the general population was 0.04% based upon the findings of an autopsy series[36]. The majority of these hemangiomas are cavernous; however, capillary lesions have been described. Although usually solitary, multiple lesions can be seen in congenital blue rubber bleb nevus syndrome[37]. Esophageal hemangiomas are usually found incidentally. When symptomatic, they are most often associated with bleeding and dysphagia. In such cases, surgical resection has been performed, but endoscopic resection has also been accomplished safely[38]. Although esophageal hemangiomas are uncommon, careful consideration during endoscopy is required to avoid the misdiagnosis of varices[39].

Dysphagia aortica

In dysphagia aortica was observed in three patients (1 man, 2 women; mean age: 68 years, range: 63-78 years). They visited our clinics with postprandial abdominal fullness and progressive dysphagia to solid meals. The esophagus begins on the right side of the thoracic aorta and then, descends and crosses the aorta anteriorly across its lower third. EGD reveals external compression of the middle-to-lower esophagus by the tortuous aorta with pulsations from the great vessels (Figure 10A).

Dysphagia aortica is a rare etiology of dysphagia resulting from the extrinsic compression of the esophagus by a thoracic aortic aneurysm or the tortuosity and elongation of the thoracic aorta (Figure 10B). The clinical findings resemble those of esophageal malignancy or esophageal motility disorders. Esophageal compression by a vascular structure is a common cause of dysphagia[40]. Non-aneurysmatic aortic dysphagia is usually observed in the elderly, especially in hypertensive women with enlarged heart and kyphosis[41,42].

The treatment of dysphagia aortica depends on symptom severity. Symptoms of mild dysphagia often improve with diet modifications (*e.g.*, by avoiding lying down immediately after taking drugs or food, eating small but frequent meals, and chewing well) and treatment of the coexistent disease, such as heart failure or hypertension. Some patients with more severe symptoms may respond to surgery[43,44].

Dysphagia aortica should be considered in the differential diagnosis of dysphagia, especially in the growing elderly population with underlying cardiovascular disease or hypertension.

CONCLUSION

Benign esophageal lesions have a lower detection rate due to the fact that most patients are asymptomatic. But it could be easily found with the widespread use of endoscopes and the increasing awareness of this disease. The majority of these benign tumors are asymptomatic, and diagnoses are often made incidentally during investigations for other complaints or symptoms. Although biopsy or excision is required for a definitive diagnosis, understanding the endoscopic appearances provides essential help for differential diagnosis. We suggest endoscopic resection of all granular cell tumors and squamous papillomas because, while rare, these lesions have malignant potential. Hyperplastic polyps could regress after anti-acid therapy such as proton pump inhibitors. We suggest that ectopic sebaceous glands and xanthoma do not need further treatment. Esophageal hemangiomas are uncommon, and careful consideration during endoscopy is required to avoid the misdiagnosis of varices. Dysphagia aortica should be considered in the differential diagnosis of dysphagia in the elderly.

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MINIREVIEWS

Novel insights into the mechanisms whereby isoflavones protect against fatty liver disease

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Abstract

Fatty liver disease (FLD) is a growing public health problem worldwide. There is an urgent requirement for alternative and natural medicine to treat this disease. As phytochemicals, isoflavones have attracted considerable attention for the prevention of FLD. Numerous studies have revealed that isoflavones protect against FLD through various pathways which modulate fatty acid β-oxidation, lipid synthesis, and oxidative stress. Recently, the aldose reductase (AR)/polyol pathway has been reported to be involved in the development of FLD by modulating hepatic fructose production, peroxisome proliferator-activated receptor (PPAR)α activity, cytochrome P450 (CYP)2E1 expression, and gut bacterial endotoxin-induced cytokine release. It has been reported that some isoflavones are potent AR inhibitors. Here, we review the anti-FLD actions of isoflavones and the proposed mechanism whereby isoflavones protect against FLD, with regard to the AR/polyol pathway. We propose that isoflavones block the AR/polyol pathway and in turn reduce fructose production and subsequent fat accumulation in the liver in diabetic or high-glucose-diet mice. In addition, in rodents with alcoholic liver disease or nonalcoholic fatty liver disease/nonalcoholic steatohepatitis, inhibition of AR by isoflavones may improve PPARα-mediated fatty acid oxidation, reduce hepatic steatosis, and attenuate CYP2E1-mediated oxidative stress or AR/gut bacterial endotoxin-mediated cytokine overproduction, to alleviate progression of FLD.

Key words: Isoflavones; Fatty liver disease; Aldose reductase; Fructose; Peroxisome proliferator-activated receptor α; Cytochrome P450 2E1; Endotoxin

Core tip: The aldose reductase (AR)/polyol pathway has recently been reported to be involved in the development of fatty liver disease (FLD) *via* various pathways. Some isoflavones have been reported to be potent AR inhibitors. Here, we review the anti-FLD actions of isoflavones and the proposed mechanism whereby isoflavones protect against FLD, with regard to the AR/polyol pathway. We propose that isoflavones block the AR/polyol pathway to suppress fructose production in the liver, improve peroxisome-proliferator-activated-receptor-α-mediated fatty acid oxidation, ameliorate cytochrome-P450-2E1-mediated oxidative stress, and attenuate AR/gut bacterial endotoxin-mediated cytokine overproduction, which in turn alle-

viates the progression of FLD.

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INTRODUCTION

Fatty liver disease (FLD) is a condition where neutral fat accumulates in liver cells, and may be accompanied by progressive inflammation of the liver. In light of the contribution of alcohol, fatty liver may be termed alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD), and the more severe forms of NAFLD as non-alcoholic steatohepatitis (NASH). It is difficult to distinguish ALD from NAFLD histologically. The histological spectrum of ALD includes steatosis, hepatitis and fibrosis, and NAFLD can mimic the entire spectrum of hepatic changes in ALD.

FLD is a growing public health problem worldwide. The prevalence of NAFLD is approximately 30% in developed countries and nearly 10% in developing nations[1]. FLD is increasingly recognized as an important cause of end-stage liver disease[2]. Current treatments for FLD focus on the factors that may cause the disease. In general, these treatments include weight loss, cholesterol management, blood glucose control, or treatment of alcoholism. Although several pharmacological agents for the prevention of FLD have been investigated, they have been found to be effective, but have side effects[3]. Thus, there is an urgent requirement for alternative and natural medicine to treat this disease. Isoflavones are phytochemicals and have been reported to prevent FLD in numerous studies through the regulation of peroxisome proliferator-activated receptors (PPARs), carbohydrate responsive element binding protein and Wnt signaling, to regulate fatty acid β-oxidation, lipid synthesis and oxidative stress[4]. Recently, the aldose reductase (AR)/polyol pathway has been reported to be involved in the development of FLD[5-7]. Of note, isoflavones such as genistein, daidzein and puerarin have been recognized as AR inhibitors[8,9]. However, only a few studies have investigated the effect of isoflavones on FLD by inhibition of AR. Thus, this article reviews the biological effects of isoflavones on FLD, and the mechanisms whereby isoflavones protect against ALD and NAFLD/NASH, with regard to the AR/polyol pathway.

CAUSES OF ALD AND NAFLD

The causes of FLD are alcoholism, toxins, inherited metabolic disorders, and certain drugs. Almost all heavy drinkers develop fatty liver. NAFLD has been consistently associated with insulin resistance and the metabolic syndrome (obesity, diabetes mellitus, hypertension, and dyslipidemia)[10]. Although many investigations have been carried out to elucidate the mechanisms of ALD development, the pathogenesis of ALD is still not fully understood. It is generally accepted that increased release of proinflammatory cytokines, induced oxidative stress, and elevated gut bacterial endotoxins play important roles in the development of ALD[11,12]. In contrast, the underlying cause of NAFLD/NASH is still not clear. However, there are several factors, which may be involved including insulin resistance[13,14], toxic inflammatory cytokines[15], oxidative stress inside liver cells[14,16], gut microbiota[17], endoplasmic reticulum stress[18], and genetics[19]. Day *et al*[20] proposed the hypothesis of "two hits" to clarify the mechanisms underlying the progression from steatosis to steatohepatitis. The first hit is insulin resistance, which causes hepatic steatosis and excess fatty acids. The second hit is oxidative stress and associated lipid peroxidation and cytokines within the liver, which may initiate progression from steatosis to steatohepatitis and ultimately to cirrhosis. Recently, Basaranoglu *et al*[21] suggested that possible candidates for the second hit included increased oxidative stress, lipid peroxidation and release of toxic products, decreased antioxidants, adipocytokines, transforming growth factor-β, Fas ligand, mitochondrial dysfunction, fatty acid oxidation by cytochrome P450s, peroxisomes, excess iron, small intestinal bacterial overgrowth, and the generation of gut-derived toxins such as lipopolysaccharide and ethanol. In addition to the two-hit hypothesis, a "multiple parallel hits" hypothesis was recently proposed by Tilg *et al*[18] to clarify the mechanisms underlying the development of liver inflammation. Many parallel hits derived from the gut and/or the adipose tissue may promote liver inflammation, such as endoplasmic reticulum stress, adipocytokines, and innate immunity.

ISOFLAVONES FOR PREVENTION OF FLD

Isoflavones are phytochemicals found in various legumes including soybean, kudzu, red clover, fava beans, alfalfa, chickpeas and peanuts. Numerous reports indicate that the consumption of isoflavones has many health benefits, including protection against menopausal symptoms, osteoporosis, cardiovascular disease, atherosclerosis, hyperlipidemia, and cancer[22,23].

Recent studies have demonstrated that isoflavones can protect against ALD or NAFLD (Table 1). The most studied isoflavones are soy isoflavones, including genistein and daidzein. Among the soy isoflavones, genistein is the most beneficial and protects against both ALD and NAFLD/NASH in rodents[24-33]. In addition to soy isoflavones, kudzu isoflavones and their main bioactive component,

Table 1 Effects of soy, kudzu and red clover isoflavones on fatty liver disease in rodents

Experimental model	Treatment	Effects	Ref.
Mice fed high-fat diet	Genistein	Alleviates NAFLD by stimulating hepatic fatty acid β-oxidation and increasing antioxidative enzyme	Lee *et al*[24]
Rats fed high-fat diet	Genistein	Prevents emergence of NASH by attenuating oxidative stress	Yalniz *et al*[25]
Rats fed MCD diet	Soy isoflavone	Prevents liver damage by decreasing lipid peroxidation in NASH model	Ustundag *et al*[26]
Rats fed high-fructose diet	Genistein	Reduces NAFLD *via* activation of antioxidant profiles and decreases IL-6 and TNF-α	Mohamed Salih *et al*[27]
Mice fed high-fat diet	Genistein	Reduces NAFLD by regulating adipocyte fatty acid β-oxidation and adipogenesis	Kim *et al*[28]
Rats fed high-fat diet	Genistein	Slows down NASH progression by inhibiting IκB-α phosphorylation, nuclear translocation of NF-κB p65 subunit, and activation of JNK	Ji *et al*[29]
Rats provided with ethanol	Genistein	Ameliorates alcoholic liver injury and liver fibrosis by reducing lipid peroxidation, recruiting the anti-oxidative defense system, inhibiting CYP2El activity, and promoting extracellular matrix degradation	Huang *et al*[30]
ApoE$^{-/-}$ mice fed high-fat diet	Genistein	Alleviates metabolic abnormalities including hypercholesterolemia and NASH in *ApoE*$^{-/-}$ mice	Kwon *et al*[31]
Mice fed high-fat diet	Daidzein	Prevents NAFLD through the direct regulation of hepatic de novo lipogenesis and insulin signaling, and the indirect control of adiposity and adipocytokines	Kim *et al*[32]
Rats fed high-fat diet	Daidzein	Reduces weight gain and fat content in liver by affecting PPARα/γ and stearoyl coenzyme A desaturase 1	Crespillo *et al*[33]
Rats fed high-fat diet	Puerarin	Reduces NAFLD *via* hepatic leptin signaling activation (leptin receptor/JAK2/STAT3)	Zheng *et al*[34]
Rats provided with ethanol	Puerarin	Prevents acute alcoholic liver injury by inhibiting oxidative stress	Zhao *et al*[35]
Mice provided with ethanol	Tectoridin	Protects against ethanol-induced liver steatosis by modulating disturbance of PPARα pathway and ameliorating mitochondrial function	Xiong *et al*[36]
Hepatocytes treated with ethanol	Puerarin	Restores viability of cells and reduces lipid accumulation in ethanol-treated hepatocytes by activating autophagy *via* AMPK/mTOR-mediated signaling	Noh *et al*[37]
Mice fed high-fat diet	*Puerariae* flower extract (isoflavone-rich)	Exerts anti-fatty liver effects by suppressing lipogenesis in the liver	Kamiya *et al*[38]
Rats provided with the Liber-DeCarli liquid diet	Puerarin	Alleviates chronic alcoholic liver injury by inhibiting endotoxin gut leakage, Kupffer cell activation, and endotoxin receptors expression	Peng *et al*[39]
db/db diabetic mice	Red clover extract (isoflavone-rich)	Reduces liver TG and cholesterol levels by activating hepatic PPARα and inhibiting hepatic fatty acid synthase	Qiu *et al*[40]
Mice fed cholesterol-enriched diet	2-heptyl-formononetin, formononetin	Induces hepatic steatosis, but decreases markers of inflammation and liver injury	Andersen *et al*[42]
Mice fed MCD diet	Red clover extract (isoflavone-rich)	Improves hepatic steatosis, but does not alleviate liver inflammation	Qiu *et al*[41]

AMPK: Adenosine 5′-monophosphate-activated protein kinase; IκB: Inhibitor of NF-κB; JAK2: Janus kinase 2; mTOR: Mammalian target of rapamycin; STAT3: Signal transducer and activator of transcription 3; NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; TNF: Tumor necrosis factor; IL: Interleukin; MCD: Methionine-choline-deficient; PPAR: Peroxisome proliferator-activated receptor.

puerarin, have received considerable attention due to their beneficial effect on ALD and NAFLD/NASH[34-39]. Red clover isoflavones have also attracted attention. We reported that red clover isoflavones can improve hepatic steatosis in db/db obese diabetic mice[40] and methionine-choline-deficient (MCD) diet-induced NASH mice[41]. However, we did not find that red clover isoflavones alleviated liver inflammation in MCD-diet-induced NASH mice. Surprisingly, formononetin, one of the major isoflavones in red clover, is reported to induce hepatic steatosis and decrease markers of inflammation and liver injury in mice fed a cholesterol-enriched diet[42]. There are few data on the effect of biochanin A, the other major isoflavone in red clover, on FLD. It is known that biochanin A can protect against CCl_4-induced liver fibrosis[43]. Therefore, the effect of biochanan A and formononetin on FLD cannot be concluded from the present studies and requires further investigation.

AR/POLYOL PATHWAY IN FLD

The polyol pathway is a glucose metabolic shunt that is defined by two enzymatic reactions catalyzed respectively by AR (EC1.1.1.21) and sorbitol dehydrogenase (SDH, EC1.1.1.14). AR catalyzes the rate-limiting reduction of glucose to sorbitol with the aid of co-factor NADPH and then SDH converts sorbitol to fructose using NAD^+[44].

It is well documented that the AR/polyol pathway is involved in the development of diabetes complications[45,46]. Elevated AR can lead to serious diseases affecting the heart and blood vessels, eyes, kidneys and nerves. AR is induced in diseased liver, other than in the above mentioned tissues that are vulnerable to complications of diabetes. AR was detected in the livers of two human subjects with ALD, but was undetected in healthy humans[47]. Moreover, AR is induced in human livers obtained

from patients undergoing liver transplantation for fulminant (acute) liver failure or end-stage liver disease from cirrhosis due to various chronic liver diseases, including ALD, chronic hepatitis B and C, primary biliary cirrhosis, autoimmune hepatitis, and hepatocellular carcinoma[48]. These studies indicate that AR may play an important role in the development of liver injuries. Recently, investigations were conducted to elucidate the role of AR in the development of FLD. Lanaspa *et al*[5] demonstrated that genetic ablation of the AR gene resolved high-glucose-diet-induced hepatic steatosis in mice. We previously demonstrated that inhibition of AR ameliorated hepatic steatosis in db/db diabetic mice[6], and lentivirus-mediated knockdown of the AR gene alleviated MCD-diet-induced NASH in db/db mice[7]. These studies confirm the involvement of AR in the development of FLD.

ISOFLAVONES PREVENT HIGH-GLUCOSE-INDUCED FATTY LIVER BY BLOCKING THE AR/POLYOL PATHWAY

Numerous reports show that isoflavones have significant AR inhibitory activity. Park *et al*[9] reported that genistein, daidzein and puerarin inhibited AR in rat lens with IC_{50} values of 4.5, 7.9 and 44.7 μmol/L, respectively, whereas formononetin exhibited weak AR inhibitor activity (IC_{50} > 100 μmol/L). Hsieh *et al*[49] reported that genistein inhibited AR in rat lens with an IC_{50} of 16.9 μmol/L, while Choi *et al*[50] reported that genistein inhibited AR in pig lens with an IC_{50} of 20 μmol/L. Moreover, tectoridin has also exhibited potent activity, with an IC_{50} value of 1.08 μmol/L[51]. Furthermore, biochanin A shows better binding interactions with AR than epalrestat, a synthetic AR-specific inhibitor, which indicates that biochanin A possesses significant AR inhibitory activity[52].

Recently, Lanaspa *et al*[5] found that mice deficient in AR were protected against fatty liver after exposure to 10% glucose for 14 wk. They demonstrated that the metabolic conversion of glucose to endogenous fructose by the AR/polyol pathway in the liver is a key step in the development of glucose-induced fatty liver in mice. In addition, we demonstrated that inhibition of AR ameliorates hepatic steatosis in db/db diabetic mice[6]. Therefore, we postulate that isoflavones can block the AR/polyol pathway and subsequently reduce fructose production in the liver and alleviate fatty liver in humans and animals with high glucose diet or in diabetic conditions. Indeed, genistein, daidzein and red clover isoflavones improve hepatic steatosis and dyslipidemia in diabetic mice, although their mechanisms of action are reported to be through different pathways[40,53].

ISOFLAVONES PREVENT FLD BY SUPPRESSING AR AND SUBSEQUENTLY IMPROVING PPARα-MEDIATED FATTY ACID OXIDATION

It is well established that PPARα, a nuclear receptor, is a central regulator for hepatic lipid catabolism[54]. It regulates the enzymes involved in fatty acid oxidation, for example, acyl-CoA oxidase (ACO), carnitine palmitoyl transferase (CPT)-1, and liver fatty acid binding protein. The ablation of PPARα gene causes the development of FLD[55,56]. Administration of PPARα agonists improves MCD-diet-induced NASH[57,58] and ethanol-induced liver injury[59]. Genistein, daidzein, biochanin A and formononetin are well known PPARα agonists[60,61]. Genistein and daidzein alleviate NAFLD in animals fed a high-fat diet by stimulating the hepatocyte and adipocyte PPARα pathway and fatty acid β-oxidation[24,28,33]. Red clover isoflavones also reduce liver triglycerides and cholesterol levels in db/db mice by activating hepatic PPARα[40]. These studies indicate that some isoflavones may act as PPARα agonists to prevent FLD.

Overexpression of AR in hepatocytes stimulates extracellular signal-regulated kinase (ERK)1/2 activation, sequentially phosphorylates hepatic PPARα at the OH group of serine 12 and 21, and reduces mRNA expression of ACO and CPT-1, two target genes transcriptionally regulated by PPARα[62]. This study indicates that AR overexpression in hepatocytes inhibits lipid degradation by suppressing PPARα activity. In diabetic db/db mice with hepatic steatosis, elevated hepatic AR also stimulates ERK1/2 activation and phosphorylates PPARα and suppresses its activity. The AR inhibitor, zopolrestat, attenuates the phosphorylation of PPARα and the suppression of PPARα activity, which improves hepatic steatosis in db/db mice[6]. These studies indicate that AR inhibitors may improve hepatic steatosis by modulating the phosphorylation of PPARα and its transcriptional activity. Indeed, genistein can reduce the level of phosphorylated PPARα and increase the mRNA expression of ACO in high-glucose-treated HepG2 cells (unreported data). Mezei *et al*[63] demonstrated that soy isoflavones modulate lipid metabolism in part *via* a PPARα-dependent mechanism in mice fed a high-fat diet. CPT-1 mRNA is consistently found to be induced by soy isoflavones in obese Zucker rats[64] and ACO mRNA is induced by soy isoflavones in Agouti (A(vy)/a) mice fed an AIN-93G diet to alleviate hepatic steatosis[65]. These studies suggest that isoflavones may improve FLD, at least in part, *via* the regulation of AR/PPARα mediated fatty acid oxidation.

ISOFLAVONES PREVENT FLD BY SUPPRESSING AR/CYP2E1-MEDIATED OXIDATIVE STRESS

Oxidative stress within the liver may act as the second hit and initiate the progression from steatosis alone to steatohepatitis and ultimately to cirrhosis[16,20]. Isoflavones as antioxidants have been well documented[66,67]. Genistein is reported to activate antioxidative enzymes and attenuate oxidative stress in animals fed a high-fat diet, thus alleviating NAFLD and preventing the emergence of NASH[24,25]. Puerarin is also reported to prevent acute alcoholic liver injury by inhibiting oxidative stress[35].

There is accumulating evidence that cytochrome P450 (CYP)2E1 plays an important role in the pathogenesis of liver tissue injury[68]. Upregulation of CYP2E1 may initiate lipid peroxidation by the production of reactive oxygen species (ROS) and promote liver inflammation[69]. Previous studies have shown that CYP2E1 activity correlates with ethanol-induced liver injury, and alcohol-induced hepatotoxicity is reduced when CYP2E1 is inhibited by inhibitors or by ablation of the CYP2E1 gene[70-73]. In addition to ALD, elevated CYP2E1 protein expression and activity are also found in both humans and animals with NAFLD/NASH and promote the progression of NAFLD/NASH[74-76].

We found that overexpression of AR in hepatocytes results in induction of CYP2E1 mRNA and protein, and simultaneously, ROS production is also induced by AR overexpression. Lentivirus-mediated knockdown of the AR gene attenuates MCD-diet-induced CYP2E1 expression, reduces the levels of lipid peroxidation, suppresses expression of proinflammatory cytokines, and alleviates NASH in db/db and C57BL/6 mice[7]. Our observation indicates that CYP2E1 expression is induced by elevated AR in fatty liver and generates ROS production, resulting in oxidative stress. AR inhibitors may alleviate steatohepatitis by attenuating CYP2E1 induction.

Several studies have revealed that isoflavones can reduce expression of CYP2E1 in healthy or diseased animals with liver injury. Soybean extract (rich in isoflavones) significantly decreases hepatic CYP2E1 expression in healthy rats[77] or in rats with high-fat-diet-induced NASH[78]. In addition, genistein significantly inhibits CYP2E1 activity and protects against alcohol-induced chronic liver injury in rats[30]. Moreover, in mice, pretreatment with puerarin prior to the administration of CCl_4 significantly suppresses the expression of CYP2E1 protein, and prevents hepatic malondialdehyde formation[79]. These studies suggest that the protective effects of isoflavones against hepatotoxicity possibly involve mechanisms related to its ability to block CYP2E1 activity. We propose that isoflavones inhibit AR activity and, at least in part, cause the subsequent suppression of CYP2E1 activity to alleviate oxidative stress and improve liver inflammation in humans and animals with ALD or NASH, although there is no direct evidence that isoflavones suppress CYP2E1 activity by blocking the AR/polyol pathway.

ISOFLAVONES PREVENT FLD BY SUPPRESSING AR/GUT BACTERIAL ENDOTOXIN-MEDIATED CYTOKINE PRODUCTION

In addition to oxidative stress, the gut bacterial endotoxin, toxic lipopolysaccharide (LPS), plays an important role in the development of alcoholic liver injury[12] or NAFLD/NASH[80]. Bacterial endotoxin reaches the liver through the portal circulation to activate hepatic Kupffer cells (special macrophages located in the liver) and stimulate their production of NO and cytokines, which subsequently cause damage to hepatocytes. Inhibition of the AR prevents nuclear factor (NF)-κB-dependent activation of tumor necrosis factor (TNF)-α, interleukin (IL)-12, IL-6, and macrophage chemoattractant protein-1 in livers of mice injected with LPS[81]. Moreover, pharmacological inhibition or siRNA ablation of AR prevents the biosynthesis of inflammatory cytokines and chemokines in LPS-activated RAW264.7 murine macrophages[81,82]. These studies indicate that inhibition of AR can prevent LPS-induced production of cytokines and chemokines in mice.

Pretreatment of RAW264.7 macrophages with genistein, luteolin, luteolin-7-glucoside and quercetin inhibits LPS-stimulated TNF-α and IL-6 release, whereas eriodictyol and hesperetin only inhibit TNF-α release[83]. Of these, luteolin and quercetin are the most potent inhibitors of cytokine production, with an IC_{50} < 1 and 5 μmol/L for TNF-α release, respectively. The cytokine-production-inhibiting potential of these flavonids is in accordance with their AR inhibitory activity (IC_{50}: luteolin 0.5-0.6 μmol/L, quercetin 3.3-7.73 μmol/L, and genistein 4.5-16.9 μmol/L against rat lens AR), suggesting that these compounds inhibit LPS-stimulated cytokine production, at least in part, through inhibition of AR activity.

Genistein is reported to have a beneficial effect on LPS-induced injury in rodent liver, RAW264.7 murine macrophages, and murine Kupffer cells. Zhao *et al*[84] reported that genistein suppresses hepatic production of LPS-induced TNF-α, IL-1β and IL-6 in rats. *In vitro* preincubation of liver slices from naïve rats with genistein suppresses LPS-induced TNF-α production in a dose-dependent manner. Both *in vivo* and *in vitro* administration of genistein suppresses LPS-induced liver proinflammatory cytokine overproduction. Lin *et al*[85] reported that genistein treatment significantly protects against

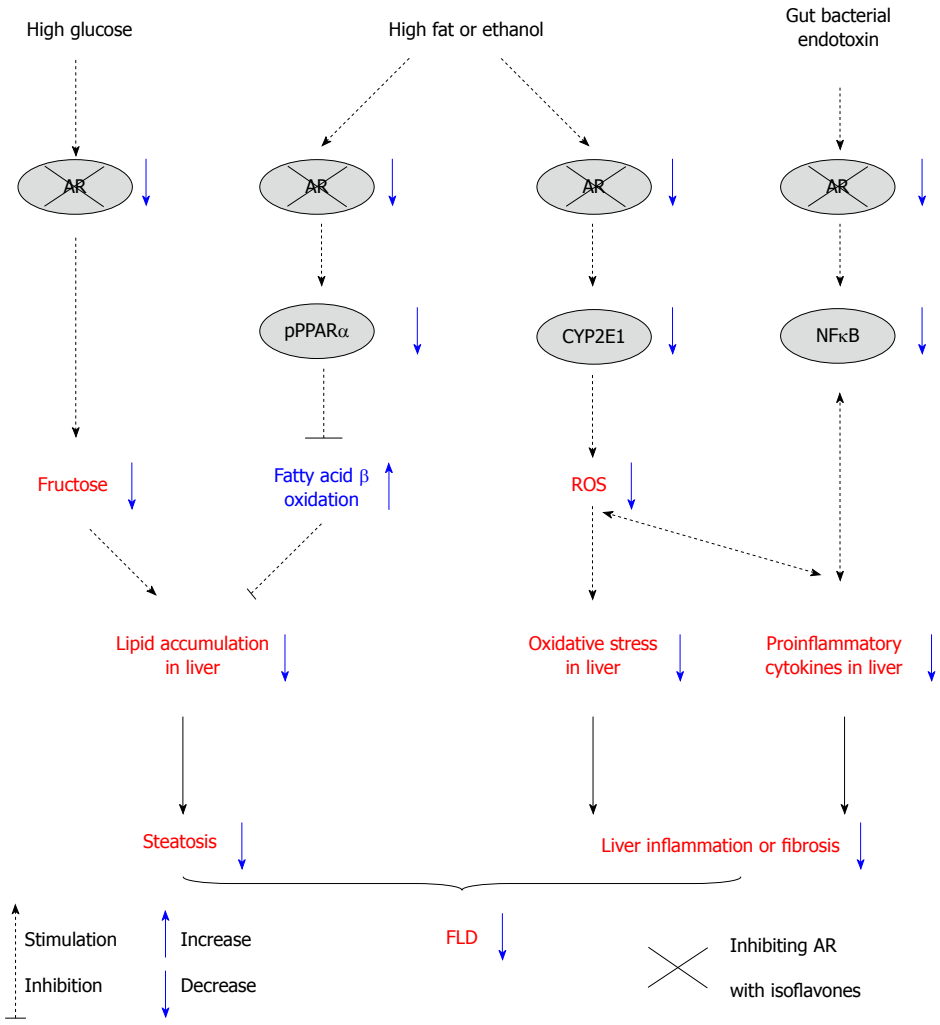

Figure 1 Proposed mechanism whereby isoflavones protect against fatty liver disease. Isoflavones inhibit the AR/polyol pathway to suppress fructose production in the liver, improve phosphorylated PPAR-α-mediated fatty acid oxidation, ameliorate CYP2E1-mediated oxidative stress, and attenuate AR/gut bacterial endotoxin-mediated cytokine overproduction, which in turn alleviates the progression of FLD. FLD: Fatty liver disease; AR: Aldose reductase; PPAR: Peroxisome proliferator-activated receptor; ROS: Reactive oxygen species.

LPS/D-galactosamine-induced liver injury in mice, and alleviates proinflammatory cytokines, including TNF-α and NO/inducible NO synthase, by inhibiting NF-κB activity.

The preventive effect of genistein on LPS-stimulated cytokine production has been found to be through inhibition of tyrosine kinase activity[86]. Genistein is a well-known inhibitor of tyrosine kinase, whereas daidzein does not inhibit tyrosine kinase activity. Genistein attenuates the liver injury caused by LPS in rats, whereas daidzein does not, which indicates the involvement of tyrosine kinase in LPS-induced liver injury. However, the AR inhibitory potential of daidzein is also weaker than that of genistein. Inhibiting AR activity is not an exclusive mechanism by which isoflavones protect against endotoxin-induced liver injury.

CONCLUSION

Collectively, isoflavones have been found to alleviate ALD and NAFLD/NASH in rodents, and these effects are partially achieved by the following mechanisms: (1) blocking the AR/polyol pathway to reduce fructose production in the liver under high-glucose conditions; (2) suppressing hepatic AR activity, which in turn improves PPARα-mediated fatty acid oxidation; (3) inhibiting AR activity and subsequently ameliorating CYP2E1-mediated oxidative stress; and (4) attenuating AR/gut bacterial endotoxin-mediated cytokine overproduction. The proposed mechanisms of action of isoflavones regarding the AR/polyol pathway are depicted in Figure 1.

Therefore, isoflavones may be useful in preventing ALD and NAFLD/NASH. Clarifying the mechanisms of action of isoflavones regarding the AR/polyol pathway will help to develop efficient anti-FLD medications. However, the literature reviewed in this paper was limited to animal models. Human data on the anti-FLD effect of isoflavones are scarce. Further clinical trials are necessary to affirm the beneficial effect of isoflavones on FLD in humans.

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ORIGINAL ARTICLE

Basic Study

Metabolic shift in liver: Correlation between perfusion temperature and hypoxia inducible factor-1α

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Abstract

AIM: To study at what temperature the oxygen carried by the perfusate meets liver requirements in a model of organ perfusion.

METHODS: In this study, we correlated hypoxia inducible factor (HIF)-1α expression to the perfusion temperature and the hepatic oxygen uptake in a model of isolated perfused rat liver. Livers from Wistar rats were perfused for 6 h with an oxygenated medium at 10, 20, 30 and 37 ℃. Oxygen uptake was measured by an oxygen probe; lactate dehydrogenase activity, lactate release and glycogen were measured spectrophotometrically; bile flow was gravitationally determined; pH of the perfusate was also evaluated; HIF-1α mRNA and protein expression were analyzed by real time-polymerase chain reaction and ELISA, respectively.

RESULTS: Livers perfused at 10 and 20 ℃ showed no difference in lactate dehydrogenase release after 6 h of perfusion (0.96 ± 0.23 *vs* 0.93 ± 0.09 mU/min per g) and had lower hepatic damage as compared to 30 and 37 ℃ (5.63 ± 0.76 *vs* 527.69 ± 45.27 mU/min per g, respectively, *P*s < 0.01). After 6 h, tissue ATP was significantly higher in livers perfused at 10 and 20 ℃ than in livers perfused at 30 and 37 ℃ (0.89 ± 0.06 and 1.16 ± 0.05 *vs* 0.57 ± 0.09 and 0.33 ± 0.08 nmol/mg, respectively, *P*s < 0.01). No sign of hypoxia was observed at 10 and 20 ℃, as highlighted by low lactate release respect to livers perfused at 30 and 37 ℃ (121.4 ± 12.6 and 146.3 ± 7.3 *vs* 281.8 ± 45.3 and 1094.5 ± 71.7 nmol/mL, respectively, *P*s < 0.02), and low relative HIF-1α mRNA (0.40 ± 0.08 and 0.20 ± 0.03 *vs* 0.60 ± 0.20 and 1.47 ± 0.30, respectively, *P*s < 0.05) and protein (3.72 ± 0.16 and 3.65 ± 0.06 *vs* 4.43 ± 0.41 and 6.44 ± 0.82, respectively, *P*s < 0.05) expression.

CONCLUSION: Livers perfused at 10 and 20 ℃ show no sign of liver injury or anaerobiosis, in contrast to livers perfused at 30 and 37 ℃.

Key words: Anaerobiosis; Hypoxia inducible factor-1α; Ischemia; Liver transplantation; Machine perfusion

Core tip: Among the techniques developed to improve the preservation of marginal organs for transplantation, hypothermic perfusion is the preferred choice. We show that it is possible to perfuse a rat liver at 20 ℃ without incurring ischemia. We evaluated liver injury, energetic status, lactate release, and hypoxia inducible factor-1α expression. Results show that symptoms of ischemia appear at temperatures > 20 ℃, whereas there is no detectable advantage below 20 ℃. These findings have interesting implications in liver preservation; maintaining the liver in a mild metabolic state could be useful for pharmacologic treatment and regeneration of the energetic status in ATP-depleted organs.

Ferrigno A, Di Pasqua LG, Bianchi A, Richelmi P, Vairetti M. Metabolic shift in liver: Correlation between perfusion temperature and hypoxia inducible factor-1α. *World J Gastroenterol* 2015; 21(4): 1108-1116 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1108.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1108

INTRODUCTION

Orthotopic liver transplantation is the treatment of choice for end-stage liver disease. The employment of this technique is limited by the shortage of viable donor organs. Recently, the donor acceptance criteria for organ retrieval have been expanded, including livers with low degrees of steatosis[1] and grafts from non-heart-beating donors[2].

The use of marginal livers for organ transplantation emphasizes a fundamental flaw of conventional cold storage: despite all improvements, marginal organs are at greater risk of preservation-associated primary nonfunction because of increased sensitivity to preservation-induced ischemia/reperfusion injury[3]. Increasing grades of donor liver steatosis were associated with worse initial poor function[4,5] due to impaired metabolism of the steatotic hepatocytes[6,7], to the crystallization of lipids during cold ischemia[8], and to an increased sensitivity to oxygen radicals during reperfusion[9]. Livers from non-heart-beating donors exhibited postoperative biliary complications[10,11] due to the superimposing effects of cold and warm ischemia[12].

In order to overcome the limits of cold storage, animal studies concerning preservation by machine perfusion are flourishing; this technique reducing ischemic injury is usually associated with organ preservation. Different settings have been tested. St Peter and colleagues showed that oxygenated, normothermic (sanguineous) machine perfusion recovers ischemic livers to a viable level[13]. In a clinical trial, Guarrera *et al*[14] demonstrated improved clinical parameters and shorter duration of hospital stay in patients who received grafts stored by hypothermic machine perfusion in comparison to patients who received grafts preserved by cold storage. Tolboom *et al*[15] showed that, in a rat liver transplantation model, the survival rate after 4 wk was 100% for animals receiving livers preserved by subnormothermic machine perfusion; on the contrary, no cold stored graft survived after transplantation.

The mechanism by which machine perfusion better preserves marginal livers is not yet fully understood, nor a rationale was given for applying particular perfusion conditions. Nonetheless, cold storage compromises the ability to reoxidize NAD(P)H through mitochondrial respiration[16], whereas machine perfusion is always associated with ATP and glycogen recovery[17,18], suggesting a decisive role of oxygenation in the control of ischemic damage during preservation. For these reasons, it is of primary importance to ensure adequate oxygenation during perfusion. The issue of oxygenation is strictly related to perfusion temperature; both the oxygen carried by the perfusate and liver oxygen requirement are strongly related to temperature, with the first decreasing and the second exponentially increasing at increasing temperatures[19].

In our previous works, we evaluated the machine perfusion at subnormothermic temperature for the preservation of ischemic[20] and steatotic[6] rat livers, in a model of *ex vivo* reperfusion. In this work, we studied how the liver responds to different perfusion conditions, with the goal of determining at what temperature the oxygen carried by the perfusate and the liver oxygen requirement meet or, from a different point of view, at what temperature the liver switches from aerobiosis to anaerobiosis, taking the road to ischemia.

We perfused rat livers at various temperatures saturating the perfusion solution with O_2:CO_2 (95%:5%). The considered temperature range allows for maintenance of homogeneous perfusion conditions, as long-term liver perfusion at 4 ℃ is usually performed at lower flow rate, and may require additives to prevent cell swelling. For these reasons, we did not include livers perfused at 4 ℃ in the experimental design. Liver injury, function, and energetic status were evaluated. The switch to anaerobic metabolism was evaluated using lactate release and mRNA/protein expression of hypoxia inducible factor (HIF)-1α, a transcription factor that precociously responds to decreases in available oxygen in the cellular environment[21].

MATERIALS AND METHODS

Animals and surgery and liver perfusion

Male Wistar rats (Harlan Laboratories Inc., Indianapolis, IN, United States) weighing 250-300 g were

allowed free access to water and food until the beginning of all experiments. The use and care of animals in this experimental study were approved by the Italian Ministry of Health and by the University Commission for Animal Care. All surgeries were performed under anesthesia, and all efforts were made to minimize suffering. Rats were anesthetized with sodium pentobarbital (40 mg/kg, *ip*) and livers were isolated as already described[20,22]. Briefly, after median laparotomy followed by bilateral subcostal incisions, the animals received 200 U of heparin per 100 g of body weight *via* the inferior vena cava (5000 IU/mL; Marvecs Services, Agrate Brianza, MI, Italy). The bile duct was cannulated with 50 G polyethylene tubing (Intramed; Becton, Dickinson, and Co., Franklin Lakes, NJ, United States), and the portal vein was cannulated with a 16 G catheter (Johnson and Johnson, New Brunswick, NJ, United States). The liver was washed out with 50 mL of modified Krebs-Henseleit buffer *via* the portal vein cannula, then was freed from ligaments, removed and placed in a jacketed chamber for perfusion at different temperatures. At the end of liver perfusion, liver samples were immediately snap frozen in liquid nitrogen and stored at -80 ℃.

Livers were divided into four experimental groups depending on perfusion temperature: 10, 20, 30 or 37 ℃ (n = 6/group). Livers were placed in an organ chamber, connected to a recirculating perfusion system, and perfused for 6 h. The perfusion medium was a modified Krebs-Henseleit buffer[18] continuously gassed with O_2:CO_2 (95%:5%). Perfusion flow was kept constant at 2.6 mL/min per gram[20].

Assays

Liver parenchyma viability was assessed through release of lactate dehydrogenase (LDH) into the effluent perfusate, as described by Bergmeyer *et al*[23]. The perfusion temperature was continuously monitored with a probe placed inside the isolated organ chamber. The portal venous pressure was continuously measured throughout the perfusion by means of a water column connected to the portal vein inflow catheter; pre-calibration was performed each time just before connecting the liver to the circuit. The basal perfusion pressure was 12-14 mmHg. Dissolved oxygen in the perfusion solution was measured with a probe (OXY 340i; WTW GmbH, Weilheim, Germany) at intervals of 1 h, both in the inlet and outlet perfusion solution; oxygen delivery rate and liver oxygen uptake rate (OUR) were calculated. The pH was continuously evaluated both in the perfusion solution reservoir and in a reference solution, consisting of the perfusion buffer kept at the same temperature and pO_2 conditions but not circulated through the liver.

Tissue ATP was measured with the luciferin-luciferase method using the ATPlite luminescence assay kit (Perkin Elmer Inc., Waltham, MA, United States) according to the manufacturer's instructions with minor changes. Briefly, frozen tissue was homogenized in ice-cold 100 mmol/L phosphate buffer with 3 mmol/L EDTA; the homogenate was immediately precipitated in 30% trichloroacetic acid and centrifuged at 3000 × g for 15 min at 4 ℃. The supernatant was diluted 50× in 100 mmol/L phosphate buffer and assayed[20].

The glycogen assay was performed as described by Bennett *et al*[24]. Frozen samples were homogenized in a solution of 10% $HClO_4$ and centrifuged at 280 × g for 15 min. The pellets were resuspended in 2 mL of deionized H_2O. Samples (0.1 mL) were mixed with 0.2 mL of 5% phenol and 1 mL of H_2SO_4. After 30 min, absorbance at 490 nm was measured[24].

HIF-1α mRNA was analyzed using real-time-PCR using total RNA isolated from frozen liver samples with TRI reagent (Sigma-Aldrich, St. Louis, MO, United States)[25]. The cDNA was generated using iScript Supermix (Bio-Rad Laboratories Inc., Hercules, CA, United States). The RNA was assayed by measuring the absorbance at 260/280 nm. HIF-1α, ubiquitin C and GAPDH gene amplification efficiencies were established by means of calibration curves (108.8, 98.6 and 97.4%, respectively). The expression of the housekeeping gene remained constant in the considered experimental group. Sequences for the primers used are: HIF-1α: 5′-ACA AGA AAC CGC CTA TGA CG-3′ (forward) 3′-TAA ATT GAA CGG CCC AAA AG-5′ (reverse); ubiquitin C: 5′-CAC CAA GAA CGT CAA ACA GGA A-3′(forward), 3′-AAG ACA CCT CCC CAT CAA ACC-5′ (reverse); GAPDH: 5′-AAC CTG CCA AGT ATG ATG AC-3′ (forward), 5′-GGA GTT GCT GTT GAA GTC GTC A-3′ (reverse). Gene expression was analyzed using Platinum Sybr Green qPCR mix UDG (Life Technologies of Thermo Fisher Scientific Inc., Waltham, MA, United States). Ubiquitin c and GAPDH were used as reference genes. The amplification was performed through two-step cycling (95-60 ℃) for 45 cycles, in an ABI prism 7000 sequence detection system (Applied Biosystems of Thermo Fisher Scientific), following the instructions of the supplier. All samples were assayed in duplicate. The results were normalized to the endogenous controls, and fold change of the gene expression was calculated using threshold cycle (Ct) values.

At the end of the preservation, the nuclear fraction was immediately isolated from fresh tissue with the Nuclear Extraction Kit (Cayman Chemical Co., Ann Arbor, MI, United States). The HIF-1α protein expression was analyzed on the nuclear fraction with an ELISA kit (HIF-1α Transcription Factor Assay Kit; Cayman). The lactate was assayed using the Lactate Colorimetric Assay Kit (BioVision Inc., Milpitas, CA, United States).

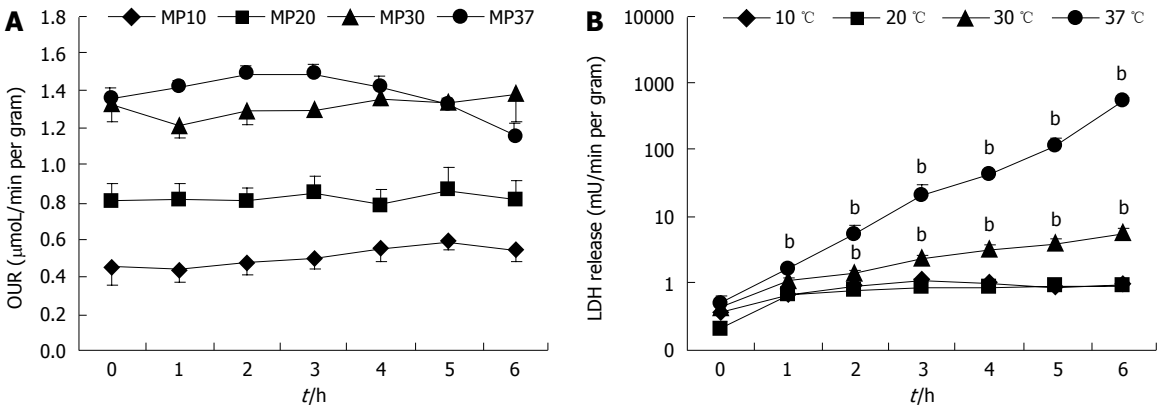

Figure 1 Liver oxygen uptake rate and lactate dehydrogenase release rate in rat livers perfused for 6. A: OUR; and B: Lactate-dehydrogenase (LDH) release rate over 6 h of perfusion at 10, 20, 30 or 37 ℃ (n = 6/group; $^bP < 0.01$ *vs* 20 ℃). OUR: Oxygen uptake rate.

Statistical analysis

Data are presented as the mean ± SE. Statistical analyses for multiple comparisons were performed using one-way analyses of variance tests with Bonferroni's corrections.

RESULTS

Liver OUR and oxygen delivery rate

In our experiments, OUR was constant during 6 h of perfusion at 10, 20, and 30 ℃. At 37 ℃, perfusion OUR dropped after 3 h, probably due to massive necrosis of the liver (Figure 1A). We observed a strong linear correlation between the basal OUR and the perfusion temperature (R^2 = 0.9979). A dependence of oxygen delivery rate on temperature was also observed; the available oxygen in the perfusion solution decreased with perfusion temperature (data not shown).

Release of LDH, portal pressure and bile production

Livers perfused at 10 and 20 ℃ showed a very low level of LDH release; livers perfused at 30 and 37 ℃ released significantly more LDH at the end of perfusion in comparison to livers perfused at 20 ℃ (Ps < 0.01). Furthermore, LDH release rate was near zero in 10 and 20 ℃ perfusion groups, suggesting a stationary condition, whereas it increased exponentially in livers perfused at 30 and 37 ℃ (Figure 1B).

At the starting time-point, portal pressure was associated with perfusion temperature. Basal pressure was higher in livers perfused at 10 ℃ (5.8 ± 0.2 mmHg), intermediate in livers perfused at 20 and 30 ℃ (4.9 ± 0.1 mmHg and 4.9 ± 0.2 mmHg, respectively), and lower at 37 ℃ (4.2 ± 0.1 mmHg). Livers perfused at 20 and 30 ℃ had identical portal pressure and differed significantly from the other groups (Ps < 0.01). During perfusion, pressure did not significantly change within each group, with the exception of the 37 ℃ group, where portal pressure significantly increased from the 3rd hour of perfusion, rising rapidly to out-of-scale values after the 4th hour (data not shown).

The increase in basal bile flow was logarithmically proportional to perfusion temperature. Basal bile flow in livers perfused at 10 and 20 ℃ was very similar, and was significantly different in comparison to livers perfused at both 30 and 37 ℃ (Ps < 0.05) (Figure 2A). Bile flow remained constant in livers perfused at 10 and 20 ℃ during the whole perfusion. On the contrary, bile flow fell rapidly after 2 h of perfusion in livers perfused at 30 ℃, and especially at 37 ℃ (Figure 2B).

ATP and glycogen in tissue

ATP and glycogen were measured in tissue samples frozen at the end of the 6-h perfusions. ATP in livers perfused at 20 ℃ was significantly higher in comparison to both 30 and 37 ℃ (Ps < 0.01). Interestingly, the ATP content in the 20 ℃ perfusion group was also higher when compared to livers perfused at 10 ℃ (P < 0.01) (Figure 2C). The 37 ℃ group had significantly lower glycogen content in comparison to the other three groups (P < 0.01); no difference was observed between the 10, 20 and 30 ℃ groups (Figure 2D).

pH and lactic acid release

We observed that there was no significant acidification during perfusion at 10 and 20 ℃. On the contrary, at 30 ℃, the pH of the perfusion solution was significantly lower in comparison to the basal values (P < 0.01); the pH also significantly dropped at 37 ℃ starting at the 3rd hour of perfusion, in comparison to the pH values at 10 and 20 ℃ for the same time points (Ps < 0.01) (Figure 3A).

To justify the pH fall at higher perfusion temperatures, we evaluated lactic acid release in the perfusion buffer as an index of anaerobiotic metabolism. Livers perfused at 10 and 20 ℃ did not release lactic acid during perfusion. In livers perfused at 30 ℃, lactic acid concentrations at the 2nd hour of perfusion were identical to the respective time points

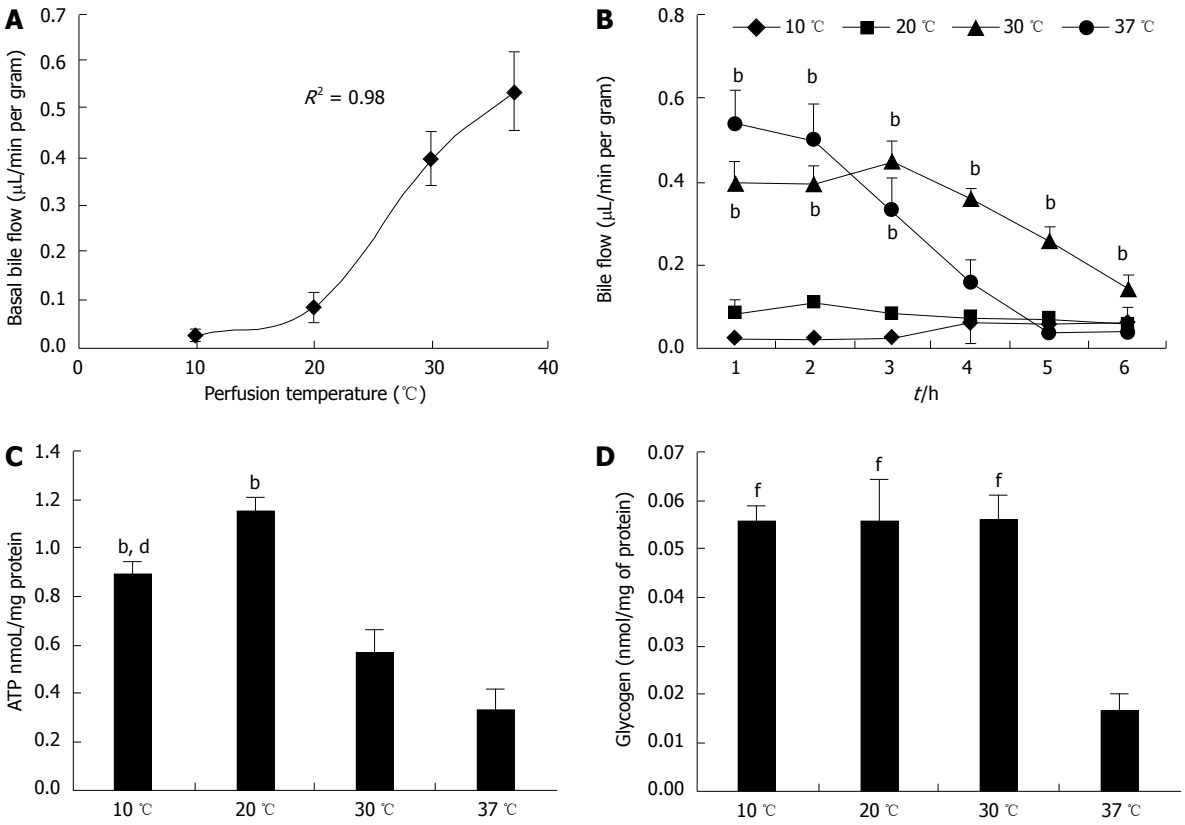

Figure 2 Bile flow, ATP and glycogen levels in rat livers perfused for 6 h. A: Basal bile flow; B: Bile flow during perfusion; C: ATP content; and D: Glycogen content in livers perfused for 6 h at 10, 20, 30 or 37 ℃ (n = 6/group; B: $^{b}P < 0.01$ *vs* 10 and 20 ℃; C: $^{b}P < 0.01$ *vs* 30 and 37 ℃, $^{d}P < 0.01$ *vs* 20 ℃; D: $^{f}P < 0.01$ *vs* 37 ℃).

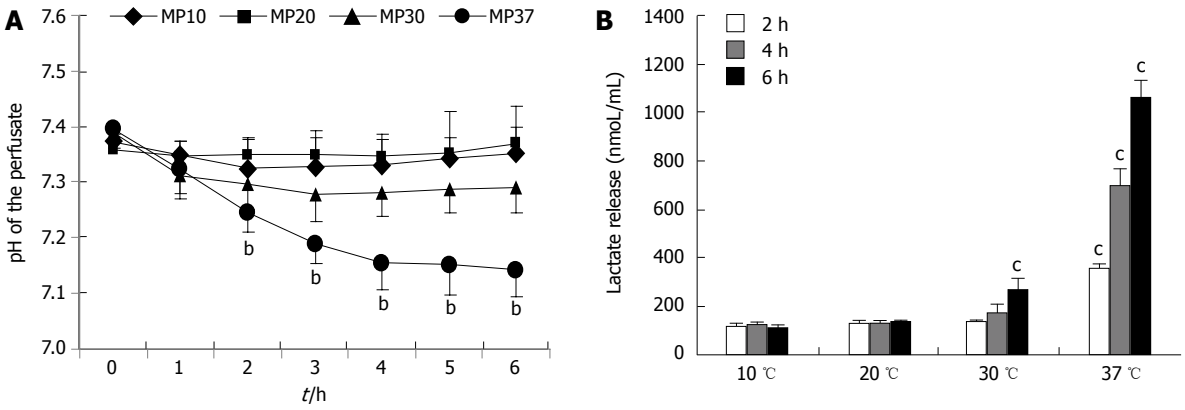

Figure 3 pH levels and lactate release in rat livers perfused for 6 h. A: Normalized pH; B: Lactic acid release in livers perfused for 6 h at 10, 20, 30 or 37 ℃ (n = 6/group; $^{b}P < 0.01$ *vs* 10 and 20 ℃; $^{c}P < 0.05$ *vs* 10 and 20 ℃).

at 10 and 20 ℃, but increased significantly in the subsequent time points (Ps < 0.05) (Figure 2B). In livers perfused at 37 ℃, lactic acid was significantly higher at the 2nd hour of perfusion, and increased dramatically during perfusion (Ps < 0.05).

HIF-1α mRNA and protein expression

HIF-1α mRNA and protein expression were assayed to identify which livers were perfused in hypoxic conditions. We observed a slight increase of HIF-1α mRNA expression in livers perfused at 30 ℃ (P < 0.05 *vs* 10 and 20 ℃) and a more accentuated rise of HIF-1α mRNA expression in livers perfused at 37 ℃ (P < 0.01 *vs* 10 and 20 ℃) (Figure 4A). Livers perfused at 10 and 20 ℃ did not show an increase in HIF-1α mRNA expression in comparison to control livers. We used an ELISA kit to evaluate HIF-1α protein expression in liver tissues at the end of perfusion, obtaining results similar to those observed for HIF-1α mRNA expression (Figure 4B).

DISCUSSION

The difficulty in perfusing the liver with acellular

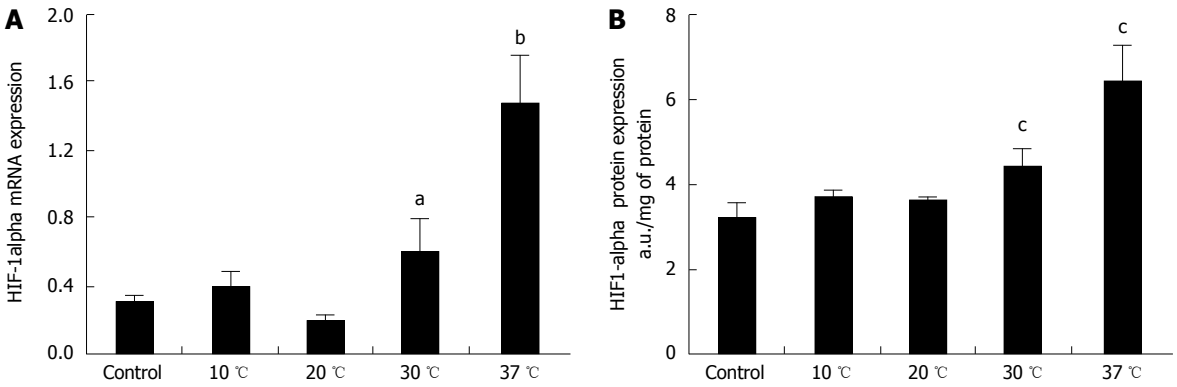

Figure 4 Hepatic hypoxia inducible factor-1α expression in rat livers perfused for 6 h. A: mRNA; and B: Protein expression of hypoxia inducible factor (HIF)-1α in livers perfused for 6 h at 10, 20, 30 or 37 ℃ (n = 6/group; $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* 10 and 20 ℃; $^{c}P < 0.05$ *vs* 10 and 20 ℃).

solutions at normothermic temperatures results from two different causes: (1) as stated by Henry's Law, oxygen solubility decreases at higher temperatures; and (2) at increasing temperature, liver metabolism increases proportionally. These two aspects act synergistically so that oxygen carried by the perfusate and liver oxygen requirement inevitably diverge at increasing temperatures. Because of this, one of the major drawbacks of acellular perfusion at normothermic temperatures is the inadequate oxygenation of the liver parenchyma, which leads to anaerobic glycolysis and acidosis[26]. In the isolated perfused rat liver model, this problem is partially solved by raising the perfusion flow at higher-than-physiologic levels; by speeding up the flow, the oxygen carried to the parenchyma will increase; on the other hand, according to Poiseuille's Law, a higher flow is not associated with an abnormal increase in physiologic portal pressure, due to the lower viscosity of acellular solutions compared to blood[26,27]. In our model, we increased the flow through the portal vein from the physiologic value of 1.7 to 2.6 mL/min per gram, obtaining a basal pressure similar to the physiologic portal pressure. Unfortunately, this procedure is not sufficient to fulfill liver oxygen requirement at 37 ℃. In our previous works, we showed that machine perfusion at subnormothermic temperature in a model of *ex vivo* reperfusion better preserves ischemic[20] and steatotic[6] rat livers with respect to conventional preservation.

The aim of the present work was to determine at what temperature the oxygen carried by the perfusate and the liver oxygen requirement meet, allowing long-term perfusion or, conversely, at what temperature liver metabolism shifts from aerobiosis to anaerobiosis. In isolated mitochondria, respiration rate increases exponentially with temperature[28]. Considering the whole organ, Fujita *et al*[29] studied the oxygen requirement at different temperatures in perfused livers, and worked out an equation showing that liver oxygen requirement increases exponentially as a function of temperature. This observation has a key implication: below a certain temperature threshold, liver oxygen requirement slightly increases with temperature, whereas above such threshold it increases dramatically. The data obtained herein suggest that this threshold temperature may lie between 20 and 30 ℃. In our perfusion model, the OUR of livers perfused at 10 and 20 ℃ was identical to the theoretical oxygen requirement according to Fujita *et al*[29], but uptake rate of livers perfused at 30 and 37 ℃ was lower than theoretical oxygen requirement, suggesting that oxygen requirements are not completely fulfilled at higher temperatures. The data obtained on liver injury and function support this hypothesis: LDH release rate was near zero in livers perfused at 10 and 20 ℃, whereas LDH increased significantly in livers perfused at 30 and 37 ℃.

The literature clearly shows that bile formation depends on the activity of various ATP-driven pumps[30,31], and consequently is strictly related to mitochondrial respiration rate. Due to dependence on temperature of both respiration rate[28,32] and enzyme activity[33], a similar bile flow dependence is expected. In our model, the basal bile flow followed this trend. Importantly, bile flow remained constant during perfusion at 10 and 20 ℃, but dramatically dropped after 2 and 3 h of perfusion in both 30 and 37 ℃ perfused livers, suggesting that liver is unable to maintain baseline bile production rate, due to oxygen deficiency. Accordingly, livers perfused at 30 and 37 ℃ contained significantly less ATP compared to livers perfused at 20 ℃. ATP content of livers perfused at 10 ℃ was significantly higher when compared to 30 and 37 ℃ groups as well, but, interestingly, was significantly lower than ATP measured in the 20 ℃ perfusion group. This difference may be explained by a lower coupling efficiency of oxidative phosphorylation, usually occurring at low temperatures[28]. Furthermore, while glycogen stores are not affected at 30 ℃, we observed a significant reduction of liver glycogen at 37 ℃.

These data suggest that livers perfused at 30 and

37 ℃ are in anaerobic conditions. Livers perfused at 20 and 10 ℃ show aerobic conditions during the whole perfusion. Accordingly, we observed that, contrary to livers perfused at 30 and 37 ℃, livers perfused at 10 and 20 ℃ did not acidify the perfusion medium. Lactic acid release is a suitable and sensitive marker of occurring anaerobic metabolism[34]. Livers perfused at 10 and 20 ℃ showed identically low lactate release with release rates near zero, demonstrating that, at these temperatures, the liver can support aerobic metabolism during the perfusion interval used in our experiments. Livers perfused at 30 ℃ showed a significantly higher lactate release rate compared to those perfused at 10 ℃ and 20 ℃. Livers perfused at 37 ℃ had dramatically higher lactate release rates, suggesting a huge difference between oxygen uptake and oxygen requirement at this temperature. Indeed, in our model of acellular perfusion, the liver cannot support aerobic metabolism at 37 ℃ and his metabolism is mainly anaerobiotic, wasting ATP and glycogen stores, and releasing great amounts of unprocessed lactic acid in the perfusate.

HIF-1α is a transcriptional activator of genes whose products, including glycolytic enzymes, are involved in systemic, local, and cellular responses to hypoxia, such as inducing alternative metabolic pathways that do not require O_2[35]. In hypoxic conditions, HIF-1α mRNA expression can increase[36,37]. To the best of our knowledge, HIF-1α mRNA and protein expression have not been assayed in a model of rat liver perfusion. We observed an increase in both HIF-1α mRNA and protein in livers perfused at 30 ℃, and a larger increase in livers perfused at 37 ℃. Interestingly, expression levels did not differ in livers perfused at 10 ℃ and 20 ℃, which were similar to controls.

These data clearly demonstrate that long term liver perfusion with simple acellular solutions is not possible above 30 ℃. Livers perfused at 37 ℃ are evidently in anaerobic conditions; livers perfused at 30 ℃ seem to be in an intermediate state, showing the first signs of distress, but not as much as livers perfused at 37 ℃, suggesting that the optimal temperature should certainly lie below 30 ℃. Should this optimal temperature necessarily be lower than 20 ℃? Our data, both those registered as a time course along perfusion period, and those assayed in the tissue, demonstrate that livers perfused at 10 and 20 ℃ exhibit quite similar and stable conditions, suggesting that the temperature where oxygen liver requirement and oxygen delivery meet is certainly below 30 ℃, but not necessarily below 20 ℃.

One of the most important applications of long-term liver perfusion is machine perfusion for organ transplantation. Recently, liver machine perfusion has been evaluated as a suitable alternative to simple cold storage, particularly for marginal organ preservation[38,39]. Furthermore, liver hypothermic machine perfusion (4-8 ℃) has recently been tested in a clinical trial with encouraging results[14,40,41]. In this trial, a starch-added solution was used to perfuse livers at 0.667 mL/min per liver g, without oxygenation. Subnormothermic perfusion has not yet been used in clinical trial, although some authors referred to the subnormothermic machine perfusion as "the way in-between" that could potentially bypass the flaws of both hypothermic and normothermic machine perfusion[42,43]. The subnormothermic temperature should allow the use of low-viscosity solutions and consequently higher flow rates, sustaining a mild liver metabolism useful for restoring energy stocks before transplantation, particularly in livers from donation after cardiac death. Moreover, the avoidance of hypothermia could be useful in fatty livers, and maintaining the liver in a mild metabolic state could be useful for genetic and immunologic manipulations before transplantation[41,42].

These data clearly show that livers perfused at 20 ℃ have no sign of anaerobiosis; therefore, reducing the perfusion temperature below 20 ℃ is unlikely to further improve this technique. On the contrary, livers perfused at 30 ℃ start to show the symptoms of lack of oxygenation. The adequate oxygenation of livers preserved by perfusion at 20 ℃ highlights this technique's concrete potential to avoid ischemic insult, the real culprit of the preservation injury observed in cold storage-transplanted organs. The subnormothermic temperature, allowing a less complicated setting, might also favor the successful translation of this technique from experimental studies into clinical practice.

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COMMENTS

Background

Cold (0-4 ℃) storage is considered the gold standard in organ preservation for transplantation. Currently, as new perfusion techniques are developed to improve the preservation of marginal organs, hypothermic machine perfusion is the preferred choice, though it presents drawbacks of cold injury, a slowed down metabolism, and a more complex setting.

Research frontiers

Clinical trials show improved clinical parameters and shorter duration of hospital stay in patients who receive grafts stored by hypothermic machine perfusion in comparison to patients who receive grafts preserved by cold storage.

Innovations and breakthroughs

Currently, new perfusion techniques have being developed for the preservation of marginal livers. The preferred technique is hypothermic machine perfusion (0-4 ℃). The authors demonstrated that livers perfused at 20 ℃ show no sign of ischemia. Therefore, reducing the perfusion temperature below 20 ℃ is unlikely to further improve this technique. On the contrary, livers perfused at 30 ℃ start to show the symptoms of lack of oxygenation.

Applications

The adequate oxygenation of livers perfused at 20 ℃ highlights this technique's concrete potential to avoid cold injury and ischemic insult during liver preservation for transplantation. Furthermore, the subnormothermic temperature, allowing a less complicated setting, might also favor the successful translation of this technique from experimental studies into clinical practice. Finally, maintaining the liver in a mild metabolic state during preservation could be useful for pharmacologic, genetic and immunologic manipulations, and for regeneration of the energy status in ATP-depleted organs, avoiding both ischemic and cold-induced insult. This possibility could be extremely suitable for the preservation of livers rejected as non heart beating donor organs and steatotic organs.

Terminology

Oxygenated machine perfusion is a technique currently under development for the preservation of organs for transplantation. This technique is aimed at reducing the ischemic injury and better preserving marginal organs, which suffer cold and ischemic injury from static cold storage, the gold standard for organ preservation.

Peer review

This manuscript showed that perfusion of livers at higher temperatures (> 30 ℃) leads to hypoxia and increased hypoxia inducible factor-1α expression.

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ORIGINAL ARTICLE

Basic Study

Effects of traditional Chinese herbal medicine San-Huang-Xie-Xin-Tang on gastrointestinal motility in mice

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Abstract

AIM: To investigate the effects of San-Huang-Xie-Xin-Tang (SHXXT), a herbal product used in traditional Chinese medicine, on gastrointestinal (GI) motility in mice.

METHODS: The *in vivo* effects of SHXXT on GI motility were investigated by measuring the intestinal transit rates (ITRs) using Evans blue in normal mice and in mice with experimentally induced GI motility dysfunction (GMD).

RESULTS: In normal ICR mice, ITRs were significantly and dose-dependently increased by SHXXT (0.1-1 g/kg). GMD was induced by injecting acetic acid or streptozotocin intraperitoneally. The ITRs of GMD mice were significantly reduced compared to normal mice, and these reductions were significantly and dose-dependently inhibited by SHXXT (0.1-1 g/kg).

CONCLUSION: These results suggest that SHXXT is a novel candidate for the development of a prokinetic agent that may prevent or alleviate GMD.

Key words: Gastrointestinal disorders; San-Huang-Xie-Xin-Tang; Motility; Gastrointestinal tract; Intestinal transit rate

Core tip: San-Huang-Xie-Xin-Tang (SHXXT), a traditional Chinese medicinal formula, is widely used in Eastern Asia, particularly to ameliorate the symptoms of gastrointestinal (GI) disorders. Our data suggest that SHXXT is a novel candidate for the development of a prokinetic agent that may prevent or alleviate GI motility dysfunctions in humans. Considering the effects of this drug on GI motility, further research is required to identify the compounds responsible for the effects of SHXXT and to determine their mechanisms of action.

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INTRODUCTION

San-Huang-Xie-Xin-Tang (SHXXT), a traditional Chinese medicinal formula, is widely used in Eastern Asia, to ameliorate the symptoms of gastrointestinal (GI) disorders related to gastritis, gastric bleeding, peptic ulcers, and abnormal GI motility[1-3]. SHXXT is composed of Coptidis rhizoma (*Coptis chinesis* Franch), Rhei rhizoma (*Rheum officinale* Baill), and Scutellariae radix (*Scutellaria baicalensis* Georgi). Coptidis Rhizoma has been previously reported to have protective effects against ischemia induced by the renal dysfunction associated with various metabolic conditions, such as hypercholesterolemic diseases[4-6]. Rhei Rhizoma has been reported to regulate hepatic and pancreatic functions, to ameliorate senility and improve cognition[7-10]. Scutellariae Radix has been suggested to have anticonvulsant activity, cytoprotective properties and anti-inflammatory effects[11-13].

In traditional Chinese medicine, SHXXT is thought to reduce heat and the effects of toxins and to promote defecation; thus, it is used to treat constipation with high fever, restlessness, thirst, and insomnia. Recently, SHXXT has been reported to possess a variety of pathophysiological and pharmacological properties, which include anti-inflammatory[14-16], anti-oxidant[17,18], anti-hypertensive[19], immunomodulatory[20], anti-apoptotic[21], and neuroprotective[22,23] effects. In addition, SHXXT is thought to inhibit gastric secretion and promote gastric muscle relaxation[1].

In our previous study, we investigated the effects of SHXXT on the pacemaker potentials of cultured interstitial cells of Cajal (ICCs) derived from the murine small intestine[24]. ICCs are GI pacemaker cells that generate rhythmic oscillations in membrane potentials known as slow waves[25-28]. SHXXT was found to modulate pacemaker potentials in ICCs *via* 5-HT_3 and 5-HT_4 receptor-mediated pathways, external Ca^{2+} influx, and Ca^{2+} release from internal stores in a mitogen-activated protein kinase dependent manner. In addition, of the ingredients in SHXXT, Coptidis rhizome and Rhei rhizome have been reported to regulate pacemaker potentials[24]. Therefore, we hypothesized that SHXXT could modulate the pacemaking activities of ICCs in the GI tract and thus has potential pharmacological relevance for the treatment of GI motility disorders. However, despite the considerable use of SHXXT to treat GI dysfunction, little is known of its *in vivo* regulatory effects on GI motility. Therefore, we performed this study to investigate the effects of SHXXT on the mouse GI tract.

MATERIALS AND METHODS

Reagents and plant materials

HPLC grade ethanol, acetonitrile, and water were purchased from Fisher Scientific (Pittsburgh, PA, United States). Baicalein (> 97.2%), wogonin (> 99.8%), and sennoside A (> 97.7%) were obtained from the Korean Food and Drug Association (O-song, South Korea). Baicalin (> 95%), berberine (> 95%), palmatine chloride hydrate (> 97%), aloe-emodin (> 95%) and emodin (> 95%) were from Sigma-Aldrich (St. Louis, MO, United States). SHXXT (Coptidis rhizoma (*Coptis chinesis* Franch) 0.23 g, Scutellariae radix (*Scutellaria baicalensis* Georgi) 0.56 g, and Rhei rhizoma (*Rheum officinale* Baill) 1.28 g: total 2.07 g) was obtained from I-World Pharm Co., Ltd. (Anyang, South Korea). The preparation and analysis of some active fractions have been previously reported[24]. To compare the prokinetic activities of the drugs tested in this study, the aqueous extract of the dried immature fruit of Poncirus trifoliata Raf. (PF) was obtained as previously described[26]. PF, one of the most popular traditional folk medicines in South Korea derived from the Rutaceae fruits, is known to have unique and potent prokinetic activities in normal and GI motility dysfunction (GMD) rodents[26,29].

Preparation of standard and sample solutions

To prepare stock solutions of standard compounds, we accurately weighed out 10 mg of each of the compounds, except for aloe-emodin and sennoside A, and dissolved them in 10 mL of methanol. Aloe-emodin was dissolved in methanol containing 1% of DMSO, and sennoside A was dissolved in an $NaHCO_3$ solution as described in The Korean Pharmacopoeia. Stock solutions were then diluted (10-fold) and filtered through a 0.2 μm syringe filter before injection. The final concentration of the standard solutions injected was 100 μg/mL. Sample solutions were made by dissolving 1.000 g of SHXXT in 10 mL of 60% methanol, diluted 10-fold, and filtered through a 0.2 μm syringe filter prior to injection. The final concentration of the sample solution injected was 10 mg/mL.

Chromatographic conditions

A smart LC system comprised of an LC800 (GL sciences, Japan) equipped with a solvent delivery unit, an autosampler, a column oven, and a UV-visible detector was used. Chromatographic separation was performed on an Inertsil ODS-4 column (2.1 mm x 150 mm, i.d. 3 μm; GL Sciences, Japan) at 40 ℃. The mobile phase consisted of water (A) and acetonitrile (B), and the gradient program used was as follows: 25% (B) for 2 min, 25%-68% (B) from 2 to 18.5 min, 68%-25% (B) from 18.5 to

20 min, and 25% (B) for 2 min. The flow rate was set at 0.3 mL/min and the injection volume was 1 μL. The detection wavelength used for standard compounds was set at 280 nm. Acquired data were processed using EZChrom Elite software (Ver. 3.3.2 SP1, Pleasanton, CA, United States).

Animals

All animals were obtained from Samtako Bio Korea Co., Ltd. Male ICR mice weighing 25-30 g were used in the study. Animals were maintained under controlled conditions (22 ± 3 ℃, humidity 52% ± 6%, and illumination from 6 a.m. to 6 p.m.). Animal care and experiments were conducted in accordance with the principles issued by the ethics committee of Pusan National University (South Korea). Animals were allowed free access to a commercial diet and tap water, but were deprived of food for 24 h before experiments. All experiments were conducted between 10 a.m. and 6 p.m.

Single oral dose toxicity of SHXXT extract in mice

SHXXT extract was administered intragastrically to mice through an orogastric tube at doses of 0, 0.5, 1, 2 or 5 g/kg delivered at 10 mL/kg. Seventy mice were tested for each dose and gender combination, and thus, a total of 60 mice were used. Each group was carefully observed for overt clinical signs and mortality at hourly intervals for 5 h after administration, and then on a daily basis for 14 d. Individual body weights were measured before dosing and on days 1, 3, 7 and 14 after administration. On day 14, the last day of observation, all animals were euthanized under ether anesthesia and necropsied.

Measurement of evans blue intestinal transit rates

The effect of SHXXT extract on intestinal propulsion was assessed by measuring the intestinal transit distance of Evans blue solution (5%, w/v, in DW). At 30 min after the intragastric administration of SHXXT extract in normal mice, Evans blue solution was intragastrically administered through an orogastric tube at a volume of 0.1 mL/kg of body weight. Animals were sacrificed 30 min after administration, and intestinal transit distance was determined by measuring the distance the Evans blue had migrated in the intestine from the pylorus. Intestinal transit rate (ITR) (%) was defined as the distance traveled by Evans blue expressed as a percentage of total small intestine length (from the pylorus to the ileal extremity). In order to minimize inter-day variations, ITRs were determined in normal and GDM mice on the same day.

Mouse model of peritoneal irritation induced by acetic acid

Peritoneal irritation was induced using acetic acid (AA) in mice 30 min after the intragastric administration of SC extract (or DW as vehicle). Peritoneal irritation was induced by an intraperitoneal injection of acetic acid (0.6%, w/v, in saline) at a dose of 10 mL/kg, as previously described[29,30]. After injecting acetic acid, mice were placed in individual cages and allowed to recover for 30 min.

Streptozotocin-induced diabetic mouse model

Male ICR mice (aged 5 wk) were used in this experiment. Mice were randomly allocated to either a control group or a diabetic group. To produce diabetes, mice were fasted overnight and, on the following day, streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO) solution was administered intraperitoneally. STZ was prepared freshly in 0.1 mol/L ice-cold citrate buffer (pH = 4.0) and administered at a dose of 200 mg/kg body weight. Control mice were intraperitoneally administered the same volume of 0.1 mol/L citrate buffer. Animals had free access to food and water and were maintained under standard housing conditions (24-27 ℃, RH: 60%-65%) under a 12 h light/dark cycle. After two months, blood was withdrawn via the tail vein after an 8 h fast and blood glucose concentrations were measured using a one-touch blood glucose monitoring system (Johnson and Johnson). Diabetes was defined as a blood glucose level > 16 mmol/L.

Statistical analysis

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

RESULTS

Acute toxicity of SHXXT extract in normal mice

To examine the effect of SHXXT extract on GI motility, we used ICR normal mice. Of the 70 mice tested, no fatality occurred during 2 wk post-administration even at a dose of 5 g/kg, indicating that the minimal lethal dose of SHTTX extract in normal mice exceeds 5 g/kg. Furthermore, no abnormal clinical signs were observed and near identical increases in body weight were observed in treated and untreated mice. In addition, no abnormal findings were evident at the 14 d necropsy. Accordingly, SHXXT extract appeared to be safe, which is consistent with its widespread use in traditional Korean medicine at single doses of 9-12 g in humans[31].

Effects of SHXXT extract on ITR in normal mice

Mean ITR (%) values for Evans blue after 30 min in normal mice are shown in Figure 1. Mean ITR for non-treated normal mice (control) was 55.8%

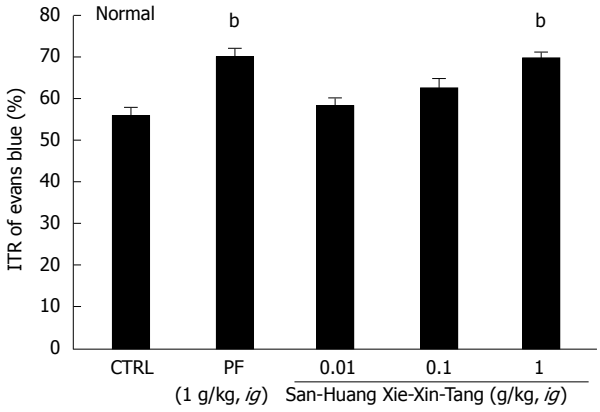

Figure 1 Effect of San-Huang-Xie-Xin-Tang extract on intestinal transit rate (%) in normal mice. Mice (n = 7 for each bar, except for control) were treated with San-Huang-Xie-Xin-Tang extract and then intragastrically administered Evans blue 30 min later. Intestinal transit rate (ITR) (%) values were determined 30 min after Evans blue administration. Bars represent mean ± SE. $^{b}P < 0.01$ *vs* control group. CTRL: Control; PF: Poncirus trifoliata Raf.

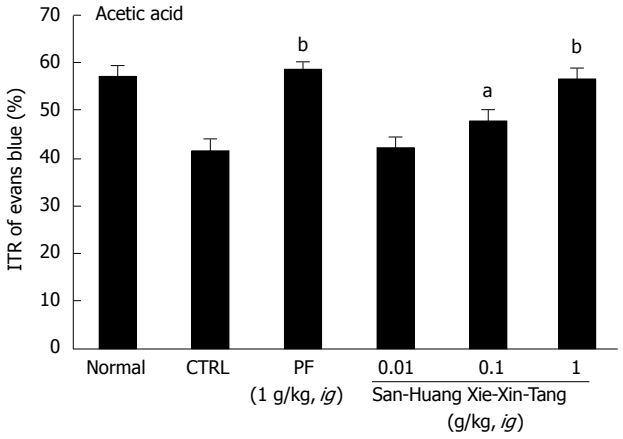

Figure 2 Effect of San-Huang-Xie-Xin-Tang extract on intestinal transit rate in acetic acid treated mice. Mice (n = 6 for each bar) were treated with San-Huang-Xie-Xin-Tang extract and then intragastrically administered Evans blue 30 min later. Intestinal transit rate (ITR) (%) values were determined 30 min after Evans blue administration. Bars represent mean ± SE. $^{a}P < 0.05$ *vs* control group; $^{b}P < 0.01$ *vs* control group. CTRL: Control; PF: Poncirus trifoliata Raf.

± 1.7%. PF significantly accelerated ITR at an intragastric dose of 1 g/kg (69.5% ± 2.3%, P < 0.01), which is consistent with previous reports[32,33]. On the other hand, the ITR values for SHXXT extract at 0.01, 0.1 and 1 g/kg were 58.0% ± 1.5%, 62.3% ± 2.1% and 69.3% ± 1.6%, respectively (P < 0.01; Figure 1).

Effects of SHXXT extract on ITR in mice with GMD

To examine the effect of SHXXT extract on GI motility, we used AA mouse and STZ-induced diabetic mouse models with experimental GMD. As expected, a significant retardation in ITR (%) was observed in the AA mouse model (41.5% ± 2.6%; Figure 2). Conversely, significant inhibition of this retardation was observed when SHXXT extract was intragastrically administered at 0.01, 0.1, or 1 g/kg

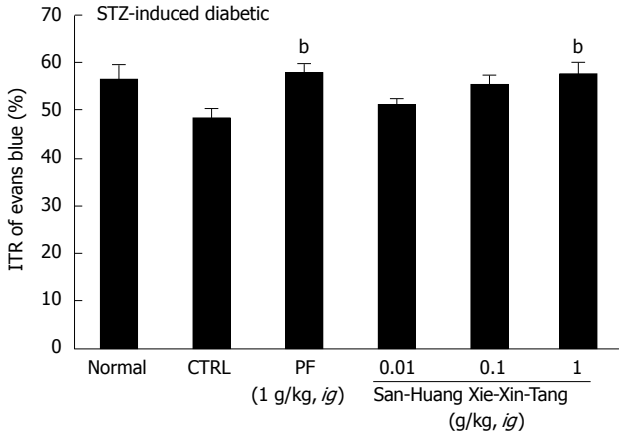

Figure 3 Effect of San-Huang-Xie-Xin-Tang extract on intestinal transit rate in streptozotocin-induced diabetic mice. Two months after administering streptozotocin (STZ), mice (n = 7 for each bar) were treated with San-Huang-Xie-Xin-Tang extract and then intragastrically administered Evans blue 30 min later. Intestinal transit rate (ITR) (%) values were determined 30 min after Evans blue administration. Bars represent mean ± SE. $^{b}P < 0.01$ *vs* control group. CTRL: Control; PF: Poncirus trifoliata Raf.

[42.3% ± 2.2%, 47.8% ± 2.1% (P < 0.05) and 56.7% ± 2.4% (P < 0.01), respectively; Figure 2]. Additionally, PF (an intragastric dose of 1 g/kg) significantly accelerated ITR in a similar manner to SHXXT in the AA mouse model (58.8% ± 1.5%, P < 0.01; Figure 2). No abnormal clinical signs or changes were observed in AA mice after the intragastric administration of SHXXT. A significant retardation in ITR (%) was also observed in STZ-induced diabetic mice (48.5 ± 2.0%; Figure 3), and significant inhibition of this retardation was observed after intragastric administration of SHXXT extract at 0.01, 0.1 or 1 g/kg [51.2% ± 1.3%, 55.5% ± 1.9% and 57.8% ± 2.4% (P < 0.01), respectively; Figure 3]. PF (an intragastric dose of 1 g/kg) significantly accelerated ITR in a similar manner to SHXXT in STZ-induced diabetic mice (58.1% ± 1.8%, P < 0.01; Figure 3). No abnormal clinical signs or changes were observed in STZ-induced diabetic mice after administering SHXXT extract at any intragastric dose. These results suggest that SHXXT extract increased ITR in mice with GMD.

Effects of coptidis rhizoma, rhei rhizoma, and scutellariae radix extract on ITR in GI motility

SHXXT is composed of Coptidis rhizoma (CR, *Coptis chinesis* Franch), Rhei rhizoma (RR, *Rheum officinale* Baill) and Scutellariae radix (SR, *Scutellaria baicalensis* Georgi). Therefore, we investigated the effects of these components in normal mice and in mouse GMD models. Mean ITR values for Evans blue during 30 min in normal mice treated with CR and RR are shown in Figures 4 and 5. CR and RR significantly accelerated ITR at intragastric doses of 1 g/kg [63.1% ± 1.4% (P < 0.05) and 59.2% ± 1.8% (P < 0.05), respectively; Figures 4A and 5A].

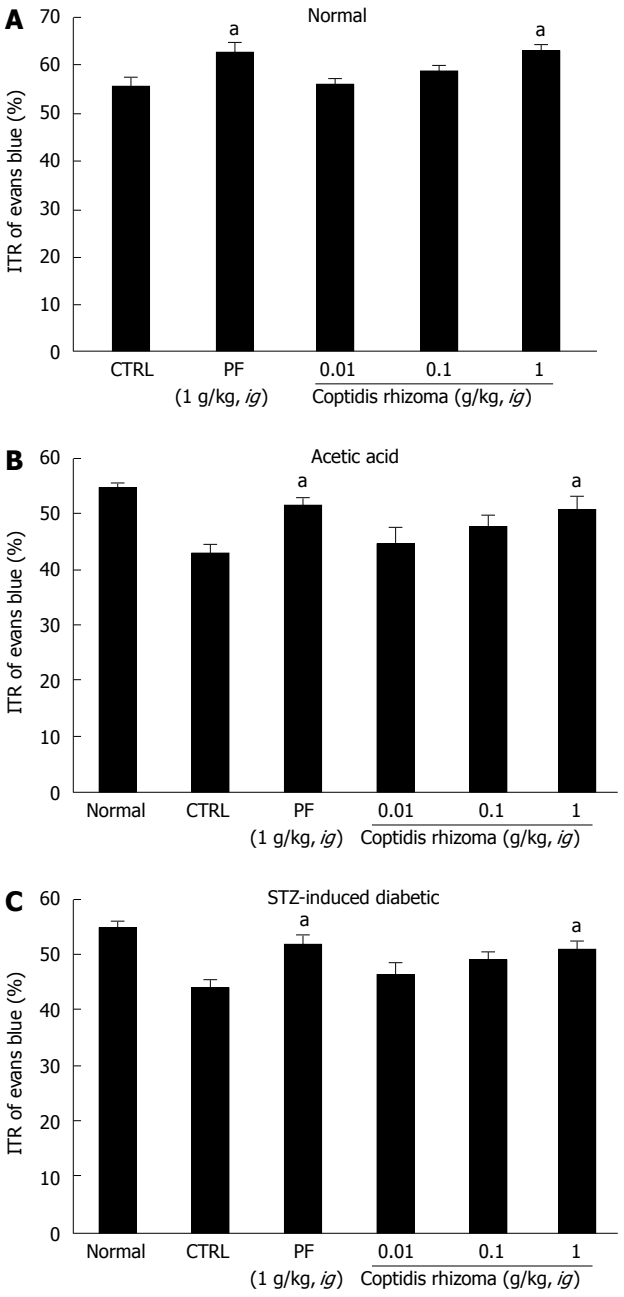

Figure 4 Effect of Coptidis rhizoma extract on intestinal transit rate in normal mice and mice with gastrointestinal motility dysfunction. Intestinal transit rate (ITR) values were determined 30 min following the intragastric administration of an Evans blue solution to normal mice (A), to mice 30 min after the induction of acetic acid (B), and to mice 2 mo after the induction of streptozotocin (STZ) (C) with Coptidis rhizoma extract. Bars represent mean ± SE. $^{a}P < 0.05$ *vs* control group. CTRL: Control; PF: *Poncirus trifoliata* Raf.

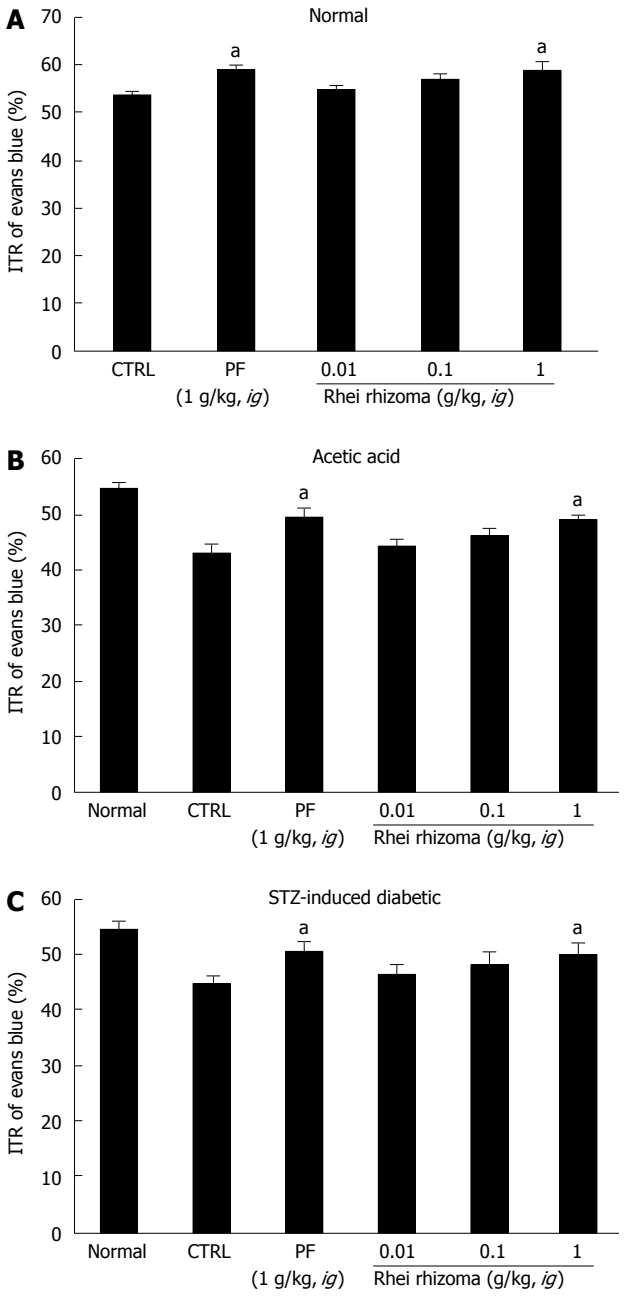

Figure 5 Effect of Rhei rhizoma extract on intestinal transit rate in normal mice and mice with gastrointestinal motility dysfunction. Intestinal transit rate (ITR) values were determined 30 min following the intragastric administration of an Evans blue solution to normal mice (A), to mice 30 min after the induction of acetic acid (B) and to mice 2 mo after the induction of streptozotocin (STZ) (C) with Rhei rhizoma extract. Bars represent mean ± SE. $^{a}P < 0.05$ *vs* control group. CTRL: Control. PF: *Poncirus trifoliata* Raf.

In addition, as expected, a significant retardation in ITR was observed in the AA and STZ-induced diabetic mouse models, and significant inhibition of this retardation was observed when CR or RR extract was intragastrically administered at 1 g/kg in the AA mouse model [51.1% ± 2.1% ($P < 0.05$) and 49.1% ± 0.8% ($P < 0.05$), respectively; Figures 4B and 5B] and in the STZ-induced diabetic mouse model [51.1% ± 1.6% ($P < 0.05$) and 50.1% ± 2.1% ($P < 0.05$), respectively; Figures 4C and 5C]. No abnormal clinical signs or changes were observed in the AA and STZ-induced diabetic mouse models after CR or RR administration. PF (an intragastric dose of 1 g/kg) significantly accelerated ITR in a similar manner to CR and RR (Figures 4 and 5). However, when SR was administered, no ITR changes were observed in normal or GDM mouse models (Figure 6). These results suggest that CR and RR increased ITR in mice.

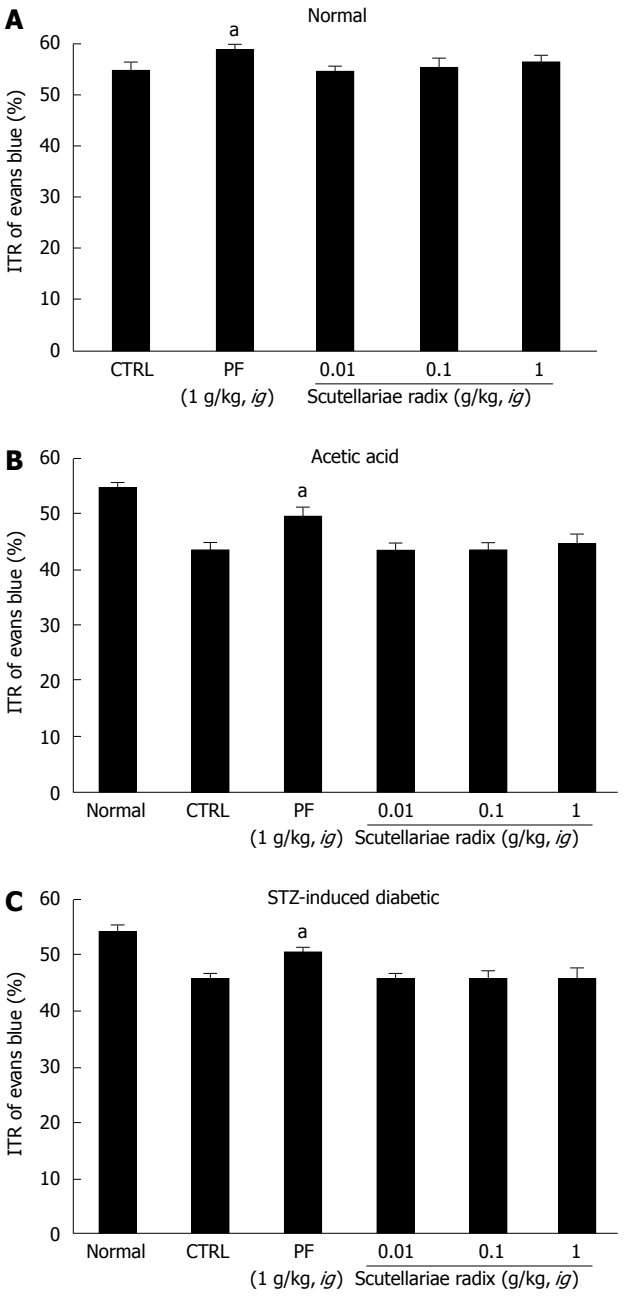

Figure 6 Effect of Scutellariae radix extract on intestinal transit rate in normal mice and mice with gastrointestinal motility dysfunction. Intestinal transit rate (ITR) values were determined 30 min following intragastric administration of an Evans blue solution to normal mice (A), mice 30 min after the induction of acetic acid (B) and to mice 2 mo after the induction of streptozotocin (STZ) (C) with Scutellariae radix extract. Bars represent mean ± SE. $^{a}P < 0.05$ *vs* control group. CTRL: Control. PF: *Poncirus trifoliata* Raf.

DISCUSSION

The purpose of the present study was to investigate the effects of SHXXT on GI motor function. The findings of this study indicate for the first time that SHXXT has potent prokinetic activity in normal and GMD mice. Although traditional oriental medicines have been widely used for the treatment of a variety of digestive dysfunctions, few studies have been conducted on their effects on GI motor functions. Fructus Aurantii, the unripe fruit of *Citrus aurantium* Linn (Rutaceae), is a Qi-regulating drug that is used in traditional Chinese medicine to improve GI function. Fructus Aurantii has been reported to enhance GI motility by altering 5-HT and vasoactive intestinal peptide expression levels in the rat GI tract[34]. Aqueous extracts from the dried mature fruits of *Aurantii nobilis* Pericarpium have been used in traditional folk medicine to treat GI motility disorders in South Korea, and have been suggested to be potential prokinetic agents for the prevention or alleviation of GMD in humans[29]. On the other hand, Crotonis Fructus is the mature fruit of *Croton tiglium* L. (Euphorbiaceae), and has been used for thousands of years in traditional Chinese medicine to treat GI diseases, such as constipation, abdominal pain, peptic ulcer, and intestinal inflammation. Crotonis Fructus has been reported to regulate GI motility *via* the activation of M3 muscarinic receptors and Ca^{2+} influx through L-type Ca^{2+} channels[35]. *Atractylodes Japonica* Koidz (Compositae) is commonly used to treat GI disorders in Korean traditional medicine. It specifically acts on the distal colon longitudinal muscles within the GI smooth muscles *via* the activation of acetylcholinergic muscarinic receptors[36]. Daikenchuto (TU-100) is a traditional Japanese (Kampo) medicine used to treat postoperative ileus, and it has been reported to increase GI motility by modulating cholinergic and serotonergic mechanisms. Furthermore, it was found to have beneficial effects on functional constipation and irritable bowel syndrome[37]. SHXXT was widely used to ameliorate the symptoms of GI disorders related to gastritis, gastric bleeding, peptic ulcers, and abnormal GI motility. In addition, SHXXT has gastric protective mechanisms and promotes gastric muscle relaxation. SHXXT has been reported to decrease phosphodiesterase activity and smooth muscle tone in the GI tract, and the extents of phosphodiesterase inhibition and smooth muscle relaxation were found to be more prominent in the lower GI tract[2,3].

In a previous study, SHXXT was found to regulate ICC pacemaker activity. Furthermore, Coptidis rhizome and Rhei rhizoma modulated ICC pacemaking activity, whereas Scutellariae radix had no effect[24]. In the present study, ITR was significantly and dose-dependently increased by SHXXT (0.1-1 g/kg) in normal mice; in GMD mice, ITR was significantly retarded *vs* normal mice. However, this retardation was significantly and dose-dependently inhibited by SHXXT (0.1-1 g/kg), demonstrating that SHXXT has a modulatory effect on GI motility.

The GI tract consists of enteric neurons, interstitial cells, immune cells and smooth muscle cells. Interstitial cells include ICCs and are platelet-derived growth factor receptor alpha-positive (PDGFRα^{+}).

They generate pacemaker activity throughout the GI tract and transduce enteric nerve signals to adjacent smooth muscle cells[38]. Though we experimented with the effects of SHXXT on only ICCs, SHXXT depolarized the pacemaker activity of ICCs and increased the ITR of GI motility. Therefore, we think that SHXXT may have a potential role in the regulation of GI motility through the modulation of ICCs. In the future, we will experiment with the effects of SHXXT on enteric neurons and smooth muscle cells. In addition, it has been shown that the pacemaker activities of ICCs in the murine small intestine are primarily due to periodic activations of nonselective cation channels[25,39] or Cl^- channels[40]. Kim *et al*[25] (2005) suggested that transient receptor potential melastatin (TRPM) 7 is required for ICC pacemaker activity in the murine small intestine, while Hwang *et al*[41] (2009) suggested that a Ca^{2+}-activated Cl^- channel (CaCC, Tmen16A, anoctamin 1 (ANO1)) is involved in ICC slow wave generation. Therefore, it has been proposed that TRPM7 and ANO1 may be important targets for the pharmacological treatment of GI motility disorders. In the future, because the TRPM7 or ANO1 channels are involved in the pacemaker activity of ICC, we will investigate which ion channel is involved in SHXXT action.

Taken together, our results suggest that SHXXT is a good candidate for the development of a gastroprokinetic agent. In addition, we suggest that the active mechanism of SHXXT on GI motility should be explored *in vivo*.

COMMENTS

Background

San-Huang-Xie-Xin-Tang (SHXXT) (composed of Coptidis Rhizoma (*Coptis chinesis* Franch), Rhei Rhizoma (*Rheum officinale* Baill), and Scutellariae Radix (*Scutellaria baicalensis* Georgi), a traditional Chinese medicinal formula, is widely used in Eastern Asia, to ameliorate the symptoms of gastrointestinal (GI) disorders. However, despite the considerable use of SHXXT to treat GI dysfunction, little is known of its *in vivo* regulatory effects on GI motility.

Research frontiers

SHXXT is a good candidate for the development of a gastroprokinetic agent.

Innovations and breakthroughs

In normal mice, intestinal transit rates (ITRs) were significantly and dose-dependently increased by SHXXT (0.1-1 g/kg). GI motility dysfunction (GMD) was induced by injecting acetic acid or streptozotocin intraperitoneally. ITRs of GMD mice were significantly reduced compared to normal mice, and these decreases were significantly and dose-dependently inhibited by SHXXT (0.1-1 g/kg).

Applications

SHXXT may be a new target or a novel candidate prokinetic agent for pharmacological treatment of GI motility disorders.

Terminology

GI motility: The movements of the digestive system, and the transit of the contents within it; ITRs: Passage of food (sometimes in the form of a test meal) through the GI tract as measured in minutes or hours; Interstitial cells of Cajal: The pacemaker cells of the GI tract.

Peer review

This study clarified the effect of SHXXT on GI motility. This is an interesting study, especially for those who reside in the Western Hemisphere and do not come in frequent contact with this substance.

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ORIGINAL ARTICLE

Basic Study

Weichang'an and 5-fluorouracil suppresses colorectal cancer in a mouse model

Li Tao, Jin-Kun Yang, Ying Gu, Xin Zhou, Ai-Guang Zhao, Jian Zheng, Ying-Jie Zhu

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Author contributions: Tao L and Yang JK designed the research; Tao L, Gu Y and Zhao AG performed the research; Zhu YJ and Zheng J analyzed the data; and Tao L, Yang JK and GuY, Zheng J wrote the paper; Zhou X performed preparation of the Chinese medicine and quality control.
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Abstract

AIM: To examine the effect of Weichang'an (WCA) and 5-fluorouracil (5-FU) on colorectal tumor and hepatic metastasis in a mouse model.

METHODS: Quantitative determination of hesperidin, the effective component in WCA decoction, was performed using HPLC. *In vitro* cytotoxicity of WCA was determined by treating HCT-116 cells with WCA diluents or serum extracted from rats that received WCA by oral gavage for 1 wk and MTT assays. Forty-eight nude mice received cecum implantation with tumor blocks subcutaneously amplified from human colon cancer cell line HCT-116. Mice were randomly divided into four treatment groups: control (CON), WCA, 5-FU and combination (WCA + 5-FU). Pathological examination of tumors consisted of tissue sectioning and hematoxylin and eosin staining. Tumor weight and size were measured, and the number of metastatic lesions was counted. Serum carcinoembryonic antigen (CEA) level was determined by ELISA. The expression levels of tumor genesis and metastasis-related proteins β-catenin and matrix metalloproteinase (MMP)-7 were measured by real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR), immunohistochemistry and immunoblotting. Cell fractionation was used to investigate intracellular distribution of β-catenin.

RESULTS: Parenchymal tumors were palpable in the abdomens of nude mice 2 wk post-implantation and orthotopic tumor formation rate was 100% in all groups. 5-FU treatment alone significantly decreased tumor weight compared to the CON group (1.203 ± 0.284 g *vs* 1.804 ± 0.649 g, $P < 0.01$). WCA treatment alone reduced the rate of metastasis (50% *vs* 100%,

$P < 0.05$). Combination treatment of WCA + 5-FU was the most effective, reducing the tumor weight (1.140 ± 0.464 g *vs* 1.804 ± 0.649 g, $P < 0.01$) and size (1493.438 ± 740.906 mm^3 *vs* 2180.259 ± 816.556 mm^3, $P < 0.05$), the rate of metastases (40% *vs* 100%, $P < 0.01$), and serum CEA levels (31.263 ± 7.421 μg/L *vs* 43.040 ± 11.273 μg/L, $P < 0.05$). Expression of β-catenin and MMP-7 was decreased in drug-treated groups compared to controls, as detected using real-time quantitative RT-PCR, immunohistochemistry and immunoblotting, respectively. Cell fractionation assays revealed that nuclear translocation of β-catenin was reduced by WCA and/or 5-FU treatments.

CONCLUSION: Combination of WCA with 5-FU significantly inhibited colon tumor growth and hepatic metastases. Decreased expression of β-catenin and MMP-7 may be important.

Key words: Colorectal cancer; Hepatic metastasis; Weichang'an formula; Orthotopic transplant nude mouse model; Chemotherapeutics 5-fluorouracil

Core tip: In this study, the anti-colon cancer activity of Weichang'an (WCA), a Chinese herbal medicine, was assessed in an orthotopic transplantation nude mouse model. Combination of WCA with 5-fluorouracil inhibited orthotopic tumor growth and hepatic metastases. Decreased expression of β-catenin and metalloproteinase-7, which are crucial proteins modulating tumor aggression, may be important for the anti-tumor properties of WCA.

Tao L, Yang JK, Gu Y, Zhou X, Zhao AG, Zheng J, Zhu YJ. Weichang'an and 5-fluorouracil suppresses colorectal cancer in a mouse model. *World J Gastroenterol* 2015; 21(4): 1125-1139 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1125.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1125

INTRODUCTION

Colorectal cancer is the third most commonly diagnosed cancer and the third most common cause of cancer-related death worldwide. Approximately one million new cases of colorectal cancer are diagnosed in both men and women around the world each year[1]. It is estimated that 50%-60% of patients diagnosed with colorectal cancer will develop hepatic metastases during the course of their disease[2-4], and unfortunately, 80%-90% of these metastases will be unresectable[5-7]. While metastatic lesions may be found throughout the body following locoregional treatments, the most frequent site of colorectal cancer metastasis is the liver[8]. The 5-year survival rate of patients with hepatic metastases and not undergoing surgical treatment is low[3,9]. Patients with unresectable hepatic metastases require multidisciplinary treatments, including surgery, chemotherapy, liver-targeting therapies, and traditional Chinese medicine (TCM). TCM has been used extensively in China for treatment of various diseases, including cancer. TCMs have a number of benefits, including decreasing the negative side effects associated with chemotherapy and radiotherapy, improving patients' immune function, and enhancing the effects of conventional cancer treatments[10-12].

Weichang'an (WCA) is a traditional Chinese formula prescribed by practitioners of TCM, based on clinical experiences and pharmaceutical screening. The principal elements comprising WCA are invigorating spleen herbs, whereas heat-clearing and detoxicating herbs, hard lump-resolving herbs and blood stasis removing herbs are adjuvant components. Previous clinical studies have indicated that patients with gastric carcinoma benefit from WCA treatment[13,14]. In addition, WCA has been shown to increase the 5-year survival rate and reduce the 1- and 2-year metastatic rates in patients with colorectal cancer[15]. However, whether WCA is effective in colorectal cancer with hepatic metastasis is still unclear.

Many studies have shown that the Wnt/β-catenin signaling pathway regulates tumor cell invasion and metastasis. In oral squamous cell carcinoma cells, the accumulation of β-catenin in the cytoplasm induced T-cell factor/lymphoid enhancing factor transcriptional activity, and increased matrix metalloproteinase (MMP)-7 expression, thereby inducing the conversion of epithelial cells to mesenchymal cells, as well as enhancing invasion and metastasis[16]. The Wnt/β-catenin pathway also plays a critical role in colorectal cancer development[17]. We hypothesized that WCA may affect the Wnt/β-catenin pathway.

To elucidate the efficacy of WCA in inhibiting colorectal cancer cell growth and hepatic metastasis and underlying mechanisms, we administrated WCA combined with 5-fluorouracil (5-FU) in an orthotopic transplant nude mouse model grafted with HCT-116 human colon cancer cells. The pathological phenotype, tumor genesis, metastatic lesions, carcinoembryonic antigen (CEA) levels and β-catenin/MMP-7 expression were detected.

MATERIALS AND METHODS

Drugs

WCA was provided by the Dispensary of TCM, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai, China. The composition of WCA includes *Pseudostellaria heterophylla* (Miq.) Pax, *Atractylodes macrocephala* koidz., *Poria cocos*

(Schw.) Wolf, *Glycyrrhiza uralensis* Fisch., *Sargentodoxa cuneata,* and *Prunella vulgaris* L. The preparation of the WCA decoction has been described previously[13,14]. The concentration of hesperidin (National Institutes for Food and Drug Control, Beijing, China) in the WCA formula was determined by HPLC (Agilent 1100; Palo Alto, CA, United States) using the following conditions: Agilent 1100 C_{18} column (250 mm × 4.6 mm, 5 μm); mobile phase, acetonitrile:water 8% HAC (18:82); wavelength, 284 nm; flow rate, 1 mL/min; and column temperature, 15 ℃. 5-FU was purchased from Shanghai Xudong Haipu Pharmaceutical Co. Ltd. (Shanghai, China). In the present study, 5-FU was used at a concentration of 2.5 mg/mL.

Animals

Male BALB/$C^{-nu/nu}$ mice, aged 4-6 wk and weighing 18-20 g, were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd. [Shanghai, China; certification NO. SCXK (Shanghai) 2012-0002]. Mice were housed under specific pathogen-free conditions and were given free access to food and water. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals, Ministry of Science and Technology, China. This study was approved by the Animal Care and Scientific Committee of the Shanghai University of traditional Chinese medicine.

Cell culture

The human colon cancer cell line HCT-116 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in McCoy's 5A modified medium (Gibco, Carlsbad, CA, United States) containing 10% heat-inactivated fetal bovine serum and supplemented with penicillin (100 U/mL) and streptomycin (100 g/mL). Cells were maintained at 37 ℃ and 5% CO_2.

MTT assay

During the logarithmic growth phase, HCT-116 cells were conventionally digested, after which the cells were seeded into a 96-well plate at a density of 2 × 10^4 cells/mL. The control group was set, and the cells were cultured at 37 ℃ in a humidified atmosphere of 5% CO_2 in air. The medium was discarded when cells had reached 70%-80% confluence. Medium containing 0, 3%, 6% and 9% WCA decoction or 5%, 10% and 20% of serum extracted from rats that received WCA by oral gavage (OG) (2 mL/d for 1 wk) was added to each well (100 μL/well). Each condition was tested in quadruplicate, and the cells were treated for 0, 24, 48 and 72 h. After treatment, 20 μL MTT reagent (Sigma, St Louis, MO, United States) was added to each well for 4 h. The culture medium was discarded, and 150 μL dimethyl sulfoxide (Sigma) was added. Formazan was dissolved by gentle agitation for 10 min. Optical density (OD) for each well was determined using a plate reader (Synergy2; Bio-tek, Winooski, VT, United States) at a wavelength of 490 nm. All experiments were performed three times.

Establishment of the colon cancer transplant nude mouse model and drug administration

HCT-116 cells (2 × 10^7) were subcutaneously injected into the right axilla of nude mice. After 3 wk, tumors were excised and cut into 1-2 mm^3 pieces using sterile techniques. Tumor blocks were transplanted into the cecum of 48 nude mice by a purse-string suture (Figure 1A and 1B). Drug administration started 7 d after tumor implantation. The 48 nude mice were randomly divided into four treatment groups (12 mice/group). Mice in the control (CON) group received saline by OG (0.5 mL/d) and by intraperitoneal (IP) injection (0.4 mL/time, once/week). Mice in the WCA group received WCA by OG (0.5 mL/d) and saline by IP injection (0.4 mL/time, once/week). Mice in the 5-FU group were given saline by OG (0.5 mL/d) and 5-FU by IP injection (50 mg/kg per time, once/week). Mice in the WCA + 5-FU group received WCA by OG (0.5 mL/d) and 5-FU by IP injection (50 mg/kg per time, once/week). All treatments lasted for 7 wk. Twenty-four hours after the final drug administration, laparotomy was performed and blood samples were collected from each animal. Tumors (both orthotopic and metastatic) and tumor-adjacent tissues were collected, and conventional pathological examination, immunohistochemistry (IHC), real-time polymerase chain reaction (PCR), and immunoblotting assays were performed.

Tumor measurement and observation

Weights of the orthotopic tumors were measured using an electronic platform balance. The width (a) and length (b) of each orthotopic tumor were measured with a caliper, and tumor size was calculated according to the following formula: tumor size (mm^3) = π × a^2 × b/6. Inhibition of tumor growth caused by drug treatment was estimated based on the following formula: inhibition rate of tumor (%) = (1 - mean tumor weight in treatment group/mean tumor weight in CON group) × 100%.

Pathological examination

Orthotopic tumors, tumor-adjacent tissues, and metastatic tumors were embedded in paraffin and cut into sections. These sections were then stained with hematoxylin and eosin (HE; Sigma) and visualized under a BH2 optical microscope (Olympus, Tokyo, Japan).

Determination of serum CEA level

Blood samples obtained from mice and were naturally

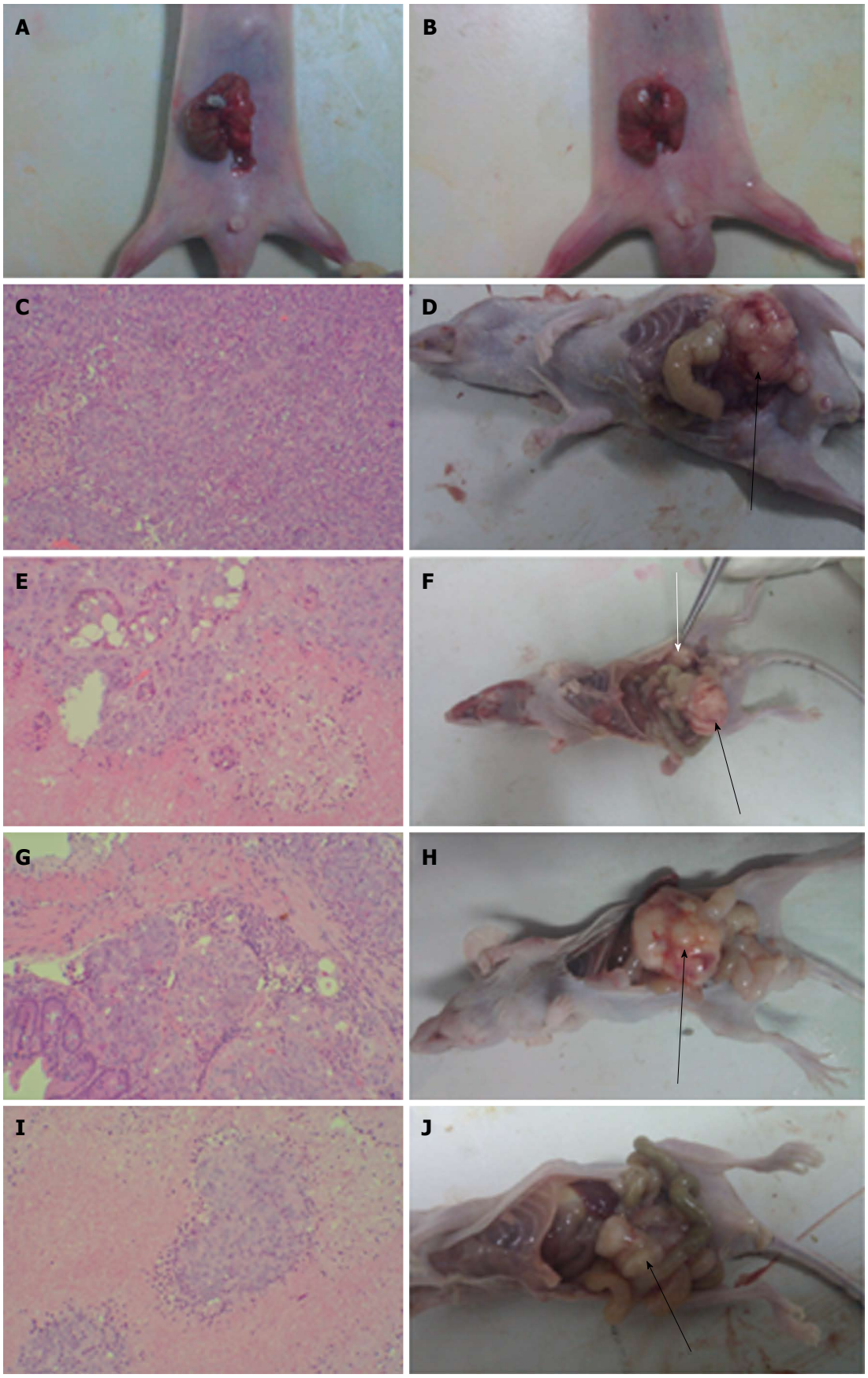

Figure 1 Pathology of primary colon cancer tissues (HE staining) and gross anatomy of nude mice implanted with human colon cancer HCT-116 cells. A, B: Tumor pieces were transplanted into the cecum of nude mice by purse-string suture; C, E, G and I: HE staining of colon cancer tissues from mice in the CON, WCA, 5-FU and WCA + 5-FU groups (magnification × 100); D, F, H and J: Gross anatomy of mice in the CON, WCA, 5-FU and WCA + 5-FU groups. Black arrows indicate orthotopic tumors, and white arrows indicate the abdominal wall tumor. WCA: Weichang'an; 5-FU: 5-fluorouracil; CON: Control.

coagulated for 20 min after collection. Samples were centrifuged at 1000 *g* for 10 min, and the supernatant was collected and stored at -20 ℃. Serum CEA levels were determined using a CEA ELISA kit (Bio-Swamp, Wuhan, China) according to the manufacturer's instructions. Absorbance was measured at 450 nm on an MK3 microplate reader (Thermo Fisher Scientific, Waltham, MA, United States).

Real-time PCR

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, United States), and the concentration and purity of RNA were determined using a UV spectrophotometer. Total RNA was reverse-transcribed into cDNA with reverse transcriptase reagents (Shanghai Jierdun Biotech Co. Ltd., Shanghai, China) according to the manufacturer's protocol. An ABI Step One Plus Real-Time-PCR System (Applied Biosystems, Foster City, CA, United States) was used with SYBR Green Master Mix (ABI) and primers (Invitrogen, Shanghai, China) for measurement of β-catenin and MMP-7. Primers were: β-catenin sense (5'-GGT TTC CCA TTG GTT CAC-3') and antisense (5'-CAT AAA TCC CGC CTA ACG-3'); and MMP-7 sense (5'-GAA CAG GCT CAG GAC TAT CTC-3') and antisense (5'-ACA TCT GGC ACT CCA CAT C-3'). GAPDH was used as a reference to obtain the relative fold change for target genes using the comparative Ct method. Primer sequences for GAPDH were: sense (5'-CGG AGT CAA CGG ATT TGG TCG TAT-3') and antisense (5'-AGC CTT CTC CAT GGT GGT GAA GAC-3'). Relative β-catenin and MMP-7 expression were estimated using the $2^{-\Delta\Delta CT}$ method.

IHC

We performed IHC on orthotopic and metastatic tumors to determine protein expression levels of both β-catenin and MMP-7. The following primary antibodies were used: rabbit monoclonal anti-β-catenin antibody (diluted 1:200, rabbit IgG1; ab79089, Abcam, Cambridge, United Kingdom) and anti-MMP-7 antibody (diluted 1:200, rabbit IgG1; ab4044, Abcam, Cambridge, United Kingdom). The secondary antibody used was a biotinylated goat anti-rabbit IgG (Beyotime Institute of Biotechnology, Shanghai, China). Sections were visualized with diaminobenzidine (DAB; Shanghai Jierdun Biotech Co. Ltd., Shanghai, China) and counterstained with HE. IHC images were captured with a digital camera (Nikon, Tokyo, Japan) and analyzed using the IMS imaging processing system (Shanghai Jierdun Biotech). Positively stained regions were counted and analyzed.

Cell fractionation

Cells were fractionated into cytosolic and nuclear fractions, with little modification[18]. Cells from both orthotopic tumor tissues and hepatic metastatic cancer tissues were collected, washed with PBS and centrifuged at 500 × *g*. Pelleted cells were resuspended in ice-cold 0.1% NP40-PBS and lysed by pipetting up and down several times. A portion of the cell suspension was kept as a whole cell lysate. The cell lysates were centrifuged at 14000 × *g* for 1 min and the supernatants were collected as a cytosolic fraction, while the pellets (nuclei) were washed twice with ice-cold 0.1% NP40-PBS. The harvested pellets were resuspended in Laemmli sample buffer (Bio-Rad), sonicated for 30 s, and collected as nuclear fractions. Equivalent proportions of two fractions were analyzed by SDS-PAGE and immunoblotting. The purity of the fractions was assessed by detecting specific subcellular marker proteins such as GAPDH as cytosolic protein and H3 as nuclear protein.

Immunoblotting

Total protein was extracted from both orthotopic tumor tissues and hepatic metastatic cancer tissues, and protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific). Immunoblotting was performed with the following primary antibodies: rabbit monoclonal anti-β-catenin antibody (diluted 1:1000, rabbit IgG1; ab79089, Abcam, Cambridge, MA, United States) and anti-MMP-7 antibody (diluted 1:300, rabbit IgG1; ab4044, Abcam, Cambridge, MA, United States). Anti-rabbit horseradish-peroxidase-conjugated secondary antibody was used at a dilution of 1:2000. Detection of GAPDH [diluted 1:1500; Cell Signaling Technology (CST), Beverly, MA, United States] and H3 (diluted 1:1000, #4499S, CST) served as internal loading controls. All blots were scanned with the Labwork imaging processing system. Protein band pixels were calculated using Gel-pro Analyzer 4.0 (Media Cybernetics, Rockville, MD, United States).

Statistical analysis

Continuous data are expressed as mean ± standard deviation (SD) and comparison of the means among four groups was performed using one-way analysis of variance (ANOVA). If Levene's test revealed homogeneity of variance, multiple comparisons were performed using Fisher's least significant difference test. If the Levene's test revealed heterogeneity of variance, the Welch and Brown-Forsythe tests were used. In this case, multiple comparisons were performed using Dunnett's T3 and Dunnett's C tests. Data with small sample size are expressed as mean ± SD and comparisons among four groups were performed using Kruskal-Wallis tests. Nemenyi tests were further used to perform multiple comparisons. Categorical data were analyzed using χ^2 tests. R 3.1 software was used to perform Nemenyi tests and other statistical analyses were performed using

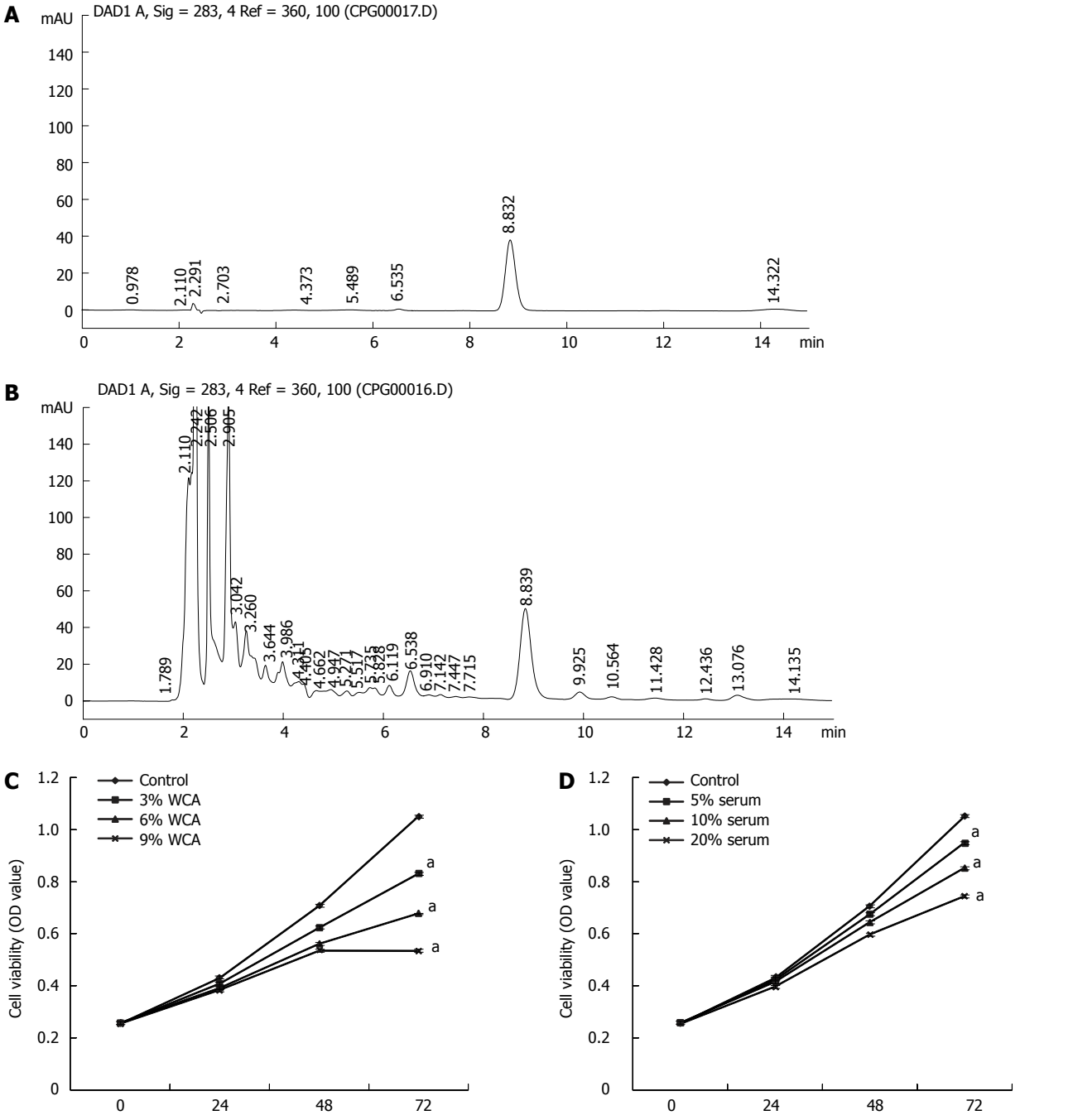

Figure 2 HPLC fingerprinting of hesperidin (A) and Weichang'an (B); HCT-116 cells were treated with different concentrations of Weichang'an (C) and different concentrations of Weichang'an-containing serum (D) *in vitro*. Proliferation of cells was detected using the MTT assay. Cell viability was expressed as optical density (OD) value (mean ± SD). $^{a}P < 0.05$ *vs* cell viability of the control group at the same time point. WCA: Weichang'an.

SPSS version 18.0 (SPSS Inc., Chicago, IL, United States). $P < 0.05$ were considered significant.

RESULTS

Determination of WCA concentration and ex vivo effects on HCT-116 cells

To normalize the concentration of WCA, the concentration of hesperidin in WCA was determined by HPLC and a standard curve (y = 1639.7x + 9.1343; correlation coefficient $r = 0.9990$) was obtained with the peak area as the Y axis and concentration of control hesperidin as the X axis. HPLC fingerprinting of control hesperidin was performed at 8.832 min (Figure 2A), and HPLC fingerprinting of WCA was done at 8.839 min (Figure 2B). Three batches of WCA had similar concentrations of hesperidin.

The human colon cancer cell line HCT-116 was treated with 0, 3%, 6% and 9% WCA decoction filtrate or with 5%, 10% and 20% of serum extracted from rats that received WCA by OG (2 mL/d for 1 wk). As shown in Figure 2C and 2D, 72 h after treatment, WCA exerted significant inhibition on HCT-116 cell proliferation ($P < 0.05$).

Table 1 Weight and size of orthotopic tumors in nude mice and tumor inhibition rates resulting from drug treatments

Group	*n*	Weight (g)	Size (mm^3)	Tumor inhibition
CON	11	1.804 ± 0.649	2180.259 ± 816.556	-
WCA	10	1.459 ± 0.431	1616.795 ± 566.260	19.087%
5-FU	11	1.203 ± 0.284[b]	1695.657 ± 656.594	33.298%
WCA + 5-FU	10	1.140 ± 0.464[b]	1493.438 ± 740.906[a]	36.802%

[a]$P < 0.05$ *vs* CON; [b]$P < 0.01$ *vs* CON. WCA: Weichang'an; 5-FU: 5-fluorouracil; CON: Control.

Mouse survival and pathogenic manifestations after tumor implantation

One mouse in each group died within 1 wk of orthotopic transplant surgery; in each instance, death may have been caused by the surgical procedure itself. Additionally, one mouse died 12 d after surgery in the WCA group, and one 10 d after surgery in the WCA + 5-FU group. Therefore, 42 mice survived the entire length of the procedure and drug administration. Parenchymal tumors were palpable in the abdomens of mice 2 wk post-surgery. Furthermore, the body weights of mice were reduced by 5 wk after surgery. At 7 wk post-surgery, marasmus and loss of appetite occurred. Additionally, feces appeared soft and red in color; mice also displayed skin casting and slow movement and activity.

Effect of WCA on orthotopic tumor growth and hepatic metastasis

All mice that underwent orthotopic transplantation surgery developed tumors. HE staining and gross anatomy of tumors are shown in Figure 1. The CON group showed obvious atypia of orthotopic cancer cells; large cancerous cells displaying karyokinesis could also be seen. Irregularly arranged tumor-giant cells were also present. Blood vessels were abundant, and there was little connective tissue found in the interstitium. Monocyte infiltration and necrosis were apparent in the tumor interstitium (Figure 1A). The WCA, 5-FU and WCA + 5FU groups showed reduced atypia of orthotopic cancer cells and the presence of large cancerous cells. Irregularly arranged tumor-giant cells were also seen. Blood vessels were abundant in the interstitium. Increased monocyte infiltration was observed in the tumor interstitium, and enlarged necrotic areas were apparent. A significant increase in the cavity-like structure could also be observed (Figure 1B, 1C and 1E). One-way ANOVA showed significant differences in the mean tumor weight among the four groups ($P = 0.010$). Moreover, the mean tumor weight in the CON group was significantly different from the mean tumor weight in both the 5-FU ($P = 0.005$) and WCA + 5-FU groups ($P = 0.003$). Treatment of mice with WCA, 5-FU and WCA + 5-FU inhibited tumor growth by 19.087%, 33.298% and 36.802%, respectively (Table 1). The average size of orthotopic tumors between the CON and the WCA + 5-FU groups was significantly different ($P = 0.031$); however, no other significant differences were identified (Table 1).

Compared with the CON group, mice in the WCA and the WCA + 5-FU groups had fewer liver, abdominal and intestinal metastases (Figure 3). Hepatic metastatic tumors appeared as nodules with massive atypia in mice belonging to the CON group. Cancer cells were large, and little connective tissue was observed in the interstitium. Infiltration and necrotic alteration of monocytes were found in the tumor interstitium. There were hepatic tissue necrosis and lymphocyte infiltration at the boundary of the tumor interstitium (Figure 3A). As for hepatic metastatic tumors in mice in the WCA group, no apparent boundary between the metastases was evident. There were obvious hepatic tissue necrosis and infiltration of both lymphocytes and monocytes (Figure 3B). In hepatic metastatic tumors in mice from the 5-FU and WCA + 5FU groups, cancer cells were irregularly arranged and an increase in the interstitium was observed. Massive bleeding has also occurred (Figure 3C and 3D). The gross metastatic rates in the WCA and the WCA + 5-FU groups were significantly lower than in the CON group ($P = 0.012$ and 0.004, respectively). However, there was no significant difference in hepatic metastases among any of the drug-treated groups (Table 2).

Effect of WCA on serum CEA level

There was no significant difference in serum CEA levels among the CON (43.040 ± 11.273 μg/L), WCA (34.282 ± 14.731 μg/L), and 5-FU (35.462 ± 11.022 μg/L) groups ($P = 0.122$). However, serum CEA levels in the WCA + 5-FU group (31.263 ± 7.421 μg/L) were significantly lower than that in the CON group ($P = 0.023$).

Effect of WCA on β-catenin and MMP-7 mRNA expression in orthotopic tumors and liver metastases

To begin to investigate the molecular mechanisms underlying metastasis in our model, we measured mRNA expression of β-catenin, a Wnt pathway regulator of cell-cell adhesion, by real-time RT-PCR. One-way ANOVA revealed significant differences in β-catenin expression in orthotopic tumors among the four groups ($P = 0.001$). Specifically, mice treated with WCA, 5-FU and WCA + 5-FU showed decreased β-catenin mRNA expression in orthotopic tumors compared to control treated mice ($P = 0$, 0.021 and 0.006, respectively; Table 3).

In liver metastases, as sample size was small (3-5 per group), Kruskal-Wallis nonparametric tests were performed. The results showed that there was no significant difference in β-catenin mRNA expression levels in the CON, WCA, 5-FU and WCA + 5-FU treatment groups ($P = 0.155$; Table 3).

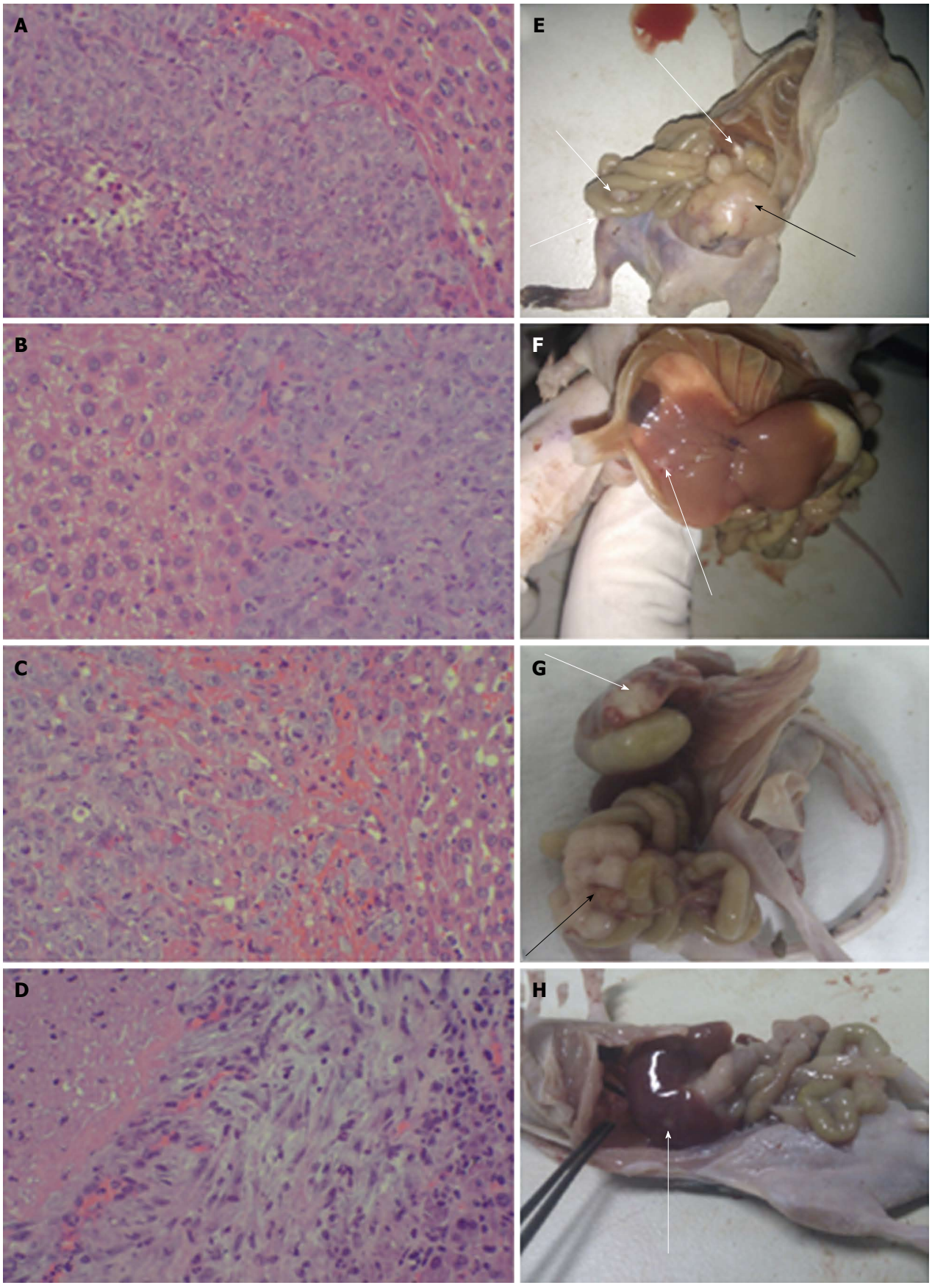

Figure 3 Hepatic metastases of colon cancer in the orthotopic transplant nude mouse model. A-D: HE staining of hepatic metastases tissues from mice in the CON, WCA, 5-FU and WCA + 5-FU groups (magnification × 200); E-H: Gross anatomy of mice in the CON, WCA, 5-FU and WCA + 5-FU groups. The black arrow indicates orthotopic tumor, the long white arrow indicates the hepatic metastatic tumor, and the short white arrow indicates the metastasis of colon cancer to the intestinal wall. WCA: Weichang'an; 5-FU: 5-fluorouracil; CON: Control.

Table 2 Cancer metastases in nude mice implanted with tumors formed from HCT-116 cell injection

Group	Mice survived, *n*	Hepatic metastases	Abdominal wall metastases	Mesenteric metastases	Splenic metastasis	Renal metastasis	Gross metastatic rate	Hepatic metastasis rate
CON	11	5	4	2	0	0	100%	45.45%
WCA	10	3	2	0	0	0	50%[a]	30%
5-FU	11	4	2	1	1	0	72.72%	36.36%
WCA + 5-FU	10	3	0	0	0	1	40%[b]	30%

[a]$P < 0.05$ *vs* CON; [b]$P < 0.01$ *vs* CON. WCA: Weichang'an; 5-FU: 5-fluorouracil; CON: Control.

Table 3 β-catenin and matrix metalloproteinase-7 mRNA levels in orthotopic and hepatic metastatic tumors in the transplant model

Group	β-catenin				MMP-7			
	Orthotopic tumors		Hepatic metastatic tumors		Orthotopic tumors		Hepatic metastatic tumors	
	n	Relative expression	*n*	Relative expression[1]	*n*	Relative expression	*n*	Relative expression[2]
CON	11	0.809 ± 0.354	5	20.588 ± 13.595	11	5.688 ± 3.255	5	1.211 ± 0.593
WCA	10	0.271 ± 0.173[b]	3	3.679 ± 3.271	10	2.236 ± 1.528[a]	3	0.030 ± 0.025[b]
5-FU	11	0.513 ± 0.261[a]	4	4.559 ± 5.491	11	2.427 ± 2.111	4	0.061 ± 0.021
WCA + 5-FU	10	0.445 ± 0.325[b]	3	5.788 ± 2.029	10	0.918 ± 1.011[b]	3	0.044 ± 0.019[a]

[1]β-catenin mRNA expression levels in hepatic metastatic tumors were presented as mean ± SD and nonparametric tests (Kruskal-Wallis tests) were performed, $P = 0.155$; [2]Matrix metalloproteinase-7 (MMP-7) mRNA expression levels in hepatic metastatic tumors were presented as mean ± SD and nonparametric tests (Kruskal-Wallis tests) were performed, $P = 0.012$. [a]$P < 0.05$ *vs* CON; [b]$P < 0.01$ *vs* CON. WCA: Weichang'an; 5-FU: 5-fluorouracil; CON: Control.

MMP-7 is an important target gene in the Wnt/β-catenin signaling pathway and the main member of the MMP family, and it was also examined in our model. The Welch and Brown-Forsythe tests showed significant differences in MMP-7 mRNA expression in orthotopic tumors among the four groups ($P = 0.001$). Moreover, MMP-7 transcript levels were significantly decreased in both the WCA and WCA + 5-FU treatment groups ($P = 0.038$ and 0.003, respectively; Table 3).

In liver metastases, MMP-7 mRNA was significantly decreased ($P = 0.012$, Kruskal-Wallis tests; Table 3) and Nemenyi tests revealed that both WCA and WCA + 5-FU treatments decreased MMP-7 mRNA expression compared to the CON group ($P = 0.001$ and 0.013, respectively; Table 3).

Effect of WCA on β-catenin and MMP-7 protein expression in orthotopic tumors and liver metastases

We measured protein expression of β-catenin and MMP-7 in both orthotopic tumors and liver metastases by IHC and immunoblotting. Figure 4A and 4B show integrated OD of β-catenin and MMP-7 protein detected by IHC in orthotopic tumors and liver metastases, respectively. As shown in Figure 4C and 4E, in both orthotopic tumors and liver metastases, β-catenin was expressed at high levels and was located in both the nucleus and cytoplasm in the CON group, and total expression of β-catenin was reduced in the WCA/5-FU/WCA + 5-FU groups compared with the CON group. As shown in Figure 4D and 4F, MMP-7 was highly expressed (brown staining) in the cytoplasm of orthotopic and liver metastatic tumors from the CON group. We also observed some membrane staining and some staining of the tumor interstitium. Expression of MMP-7 was reduced in the WCA, 5-FU and WCA + 5-FU groups compared with the CON group. The structure of the tumor tissue was also destroyed by massive necrotic areas.

One-way ANOVA analysis of IHC staining showed a significant difference in β-catenin protein expression in orthotopic tumors among the four groups ($P = 0.012$, 10 or 11 per group). Furthermore, β-catenin protein was decreased in all treatment groups compared with CON (WCA, $P = 0.003$; 5-FU, $P = 0.033$; WCA + 5-FU, $P = 0.008$; Figure 4A).

Levene's test revealed heterogeneity of variance in MMP-7 protein levels in the orthotopic tumors, and the Brown-Forsythe test showed significant differences in MMP-7 protein expression in the orthotopic tumors among the four groups ($P = 0.020$, 10 or 11 per group). Pairwise comparisons showed no significant difference between groups (all $P > 0.05$). However, a decreasing trend was observed in both the WCA group (776.30 ± 124.030) and the WCA + 5-FU group (907.30 ± 359.492) compared with the CON group (1663.73 ± 975.557; Figure 4A).

β-catenin protein expression in hepatic metastases detected using IHC was significantly different among the four groups [CON group (1272.40 ± 234.658); WCA group (982.67 ± 17.039); 5-FU group (949.75 ± 86.083); and WCA + 5-FU group (838.00 ± 46.936); $P = 0.025$ using the Kruskal-Wallis test, Figure 4B, 3-5 per group]. Pairwise comparisons using Nemenyi tests revealed that the WCA + 5-FU treatment decreased β-catenin expression compared to the CON group ($P = 0.002$).

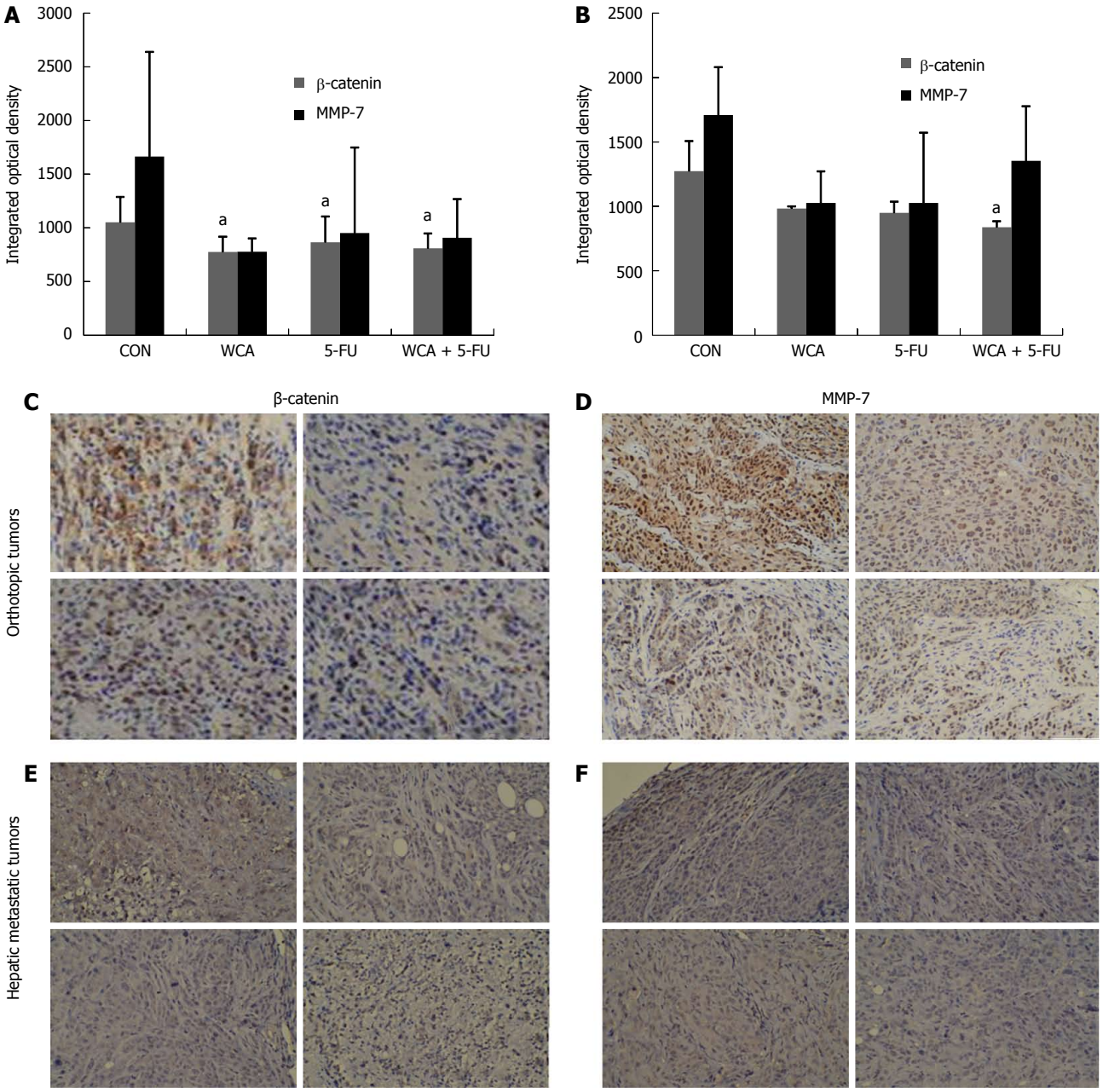

Figure 4 Immunohistochemistry detection of β-catenin and matrix metalloproteinase-7 in orthotopic tumors and hepatic metastatic tumors. A, B: Integrated optical density (OD) of β-catenin and matrix metalloproteinase (MMP)-7 protein expressed in orthotopic tumors (A) or hepatic metastatic tumors (B) from mice in the CON, WCA, 5-FU and WCA + 5-FU groups. Data are presented as mean ± SD and $^{a}P < 0.05$ *vs* corresponding CON group; C: IHC staining for β-catenin in orthotopic tumors (magnification × 200); D: IHC staining for MMP-7 in orthotopic tumors (magnification × 200); E: IHC staining for β-catenin in hepatic metastatic tumors (magnification × 200); F: IHC staining for MMP-7 in hepatic metastatic tumors (magnification × 200). WCA: Weichang'an; 5-FU: 5-fluorouracil; CON: Control.

MMP-7 expression in hepatic metastases was not significantly different among the groups (P = 0.113 using Kruskal-Wallis test, 3-5 per group), while a trend toward drug-mediated decrease in MMP-7 protein expression was observed in hepatic metastases by IHC (1709.00 ± 371.485, 1026.67 ± 245.194, 1026.00 ± 546.233 and 1353.00± 421.138 in the CON, WCA, 5-FU and WCA + 5-FU groups and mean rank 11.40, 5.67, 4.75 and 9.0, respectively).

Figure 5A and B show gray scale scanning of β-catenin and MMP-7 protein detected by western blot in orthotopic tumors and liver metastases (3 per group), respectively. β-catenin expression was significantly different among the four groups in both orthotopic and hepatic metastatic tumors (P = 0.043 and P = 0.041 by Kruskal-Wallis test, respectively). Pairwise comparisons using Nemenyi tests showed decreased expression of β-catenin in the 5-FU and WCA + 5-FU groups compared with the CON group (P = 0.023 and P = 0.043, respectively) in orthotopic tumors, while in hepatic metastatic tumors, β-catenin expression in the WCA + 5-FU group was decreased compared with the CON group (P =0.012).

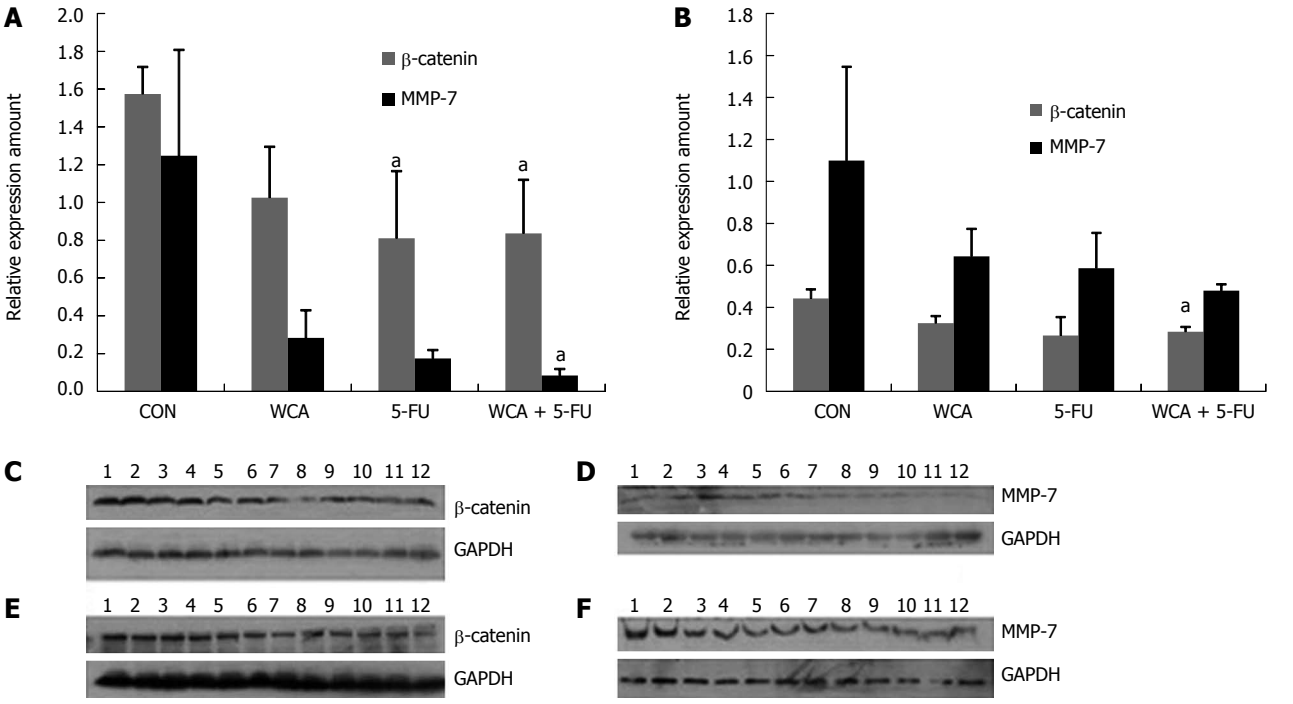

Figure 5 Western blot analysis of β-catenin and matrix metalloproteinase-7 protein expression in orthotopic and hepatic metastatic tumors. A, B: Quantification of β-catenin and matrix metalloproteinase (MMP)-7 expression in orthotopic tumors (A) and hepatic metastatic tumors (B). Data were presented as mean ± SD and $^{a}P < 0.05$ *vs* corresponding CON group; C: β-catenin expression in orthotopic tumors; D: MMP-7 expression in orthotopic tumors; E: β-catenin expression in hepatic metastatic tumors; F: MMP-7 expression in hepatic metastatic tumors. Lanes 1-3, CON group; 4-6, WCA group; 7-9, 5-FU group; and 10-12, WCA + 5-FU group. WCA: Weichang'an; 5-FU: 5-fluorouracil; CON: Control.

Using Kruskal-Wallis tests, MMP-7 showed a difference in orthotopic tumors among the four groups ($P = 0.033$) but not in hepatic metastatic tumors ($P = 0.069$). Pairwise comparisons using Nemenyi tests showed decreased expression of MMP-7 in the WCA + 5-FU group compared with the CON group ($P = 0.001$) in orthotopic tumors.

Effect of WCA on intracellular distribution of β-catenin protein

To clarify whether the intracellular distribution of β-catenin was affected by WCA, we performed cell fractionation and detected β-catenin in the cytosolic and nuclear fractions in orthotopic tumors and hepatic metastatic tumors, respectively. The immunoblotting images and quantitative diagrams are shown in Figure 6.

In orthotopic tumors (Figure 6A), Kruskal-Wallis tests showed significant cytosolic and nuclear β-catenin expression ($P = 0.019$ and $P = 0.033$, respectively). Pairwise comparisons using Nemenyi tests revealed that WCA + 5-FU treatment decreased cytosolic β-catenin expression compared to the CON group ($P < 0.001$), while WCA and WCA + 5-FU decreased nuclear β-catenin expression compared to the CON group ($P = 0.043$ and $P = 0.012$, respectively).

In hepatic metastatic tumors (Figure 6B), Kruskal-Wallis tests revealed that there was a significant difference in cytosolic ($P = 0.016$) or nuclear ($P = 0.031$) β-catenin expression among groups, and Nemenyi tests showed that WCA + 5-FU treatment significantly decreased cytosolic ($P < 0.001$) and nuclear ($P = 0.001$) β-catenin.

These data showed that WCA/5-FU treatments not only depressed β-catenin expression but also inhibited nuclear translocation both in orthotopic tumors and hepatic metastatic tumors.

DISCUSSION

Colorectal cancer most frequently metastasizes to the liver; an event that is the primary cause of death in patients with this disease[8,19]. Those with unresectable colorectal hepatic metastases have a median survival of 6.9 mo and a 5-year survival rate close to zero[20,21]. Therefore, it is critical to understand better the mechanisms underlying the process of metastasis development from colorectal cancer to the liver, in order to prolong patient survival and improve quality of life.

Quantitative determination of WCA was conducted using HPLC detection of hesperidin, which is a citrus flavonoid known to be active against some oxidative-stress-mediated diseases. Hesperidin found in orange peel is a flavanone glycoside consisting of the flavone hesperidin bound to the disaccharide rutinose. The sugar group makes hesperidin more water-soluble than hesperitin; another compound in orange peel[22]. Hesperidin may moderately

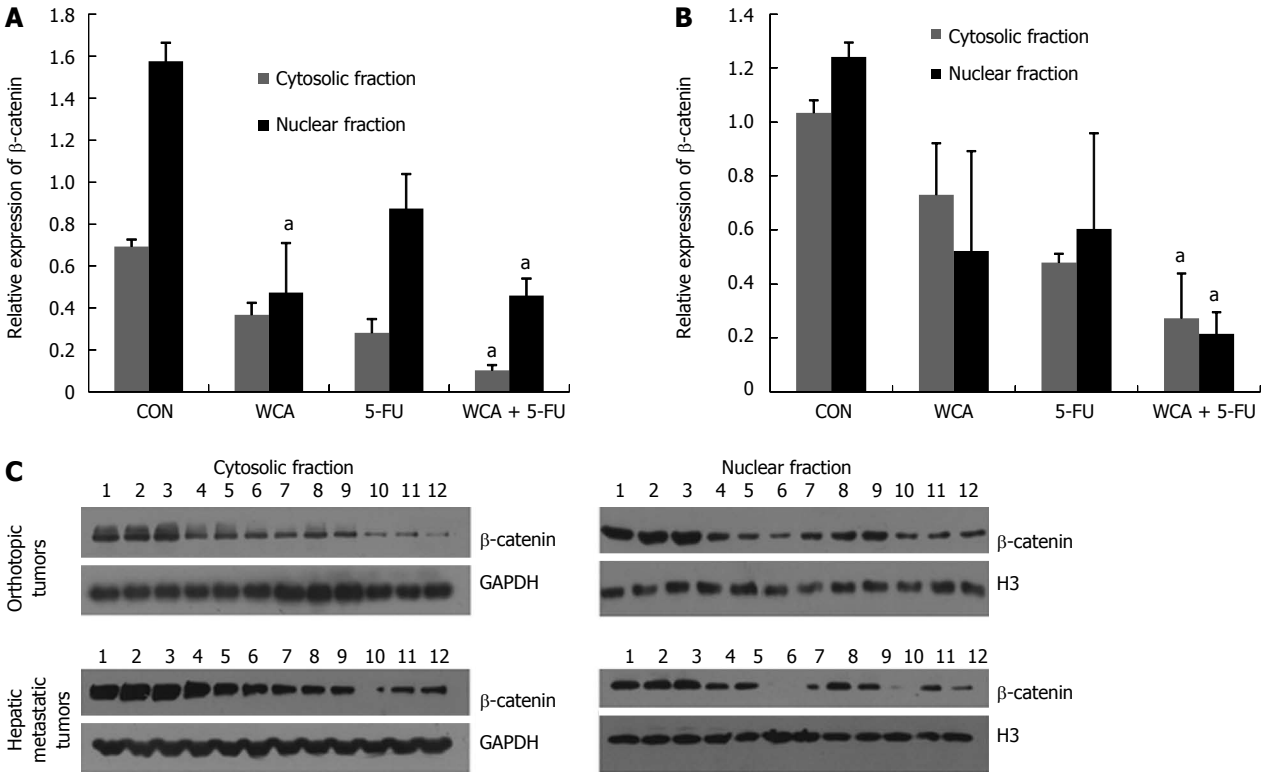

Figure 6 Cell fractionation and western blot analysis of β-catenin protein expression in both orthotopic tumors and hepatic metastatic tumors. A, B: Quantification of β-catenin expression in cytosolic fraction and nuclear fraction in orthotopic tumors (A) and in hepatic metastatic tumors (B). Data were presented as mean ± SD and $^{a}P < 0.05$ *vs* corresponding CON group; C: β-catenin expression in cytosolic fraction and nuclear fraction in orthotopic tumors and hepatic metastatic tumors. Lanes 1-3, CON group; 4-6, WCA group; 7-9, 5-FU group; and 10-12, WCA + 5-FU group. WCA: Weichang'an; 5-FU: 5-fluorouracil; CON: Control.

stimulate the gastrointestinal tract, promote the secretion of digestive enzymes, remove intestinal pneumatosis, and invigorate the stomach to relieve phlegm. Exogenous hesperidin has been shown to influence a wide variety of biological functions, such as inducing apoptosis and suppressing proliferation of human cancer cells[23], as well as inhibiting tumor development in various tissues, including colon cancer[24,25]. In WCA, hesperidin cooperates with other components to exert spleen invigorating, heat clearing, detoxicating and hard lump resolving effects. Hesperidin is a stable and controllable component, and HPLC detection of hesperidin facilitates the monitoring of WCA concentration.

In our current study, orthotopic colon tumors grown in 100% of the mice. Importantly, tumors displayed pathogenic characteristics similar to those observed in clinical cases of advanced colorectal cancer[26]. The mean weights of the orthotopic tumors in the 5-FU and WCA + 5-FU groups were significantly lower than in the CON group (both $P < 0.01$). Additionally, the mean size of orthotopic tumors in the WCA + 5-FU group was significantly smaller than in the CON group ($P < 0.05$). Treatment with WCA, 5-FU, or their combination resulted in orthotopic tumor inhibition of 19.09%, 33.30% and 36.80%, respectively, compared with controls. We detected hepatic metastases in a third of the mice used in the study. Overall, the occurrence of hepatic metastasis in the CON group was 45.45% and 36.36% in the 5-FU group, 30% in the WCA group, and 30% in the WCA + 5-FU group. Although the percentage of hepatic metastasis was lower in the three treatment groups compared with the CON group, statistical significance was not reached. This should be followed up by additional studies with larger sample sizes to determine if the drug-mediated decrease in metastatic rate is significant. We also found a significantly lower gross metastatic rate in the WCA group (50%) and in the WCA + 5-FU group (40%) compared with the CON group (100%). Upon treatment by OG, WCA is primarily absorbed by the intestine, resulting in a high concentration of WCA in the liver. This likely reduces hepatic metastasis of colon cancer. Furthermore, administration of WCA combined with abdominal 5-FU chemotherapy reduces peritoneal metastasis of the tumor cells.

Previous research has established that deregulated Wnt signaling is involved in the development of tumors and closely correlates with the invasiveness and metastatic potential of colorectal cancer[27]. β-catenin is a key downstream mediator of the Wnt signaling pathway[28,29]. Nuclear accumulation of β-catenin at the invasive front of tumors and within blood vessels is strongly associated with

hepatic metastasis and may be a useful predictor of hepatic metastasis in colorectal cancer[30-32]. MMP-7 is transcribed in response to active Wnt-β-catenin signaling. Once translated into a functional protein, MMP-7 assists in the degradation of extracellular matrix, which is a critical event in tumor invasion and metastasis. IHC expression of MMP-7 has been shown to correlate with Dukes' classification in colorectal cancer specimens, and introduction of MMP-7 into colorectal cancer cells markedly upregulates their *in vivo* invasive and metastatic potential[33]. Furthermore, MMP-7 promotes hepatic metastasis in colorectal cancer[34]. Research has also shown that elevated MMP-7 expression is related to decreased survival, and it is considered as a predictor of disease recurrence and hepatic metastasis[35].

The present study showed that administration of WCA significantly reduced the expression of β-catenin and MMP-7 mRNA in orthotopic tumors and in hepatic metastatic tumors in mice. Similarly, WCA, alone and in combination with 5-FU, reduced β-catenin and MMP-7 protein expression in orthotopic and metastatic tumors. Some results detected using immunoblotting, IHC and real-time PCR did not show significant differences. The orthotopic transplant model used in this study is a relatively natural nude mouse model of hepatic metastases, and the metastasis rate is not very high. Only 3-5 animals in each group developed liver metastases, which is not statistically powerful. In a future study, we will increase the sample size and this problem may be resolved.

CEA is often expressed in colorectal cancer and mediates the metastasis of these cancerous cells from the colon to the liver[36]. CEA binds to receptors on the surface of Kupffer cells and promotes the release of interleukin (IL)-1, IL-6, and tumor necrosis factor-α[37]. In turn, these molecules increase the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 by endothelial cells, thereby reinforcing the adhesion between the tumor cells and endothelial cells. Recent reports have shown that measurement of serum CEA levels, in combination with CA199 and CA125, can serve as an important predictor of hepatic metastasis of colorectal cancer[36]. Moreover, serum CEA levels in combination with imaging techniques may accurately predict tumor recurrence following radical surgery of colorectal hepatic metastases[38]. Importantly, our data showed that administration of WCA and 5-FU effectively reduced serum CEA levels in the orthotopic transplant nude mouse model, which may be associated with the decreased rate of hepatic metastasis that we observed.

Pathological analysis revealed that the degree of atypia in both orthotopic tumors and hepatic metastases was significantly lower in the WCA group compared with the CON group. The difference between the CON group and the WCA + 5-FU group was even more striking. Considering the relatively low rate of adverse effects of TCM, the combination of WCA with 5-FU may present a good strategy in colon cancer treatment.

Limitations of this study included the relatively small number of animals in each group, unclear exact components in WCA, and inadequate assessment of the immunological effects of WCA. In addition, different treatment strategies need to be investigated to optimize the effect of WCA. Furthermore, how WCA affects the angiogenesis of metastatic tumor, tumor microenvironment and immunological response requires further research.

In summary, combination of the TCM WCA with 5-FU inhibited colon tumor growth and hepatic metastasis in an orthotopic transplant nude mouse model. Decreased expression of β-catenin and MMP-7 may be important for the anti-tumor properties of WCA and 5-FU.

COMMENTS

Background

It is estimated that 50%-60% of patients diagnosed with colorectal cancer will develop hepatic metastases and the most frequent site of metastasis is the liver. Eighty to ninety percent of these metastases will be unresectable and require multidisciplinary treatments, including chemotherapy, liver-targeting therapies and traditional Chinese medicine (TCM). TCM has a number of benefits, including decreasing the negative side effects associated with chemotherapy and radiotherapy, improving patients' immune function, and enhancing the effects of conventional cancer treatments.

Research frontiers

A previous clinical study has indicated that patients with gastric carcinoma benefit from Weichang'an (WCA) treatment. In addition, WCA has been shown to increase the 5-year survival rate and reduce the 1- and 2-year metastatic rates in patients with colorectal cancer.

Innovations and breakthroughs

We demonstrated that combination of WCA with 5-fluorouracil (5-FU) suppressed colon tumor growth and hepatic metastases, reducing the tumor weight and size, the rate of metastases and serum carcinoembryonic antigen levels. The authors also confirmed that expression levels of β-catenin and matrix metalloproteinase (MMP)-7 are involved in the process of metastasis from colorectal cancer to the liver, as well as the inhibitory effect of WCA and 5-FU.

Applications

This study provided a new therapeutic strategy for colorectal cancer with hepatic metastasis and may greatly contribute to prolong patient survival and improve their quality of life.

Terminology

Orthotopic transplant nude mouse model: HCT-116 cells were subcutaneously injected into the right axilla of nude mice. After 3 wk, tumors were excised and cut into 1-2-mm^3 pieces using sterile techniques. Tumor blocks were transplanted into the cecum of nude mice by a purse-string suture.

Peer review

The authors analyzed the effect of the TCM WCA on colorectal carcinoma in a clinically interesting orthotopic model. Their major finding was a reduction in tumor weight and size as well as the number of metastases when WCA was combined with 5-FU. They also speculate that decreased expression of β-catenin and MMP-7 may be involved in the anti-tumorigenic properties of WCA. The design, technical performance and some of the conclusions made in this study are comprehensible. The authors address a clinically very relevant topic. The manuscript is overall written in good English.

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ORIGINAL ARTICLE

Basic Study

Loss of stromal caveolin-1 expression in colorectal cancer predicts poor survival

Zhi Zhao, Fang-Hai Han, Shi-Bin Yang, Li-Xin Hua, Jian-Hai Wu, Wen-Hua Zhan

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Author contributions: Zhao Z and Han FH contributed equally to this work; Zhao Z and Han FH designed the research; Zhao Z performed the research; Yang SB, Hua LX and Wu JH analysed the data; Zhao Z wrote the paper; Han FH and Zhan WH edited the paper.

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Abstract

AIM: To investigate the clinicopathological significance and prognostic value of caveolin-1 (CAV-1) in both tumor and stromal cells in colorectal cancer (CRC).

METHODS: A total of 178 patients with CRC were included in this study. The correlation between CAV-1 expression and clinicopathologic features and survival was studied.

RESULTS: CAV-1 expression was detected in tumor and stromal cells. The expression of stromal CAV-1 was closely associated with histological type (P = 0.022), pathologic tumor-node-metastasis stage (P = 0.047), pathologic N stage (P = 0.035) and recurrence (P = 0.000). However, tumor cell CAV-1 did not show any correlation with clinical parameters. Additionally, the loss of stromal CAV-1 expression was associated with shorter disease-free survival (P = 0.000) and overall survival (P = 0.000). Multivariate analysis revealed that the loss of stromal CAV-1 expression was an independent prognostic factor for both overall survival (P = 0.014) and disease-free survival (P = 0.006).

CONCLUSION: The loss of stromal CAV-1 expression in CRC was associated with poor prognosis and could be a prognostic factor for CRC patients.

Key words: CAV-1; Stroma; Colorectal cancer; Prognosis

Core tip: Caveolin-1 (CAV-1), an essential structural protein of the endocytic caveolae plasma membrane, plays a major role in modulating tumorigenic processes. Recent studies have revealed that the loss of stromal CAV-1 results in an activated tumor microenvironment and is significantly related to tumor recurrence and a poor prognosis for many tumors. However, the association between CAV-1 and colorectal cancer remains unknown. In our study, we observed CAV-1 expression in both tumor and stromal cells. Our results demonstrate that the loss of stromal CAV-1 is an independent predictor of poor overall survival and disease-free survival, whereas CAV-1 expression in tumor cells has no prognostic value.

Zhao Z, Han FH, Yang SB, Hua LX, Wu JH, Zhan WH. Loss of stromal caveolin-1 expression in colorectal cancer predicts poor survival. *World J Gastroenterol* 2015; 21(4): 1140-1147 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1140.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1140

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer mortality in the United States, with an estimated 142820 new cases and 50830 deaths per year[1]. Over the past ten years, the rate of death from CRC has declined by 3%, but 30% to 40% of CRC patients will develop distant metastases, and 50% will die of CRC recurrence[2].

The tumor microenvironment has recently been recognized to play an important role in determining tumor initiation and progression[3-6]. The tumor microenvironment is composed of immune cells, stromal cells (including cancer-associated fibroblasts (CAFs), adipocytes, and bone marrow-derived progenitors), and the vasculature[5,7]. Under normal physiological conditions, the stroma serves as a critical barrier to prevent malignant transformation. However, during neoplastic tumorigenesis, the stroma facilitates tumor progression and metastasis in response to molecular signals derived from carcinoma cells and other host cell types.

Caveolin-1 (CAV-1), an essential structural protein of endocytic caveolae plasma membrane invaginations, is present in most mammalian cells, such as adipocytes, endothelial cells, pneumocytes, fibroblasts, and smooth muscle cells[8,9]. CAV-1 plays a major role in modulating tumorigenic processes through its various functions, such as gene regulation, membrane trafficking, and signal transduction[10-12]. However, CAV-1 shows a compartment-dependent role in tumors. Furthermore, the role of CAV-1 is controversial in epithelial tumor cells. Several authors have proposed that high CAV-1 expression in tumor cells predicts poor survival[13,14]. In contrast, El-Gendi *et al*[15] reported that CAV-1 expression in tumor cells has no prognostic value. Recent studies have revealed that the loss of CAV-1 in the tumor stroma results in an activated tumor microenvironment and is significantly related to early tumor recurrence, metastasis, and poor clinical outcome in breast cancer, prostate cancer, and other solid tumors[16-19].

However, the association between CAV-1 and colorectal cancer remains unknown. Thus, to explore the relationship between CAV-1 expression and colorectal cancer, we analyzed CAV-1 expression in CRC stromal cells and tumor cells in a series of human CRC tissue sections.

MATERIALS AND METHODS

Patients and specimens

Colorectal tissue samples were obtained from the pathology department at the First Affiliated Hospital of Sun Yat-sen University between May 2004 and November 2009. The patients included in this study fulfilled the following criteria: (1) pathologically confirmed diagnosis of colorectal adenocarcinoma; (2) no treatment prior to curative excision of the primary tumor; (3) no previous malignancy or second primary tumor; (4) no severe coincident diseases; and (5) the availability of clinical information and follow-up data. The tumors were classified according to the American Joint Committee on Cancer staging manual (7th edition).

All patients were followed every 3 mo for the first year, every 6 mo from the second to fifth years and every 12 mo after 5 years. The study protocol was approved by the Ethics and Scientific Committee of the First Affiliated Hospital of Sun Yat-sen University and conforms to the Declaration of Helsinki. All patients and their families provided written informed consent prior to surgery for their information to be stored in the hospital database and used for research.

Immunohistochemistry

Tissue section immunohistochemistry was performed according to the instructions provided by the manufacturer. Briefly, 4 μm formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated through a graded alcohol series. Antigen retrieval was performed in 10 mmol/L sodium citrate (pH 6.0) for 5 min using a pressure cooker and the sections were allowed to cool for 45 min at room temperature. After blocking with 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase, the slides were washed three times with phosphate-buffered saline and incubated with 10% goat serum for 30 min at room temperature. The sections were then incubated with an anti-CAV-1 antibody (rabbit monoclonal, dilution 1:100, Cell Signaling Technology, CST) overnight at 4 ℃, and the slides were then incubated with an HRP-conjugated sheep anti-rabbit secondary antibody (GTVision; Shanghai, China) for 30 min at room temperature. Immunoreactivity was revealed using 3,3′-diaminobenzidine and counterstained with Mayer's hematoxylin. A known positive tissue sample (breast cancer slide) was used as a positive control.

Evaluation of immunostaining

The staining of stromal CAV-1 was scored semi-quantitatively as negative (0, no staining), weak (1, either diffuse weak staining or strong staining in less than 30% of the stromal cells) or strong (2, strong

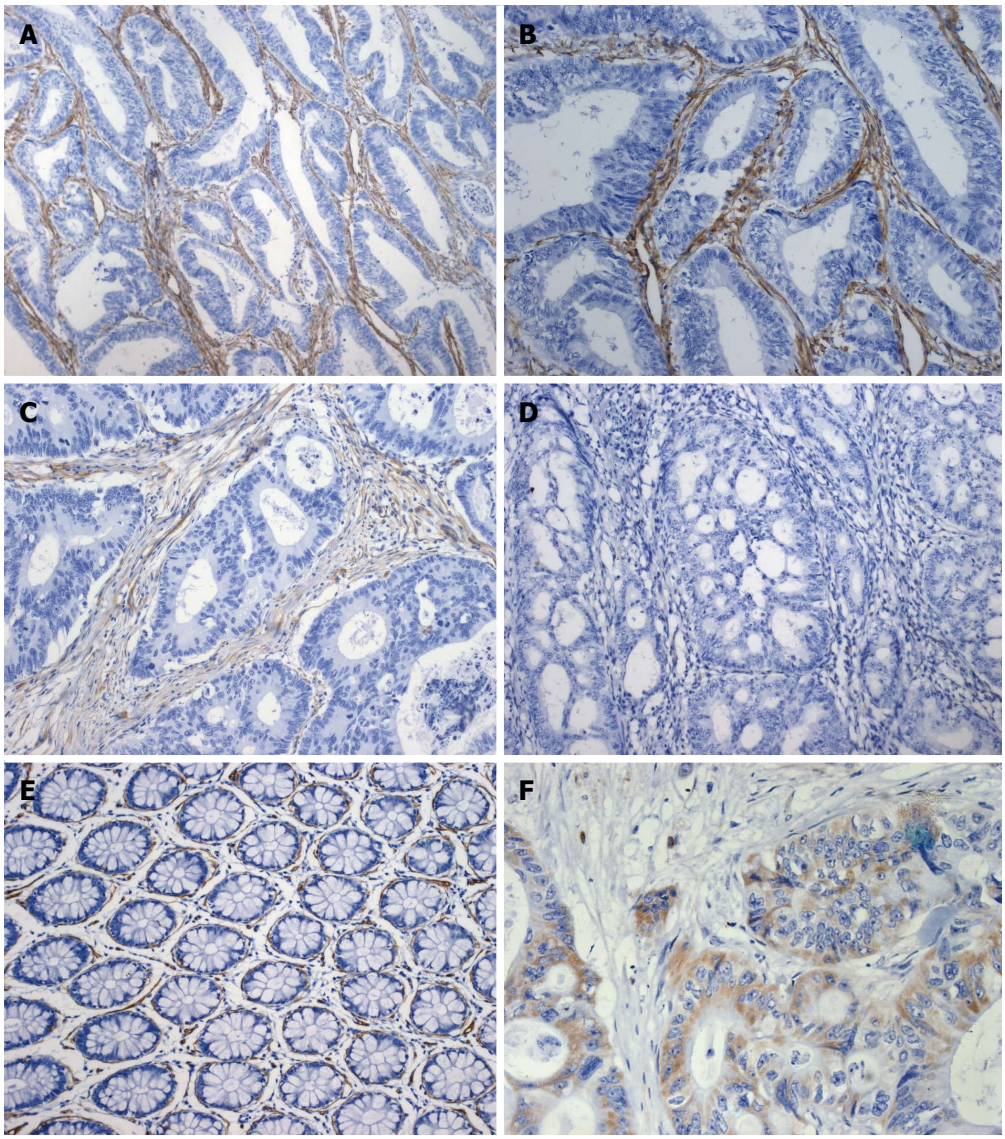

Figure 1 Immunohistochemical staining for caveolin-1. High caveolin-1 (CAV-1) expression in colorectal cancer stroma (A: × 200; B: × 400). Panel C (× 200) shows moderate stromal CAV-1 expression, and panel D (× 200) shows negative stromal CAV-1 expression. Panel E (× 200) depicts normal colorectal tissue. Panel F (× 400) demonstrates CAV-1 expression in colorectal tumor cells.

staining of 30% or more of the stromal cells)[20]. We also evaluated CAV-1 expression in tumor cells. Any expression in tumor cells was considered to be positive CAV-1 staining[20]. The immunostaining results were evaluated by two independent pathologists.

Statistical analysis

All statistical calculations were carried out using SPSS 17.0 statistical software. The associations between CAV-1 expression and various clinical parameters were evaluated using the Mann-Whitney test, Spearman's test or χ^2 test. The Kaplan-Meier test was used to evaluate disease-free survival (DFS) and overall survival (OS), and the survival curve was compared using the Log rank test. Multivariate Cox regression models were applied to evaluate CAV-1 expression and other prognostic factors with respect to DFS and OS. All *P*-values are two-sided and were considered statistically significant at the level of < 0.05.

RESULTS

The present study included 178 matched colorectal cancer tissues and 30 paraneoplastic normal tissues, which were more than 5 cm from the primary tumor sites. Of the 178 CRC patients, 103 were male (57.9%) and 75 (42.1%) were female. The median age was 54 years (range: 24-85 years). The median follow-up period was 50 (range: 9-127) months for all patients.

CAV-1 expression was observed in both the tumor and stromal cells. Additionally, adipocytes, endothelial cells, and perineurial cells showed strong CAV-1 expression and served as internal positive controls.

Table 1 Association of stromal caveolin-1 expression with clinicopathologic parameters *n* (%)

Variable	Cav-1			*P*-value
	0	1	2	
Age (yr)				0.095[1]
≤ 50	22 (33.8)	19 (29.2)	24 (36.9)	
> 50	27 (23.9)	31 (27.4)	55 (48.7)	
Gender				0.404[1]
Male	26 (25.2)	29 (28.2)	48 (46.6)	
Female	23 (30.7)	21 (28.0)	31 (41.3)	
Tumor size (cm)				0.688[1]
< 5	28 (27.7)	30 (29.7)	43 (42.6)	
≥ 5	21 (27.3)	20 (26.0)	36 (46.8)	
Tumor location				0.378[1]
Colon	32 (30.8)	28 (26.9)	44 (42.3)	
Rectum	17 (23.3)	22 (30.1)	34 (46.6)	
Histological type				0.022[2,a]
Well	1 (12.5)	4 (50.0)	3 (37.5)	
Moderate	31 (23.8)	35 (26.9)	64 (49.2)	
Poor	17 (42.5)	11 (27.5)	12 (30.0)	
Pathologic T stage				0.118[2]
T1	1 (50.0)	0 (0)	1 (50.0)	
T2	0 (0)	5 (35.7)	9 (64.3)	
T3	45 (29.2)	44 (28.6)	65 (42.2)	
T4	3 (37.5)	1 (12.5)	4 (50.0)	
Pathologic N stage				0.035[2,a]
N0	10 (16.4)	20 (32.8)	31 (50.8)	
N1	23 (29.1)	23 (29.1)	33 (41.8)	
N2	16 (42.1)	7 (18.4)	15 (39.5)	
Pathologic TNM stage				0.047[2,a]
I	0 (0)	4 (50.0)	4 (50.0)	
II	10 (18.9)	16 (30.2)	27 (50.9)	
III	39 (33.3)	30 (25.6)	48 (41.0)	
Tumor cell Cav-1				0.697[1]
Negative	26.7 (61.8)	29.1 (63.8)	76 (44.2)	
Positive	3 (50.0)	0 (0)	3 (50.0)	
Recurrence				< 0.001[1,a]
No		28 (24.1)	68 (58.6)	
Yes		21 (36.2)	10 (17.2)	

[1]Mann-Whitney test; [2]Spearman's test; [a]$P < 0.05$, stromal CAV-1 *vs* clinical variables. CAV-1: Caveolin-1; TNM: Tumor-node-metastasis.

Table 2 Association between tumor cell caveolin-1 expression and clinicopathologic parameters *n* (%)

Variable	Cav-1		*P*-value
	Negative	Positive	
Age (yr)			1.000
≤ 50	63 (96.9)	2 (3.1)	
> 50	109 (96.5)	4 (3.5)	
Gender			0.698
Male	100 (97.1)	3 (2.9)	
Female	72 (96.0)	3 (4.0)	
Tumor size (cm)			0.237
< 5	96 (95.0)	5 (5.0)	
≥ 5	76 (98.7)	1 (1.3)	
Tumor location			0.694
Colon	101 (97.1)	3 (2.9)	
Rectum	71 (95.9)	3 (4.1)	
Histological type			0.498
Well	8 (100)	0 (0)	
Moderate	124 (95.4)	6 (4.6)	
Poor	40 (100.0)	0 (0)	
Pathologic T stage			0.073
T1	2 (100.0)	0 (0)	
T2	14 (100)	0 (0)	
T3	150 (97.4)	4 (2.6)	
T4	6 (75.0)	2 (25.0)	
Pathologic N stage			0.304
N0	57 (93.4)	4 (6.6)	
N1	77 (97.5)	2 (2.5)	
N2	38 (100)	0 (0)	
Pathologic TNM stage			0.158
I	8 (100)	0 (0)	
II	49 (92.5)	4 (7.5)	
III	115 (98.3)	2 (1.7)	
Stromal Cav-1			0.208
0	46 (93.9)	3 (6.1)	
1	50 (100)	0 (0)	
2	76 (96.2)	3 (3.8)	
Recurrence			0.180
No	110 (94.8)	6 (5.2)	
Yes	58 (100)	0 (0)	

CAV-1: Caveolin-1; TNM: Tumor-node-metastasis.

Analyses for CAV-1 expression in the stroma revealed positive expression in 129 patients (72.5%) and negative expression in 49 patients (27.5%). Of the 129 positive cases, 50 (28.1%) had a score of 1, and 79 (44.4%) had a score of 2. Representative examples are shown in Figure 1. The correlations between stromal CAV-1 and clinical variables are listed in Table 1. The expression of stromal CAV-1 was closely associated with histological type ($P = 0.022$), pathologic tumor-node-metastasis (TNM) stage ($P = 0.047$), pathologic N stage ($P = 0.035$) and recurrence ($P < 0.001$). However, there was no significant correlation between the expression level of CAV-1 and age, gender, tumor size, tumor location, pathologic T stage, or tumor cell CAV-1.

Interestingly, with regard to CAV-1 expression in tumor cells, we only observed 6 tissues that were positive for expression in our study. However, as presented in Table 2, tumor cell CAV-1 did not showed any correlation with clinical parameters.

Survival analysis

Next, we evaluated the prognostic value of CAV-1 expression in colorectal cancer. Figure 2 shows that the patients who were positive for stromal CAV-1 expression had significantly longer overall survival and disease-free survival ($P < 0.001$ for both) than the patients who were negative. Of note, the patients with high levels of stromal CAV-1 (score = 2) had a good prognosis, with 89.8% of the patients surviving the follow-up period. Similarly, 77.4% of the patients with moderate stromal CAV-1 staining (score = 1) survived. In contrast, 46.9% of the patients who were negative for stromal CAV-1 expression (score = 0) survived. The 5-year survival rates showed similar patterns. CRC patients with high stromal CAV-1 had a good 5-year survival rate (92%), whereas CRC patients with moderate or absent stromal CAV-1 expression had progressively worse 5-year survival rates (61% and 46%, respectively). We also analyzed the prognostic significance of CAV-1 expression

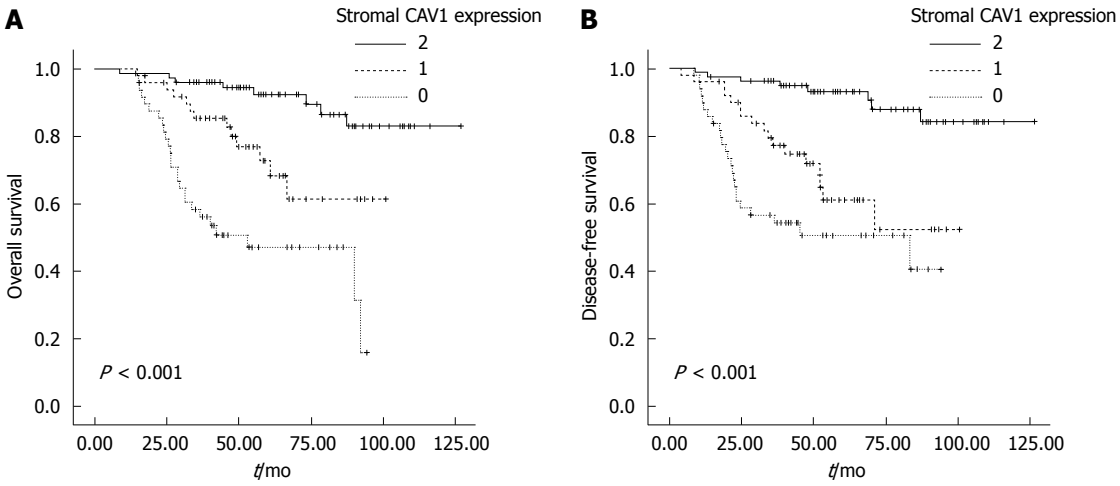

Figure 2 Kaplan-Meier curves of overall survival and disease-free survival. Patients with high stromal caveolin-1 expression have good overall survival (A) and disease-free survival (B).

Table 3 Univariate and multivariate analyses of prognostic factors in colorectal cancer with respect to overall survival and disease-free survival

Parameter	Univariate analysis *P*-value	Multivariate analysis HR (95%CI)	*P*-value
OS			
Age (> 50)	0.185	-	1.632
Gender	0.794	-	1.431
Tumor location	0.384	-	1.414
Tumor cell Cav-1	0.216	-	0.742
Stromal Cav-1	< 0.001[a]	0.578 (0.373-0.895)	0.014[a]
Tumor size	0.443	-	0.382
Histological type	0.533	-	0.666
Pathological T stage	0.067	-	0.347
Pathological N stage	< 0.001[a]	-	0.868
TNM	0.025[a]	-	0.378
Recurrence	< 0.001[a]	2.065 (1.121-3.803)	< 0.001[a]
DFS			
Age (> 50)	0.165	-	0.218
Gender	0.664	-	0.833
Tumor location	0.061	-	0.325
Tumor cell Cav-1	0.189	-	0.705
Stromal Cav-1	< 0.001[a]	0.546 (0.353-0.844)	0.006[a]
Tumor size	0.603	-	0.511
Histological type	0.478	-	0.553
Pathological T stage	0.171	-	0.888
Pathological N stage	0.002[a]	-	0.510
TNM	0.184	0.451 (0.233-0.874)	0.018[a]
Recurrence	< 0.001[a]	1.863 (1.106-2.434)	< 0.001[a]

[a]$P < 0.05$, univariate analysis *vs* multivariate analysis. CAV-1: Caveolin-1; TNM: Tumor-node-metastasis; OS: Overall survival; DFS: Disease-free survival.

in tumor cells, but no prognostic significance for either OS or DFS was found ($P = 0.216$ and 0.189, respectively).

A univariate analysis of possible prognostic indicators identified stromal CAV-1, pathologic N stage, pathologic TNM stage and recurrence as indicators of OS, and stromal CAV-1, pathologic N stage and recurrence as indicators of DFS (Table 3). In a multivariable analysis, stromal CAV-1 expression was a significant independent prognostic factor for OS and DFS ($P = 0.006$, HR = 0.546, 95%CI: 0.353-0.844; $P = 0.014$, HR = 0.578, 95%CI: 0.373-0.895, respectively). Moreover, the results also showed that recurrence was an independent prognostic factor for OS and that TNM and recurrence were independent prognostic factors for DFS (Table 3).

DISCUSSION

In this study, we evaluated the expression of CAV-1 in the stroma and tumor cells of CRC. Our results demonstrated that the loss of CAV-1 expression in the stroma is a strong and independent predictor of poor OS and DFS, whereas CAV-1 expression in tumor cells had no prognostic value. Moreover, stromal CAV-1 expression was closely associated with histological type ($P = 0.022$), pathologic TNM stage ($P = 0.047$), pathologic N stage ($P = 0.035$) and recurrence ($P < 0.001$). However, CAV-1 expression in tumor cells did not show any correlation with the clinical parameters.

Tumors, which are composed of tumor cells and stromal cells, grow within a complex tumor microenvironment. CAFs were recently demonstrated to promote tumor initiation, prevent cancer cell apoptosis, induce cancer cell proliferation, and stimulate tumor angiogenesis by secreting a large amount of growth factors, extracellular matrix components, and matrix metalloproteinases[4,6]. Although the underlying mechanisms are not fully elucidated, the loss of CAV-1 in the stroma plays a major role.

Witkiewicz *et al*[16] reported that the absence of stromal CAV-1 expression predicts early tumor recurrence and poor clinical outcome in human breast cancer. Di Vizio *et al*[21] found that the loss of stromal CAV-1 expression was closely related

to disease progression and metastasis. Jia *et al*[18] indicated that the down-regulation CAV-1 expression in the stroma predicts lymph node metastases, early tumor recurrence, and poor prognosis in esophageal squamous cell carcinoma. Consistent with previous studies, we found that the loss of CAV-1 expression in the stroma is a strong and independent predictor of a poor prognosis in CRC. In our cohort, the patients with high levels of stromal CAV-1 had a better prognosis, with 89.8% surviving the follow-up period. Similarly, 77.4% of the patients with moderate stromal CAV-1 staining survived. In contrast, 46.9% of the patients who were negative for stromal CAV-1 expression survived. We found a similar pattern for the 5-year survival rates. CRC patients with high stromal CAV-1 had a good (92%) 5-year survival rate. In contrast, CRC patients with moderate levels or absent CAV-1 expression in the stroma had progressively worse 5-year survival rates (61% and 46%, respectively). These results suggest that stromal CAV-1 expression could be used as an important prognostic factor for CRC patients.

In accordance with previous studies, we also found that the absence of CAV-1 expression in the stroma was significantly associated with early tumor recurrence[17,20]. Patients who had lower stromal CAV-1 expression were more likely to experience tumor recurrence. This finding highlights the role of the tumor stroma in determining disease recurrence in CRC patients. Therefore, new drugs that target the tumor stroma may have unexpected effects in CRC therapies.

We also found that the loss of stromal CAV-1 expression was significantly associated with lymph node invasion. Thus, the stromal CAV-1 status may serve as a predictor of lymph node invasion. CAV-1 expression in the stroma of a biopsy sample may also be useful in deciding whether endoscopic surgery should be performed for patients with early CRC. Moreover, considering that the loss of CAV-1 expression in the stroma may predict a high risk of lymph node invasion, it would also be informative when choosing a postoperative therapy for patients with no lymph node invasion.

The role of CAV-1 in tumor cells is controversial. Steffens *et al*[14] reported that low CAV-1 expression in renal cell carcinoma predicts a good clinical outcome compared with patients with high CAV-1 expression. However, El-Gendi *et al*[15] reported that CAV-1 expression in tumor cells has no prognostic value. Additionally, Friedrich *et al*[22] reported that CAV-1 deficiency in Apc (min/+) mice facilitates colorectal initiation. Luo *et al*[23] suggested that CAV-1 could be a biomarker of early-onset CRC. In our study, we also examined CAV-1 expression in epithelial tumor cells in the same patients. However, we only found 6 patients with positive tumor cell expression, and our results revealed no significant correlation between tumor cell CAV-1 and clinical characteristics or prognosis. The discrepancy could be explained by the tumor specificity of CAV-1 expression. Additionally, variation in scoring methods could explain some of the observed discrepancies.

Although the role of CAV-1 in the tumor stroma is not fully understood, recent findings support the hypothesis that CAV-1 plays a crucial role in the stroma. Koleske *et al*[24] were the first to find that CAV-1 functions as a tumor suppressor in fibroblasts. The reduction of CAV-1 levels by the constitutive activation of oncogenes, such as c-Myc, v-Src, H-Ras (G12V), and Neu/ErB2, can significantly promote tumor growth[25-27]. The tumor suppressor p53 can also transcriptionally regulate stromal CAV-1 expression and p53 inactivation induces CAV-1 down-regulation and promotes tumor progression[28]. In a xenograft model, CAV-1-deficient cancer-associated fibroblasts were found to promote both tumor growth and angiogenesis[29]. Several studies have found that the loss of CAV-1 in fibroblasts is sufficient to induce the conversion of benign stromal fibroblasts to tumor-associated fibroblasts *via* the TGF-β pathway[30,31]. In turn, CAFs promote tumor initiation, progression, and prevent cancer cell apoptosis. Recently, Pavlides *et al*[32] proposed a new tumor metabolism model, the Reverse Warburg Effect. In this model, the loss of CAV-1 causes stromal CAFs to undergo autophagy and aerobic glycolysis. As a consequence, the CAFs secrete energy-rich metabolites and chemical building blocks to adjacent tumor cells to prompt growth. The results of the present study support this hypothesis and indicate that the loss of CAV-1 expression in CAFs plays an important role in supporting tumor growth.

There are several limitations to this study. This research is a retrospective study, and there are limitations to any retrospective data collection, which is prone to bias. The relatively small sample size and the reliability of immunohistochemical techniques are other limitations.

In conclusion, our results suggest that the loss of CAV-1 expression in the stroma could be a predictor of poor clinical outcomes. As such, stromal CAV-1 levels could be used as a valuable biomarker for stratifying CRC patients into high-risk and low-risk groups at diagnosis; this information could be used to provide a more personalized approach to postoperative therapy. Further studies are needed to elucidate the mechanisms of stromal CAV-1 reduction and the tumor-stroma cross-talk that is crucial for tumor growth and metastasis.

COMMENTS

Background

Caveolin-1 (CAV-1), an essential structural protein of the endocytic caveolae plasma membrane, plays a major role in modulating tumorigenic processes. Recent studies have revealed that the loss of stromal CAV-1 results in an

activated tumor microenvironment and is significantly related to early tumor recurrence, metastasis, and poor prognosis in breast cancer, prostate cancer, and other solid tumors. However, the association between CAV-1 and colorectal cancer (CRC) remains unknown.

Research frontiers

Tumors are composed of tumor cells and stromal cells. Stromal cells have recently been recognized to play an important role in determining tumor initiation and progression. Under normal physiological conditions, the stroma serves as a critical barrier to prevent malignant transformation. However, during neoplastic tumorigenesis, the stroma facilitates tumor progression and metastasis in response to molecular signals derived from carcinoma cells and other host cell types. CAV-1, an essential structural protein of endocytic caveolae plasma membrane invaginations, is present in most mammalian cells, such as adipocytes, endothelial cells, fibroblasts, and smooth muscle cells. CAV-1 plays a major role in modulating tumorigenic processes. Recent studies have revealed that the loss of CAV-1 in the tumor stroma results in an activated tumor microenvironment and is significantly related to early tumor recurrence, metastasis, and poor clinical outcome in breast cancer, prostate cancer and other solid tumors.

Innovations and breakthroughs

This study is the first to report CAV-1 expression in both tumor and stromal cells of colorectal cancer. Moreover, our results demonstrate that the loss of CAV-1 expression in the stroma is a strong and independent predictor of poor overall survival and disease-free survival, whereas CAV-1 expression in tumor cells has no prognostic value. Additionally, CAV-1 expression in the stroma was closely associated with histological type, pTNM stage, pN stage and recurrence. Conversely, CAV-1 expression in tumor cells did not show any correlation with clinical parameters.

Applications

The results suggest that stromal CAV-1 levels could be used as a valuable biomarker for stratifying CRC patients into high-risk and low-risk groups at diagnosis; this information could be used to provide a more personalized approach to postoperative therapy.

Terminology

The stroma is a component of tumors. During neoplastic tumorigenesis, the stroma facilitates tumor progression and metastasis. Caveolin-1, an essential structural protein of endocytic caveolar plasma membrane invaginations, is present in most stromal cells.

Peer review

In the manuscript "Loss of stromal caveolin-1 expression in colorectal cancer predicts poor survival", the authors found loss of stromal CAV-1 was closely associated with histological type, pathologic TNM stage, pathologic N stage and recurrence in colorectal cancer. The higher stromal CAV-1 expression, the better patients' survival. It indicates that CAV-1 could be a prognostic factor for patients with CRC.

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ORIGINAL ARTICLE

Basic Study

β-escin reverses multidrug resistance through inhibition of the GSK3β/β-catenin pathway in cholangiocarcinoma

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Abstract

AIM: To develop a safe and effective agent for cholangiocarcinoma (CCA) chemotherapy.

METHODS: A drug combination experiment was conducted to determine the effects of β-escin in combination with chemotherapy on CCA cells. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay was performed to determine the effects of β-escin and common chemotherapeutics on the proliferation of human CCA cells (QBC939, Sk-ChA-1, and MZ-ChA-1). Immunocytochemistry was used to detect the expression of P-glycoprotein (P-gp) protein. Luciferase reporter assay was used to detect the activation of the Wnt/β-catenin pathway. The protein levels of P-gp, pS9-GSK3β, pT216-GSK3β, GSK3β, β-catenin, and p-β-catenin were further confirmed by western blotting.

RESULTS: The drug sensitivity of QBC939 and QBC939/5-fluorouracil (5-FU) cells to 5-FU, vincristine sulfate (VCR), or mitomycin C was significantly enhanced by β-escin compared with either agent alone ($P < 0.05$). In addition, the combination of β-escin (20 μmol/L) with 5-FU and VCR was synergic with a combination index < 1. Further investigation found that the mRNA and protein expression of P-gp was down-regulated by β-escin. Moreover, β-escin induced GSK3β phosphorylation at Tyr-216 and dephosphorylation at Ser-9, resulting in phosphorylation and degradation of β-catenin. Interestingly, activation of the GSK3β/β-catenin pathway induced by Wnt3a resulted in up-regulation of P-gp, which was effectively abolished by β-escin, indicating that β-escin down-regulated P-gp expression in a GSK3β-dependent manner.

CONCLUSION: β-escin was a potent reverser of P-gp-dependent multidrug resistance, with said effect likely being achieved *via* inhibition of the GSK3β/β-catenin pathway and thus suggesting a promising strategy of developing combination drugs for CCA.

Key words: β-escin; Multi-drug resistance; P-glycoprotein; GSK3β; Cholangiocarcinoma

Core tip: In our study, we received interesting and challenging results concerning the role of β-escin in reversing the multidrug resistance of cholangiocarcinoma (CCA). β-escin could enhance drug sensitivity of cholangiocarcinoma cells to common chemotherapeutics. 5-Fluorouracil, vincristine sulfate, or mitomycin C significantly reduced cell proliferation when combined with β-escin. In the molecular study, we found that β-escin could down-regulate P-gp expression *via* inhibiting the activation of GSK3β/β-catenin pathways. This study might offer a possible molecular basis for the further development of combinations of β-escin with common agents as a novel therapeutic approach for multidrug resistant CCA patients.

Huang GL, Shen DY, Cai CF, Zhang QY, Ren HY, Chen QX. β-escin reverses multidrug resistance through inhibition of the GSK3β/β-catenin pathway in cholangiocarcinoma. *World J Gastroenterol* 2015; 21(4): 1148-1157 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1148.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1148

INTRODUCTION

Cholangiocarcinoma (CCA) is the second most common primary hepatobiliary cancer, and originates from the biliary epithelium[1]. Incidence and mortality rates of CCA have been increasing worldwide over time[2], with the incidence rising by 22% between 1979 and 2004, accompanied with a 39% increase in mortality[3]. Surgical resection is the best available and potentially curative therapy for CCA[4]. However, it is difficult to make an early diagnosis of CCA patients[5], as most patients with CCA are not resectable at the time of diagnosis[6], and the results of surgical resection tend to be disappointing due to recurrence[7]. Hence, there is an urgent need for effective therapeutic strategies and drugs to combat this lethal tumor.

The problem of multidrug resistance (MDR), which affects a superfamily of ATP-dependent transporters, is a major obstacle to successful cancer therapy. One of the main reasons for MDR is the over-expression of the membrane pump, an active efflux pump affecting the pharmacokinetic profiles of drugs in cancer cells[8]. P-glycoprotein (P-gp) has been implicated in MDR in the pathogenesis of CCA[9]. Recent evidence that inhibition of P-gp could lead to an avoidance of drug resistance and the elimination of tumor cells[10] underscores the importance of the identification of different potential P-gp-mediated MDR reversal agents (or chemosensitizers) which may lead to strategies for developing improved target-based drugs for CCA therapy. P-gp modulators that have thus been intensively studied as prospective MDR reversers.

The ongoing search for P-gp inhibitors, applied in combination with anticancer drugs, is urgent. Plant-based agents capable of inhibiting P-gp with minimal adverse side effects are being increasingly utilized in drug discovery and development programs[11]. β-escin, the major active compound in extracts from the seeds of the horse chestnut (*Aesculus hippocastanum*), has shown clinically significant anti-inflammatory activity[12], as well as inhibitory effects on colon cancer, lung cancer, leukemia, CCA, and hepatocellular carcinoma[13-17]. β-escin has received extensive attention because of its potent induction of apoptosis and inhibition of cancer cell growth[13]. Several studies have elucidated that β-escin has a synergistic interaction with chemotherapeutics on cancer cells, such as the synergistic effects of β-escin and 5-FU on human hepatocellular carcinoma[18], β-escin potentiating the antitumor activity of gemcitabine on pancreatic cancer[19], and the synergistic effect of combinations of β-escin and the monoterpenes alpha-pinene, thymol, menthol in HeLa, and Cos7 cells[20]. Compelling evidence has spurred on efforts to search for the mechanisms by which β-escin regulates diverse biological functions.

In this study, we sought to investigate the mechanisms of β-escin that improve the sensitivity of CCA cells to common chemotherapeutics. We found that β-escin suppressed P-gp expression *via* the inhibition of the GSK3β/β-catenin pathways. Our results may provide a reference for gaining additional insight into the potential synergistic effects of β-escin in combination with chemotherapeutics on CCA cells, and to find a better method for the further development of anticancer drugs.

MATERIALS AND METHODS

Reagents and antibodies

Vincristine sulfate (VCR), 5-fluorouracil (5-FU), mitomycin C (MMC), cisplatin (CDDP), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and Wnt3a were purchased from Sigma-Aldrich (Indianapolis, IN, United States). β-escin was purchased from Aladdin Chemistry Co (Shanghai, China). RPMI-1640 medium and fetal calf serum were purchased from Gibco (Grand Island, NY, United States). Monoclonal antibodies against β-catenin, p-β-

catenin, P-gp, and β-actin were purchased from Santa Cruz Biotechnology (San Jose, CA, United States). Polyclonal GSK3β, pS9-GSK3β, and pT216- GSK3β antibodies were from Abcam Ltd (Cambridge, United Kingdom). Goat anti-rabbit and anti-mouse secondary antibodies conjugated to horseradish peroxidase were purchased from Thermo Scientific Pierce Co. Ltd (Rockford, IL, United States). Polyvinylidene difluoride (PVDF) membranes were from Millipore (Billerica, MA, United States). EliVision Plus Kit was from Maixin Bio (Fuzhou, China). Dual-Luciferase Reporter Assay Kit was from Promega (Madison, WI, United States).

Cell culture and treatment

The human CCA cell line QBC939 was kindly provided by Professor Shu-Guang Wang from Southwest Hospital, Third Military Medical University, Chongqing, China. The human cholangiocarcinoma MDR cell line QBC939/5-FU was established in our lab. The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL of ampicillin, and 100 U/mL of streptomycin sulfate at 37 ℃ in a humidified atmosphere under 5% CO_2. The cells were treated with culture medium containing various concentrations of drugs after 24 h seeding.

Cell proliferation assay

Cell proliferation was analyzed by MTT method. The cells were seeded at 5 × 10^3 per well into 96-well plates overnight. After treatment of the cells with a series of concentrations of 5-FU, CDDP, VCR, MMC, and β-escin, alone or in combination for 24 h, 20 μL MTT (5 mg/mL) was added to each well, and the cells were cultured for another 4 h at 37 ℃. Formazan crystals were then were dissolved in DMSO. The absorbance was measured at 490 nm using an ELISA microplate reader. The experiment was performed in triplicate.

Drug combination experiment

Combination effect was analyzed using the combination index (CI) method. The CI was defined by the following equation: CI= $(OD490)_{AB}/[(OD490)_A + (OD490)_B]$, where $(OD490)_{AB}$ was the absorbance of the drug A and B combined treated group, whereas $(OD490)_A$ and $(OD490)_B$ were that of the drug A or B alone treated group. Parameter CI values > 1 indicated antagonism, CI values = 1 indicated additivity, CI values < 1 indicated synergy, and CI values < 0.7 indicated significant synergy. Each represented CI ratio was the mean value derived from at least three independent experiments.

qPCR

Total RNA was extracted and reverse transcribed. The PCR primers are as follows:

MRP1, forward primer ATCTCTCCCGACATGACCGA, reverse primer CACACACTAGGGCTACCAGC; MRP2, forward primer CTGCCACTTTGTTTTGAGCA, reverse primer TACAAGGGCCAGCTCTATGG; MRP3, forward primer CTCCAAGTTCTGGGACTCCA, reverse primer CAGGTGGGAGAGGATGATGT; P-gp, forward primer GACATCCCAGTGCTTCAGG, reverse primer GCCACTGAACATTCAGTCG; GAPDH, forward primer CACATGGCCTCCAAGGAGTAAG, reverse primer TGAGGGTCTCTCTCTTCCTCTTGT. Real-time PCR was performed using a fluorescent temperature cycler (LC480 Real Time PCR System, Roche Co., Ltd). GAPDH was used as an internal standard.

Western blot analysis

Cell lysates containing equal amounts of proteins were separated on 10% SDS-polyacrylamide gels and electrotransferred onto PVDF membranes, which were blocked in 5% milk in PBST (NaCl 137 mmol/L, KCl 2.7 mmol/L, Na_2HPO_4 10 mmol/L, KH_2PO_4 2 mmol/L, and 0.05% Tween-20) for 1 h, with the primary indicated antibody then being incubated overnight at 4 ℃. After three washes in PBST, blots were incubated with horseradish peroxidase-conjugated secondary antibody and visualized by chemiluminescence. β-Actin was used as an internal control.

Luciferase reporter assay

QBC939 cells and QBC939/5-FU cells (1.0 × 10^5 cells/well) were seeded in 24-well plates and incubated overnight before transfection. The cells were co-transfected with reporter plasmid 200 ng pTOP-Flash or pFOP-Flash and 200 ng β-galactosidase (β-gal) using Lipofectamine 2000. TCF-responsive TOP-Flash reporter contains three TCF binding sites and the corresponding FOP-Flash contains three mutated TCF sites[21]. The indicated cells treated with β-escin (20 μmol/L) and Wnt3a (50 ng/mL) alone or together for 4 h were analyzed for luciferase activity using a Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-gal activity. The experiment was performed in triplicate.

Immunocytochemistry

After overnight culture, QBC939 and QBC939/5-FU cells were treated with 20 μmol/L β-escin for 0.5 h, fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 20 min, and then incubated with primary P-gp antibody overnight at 4 ℃, followed by incubation with horseradish peroxidase-conjugated secondary antibody for 30 min at room temperature. Images were collected and analyzed using an inverted fluorescence microscope (Leica, Barcelona, Spain). The experiment was performed in triplicate.

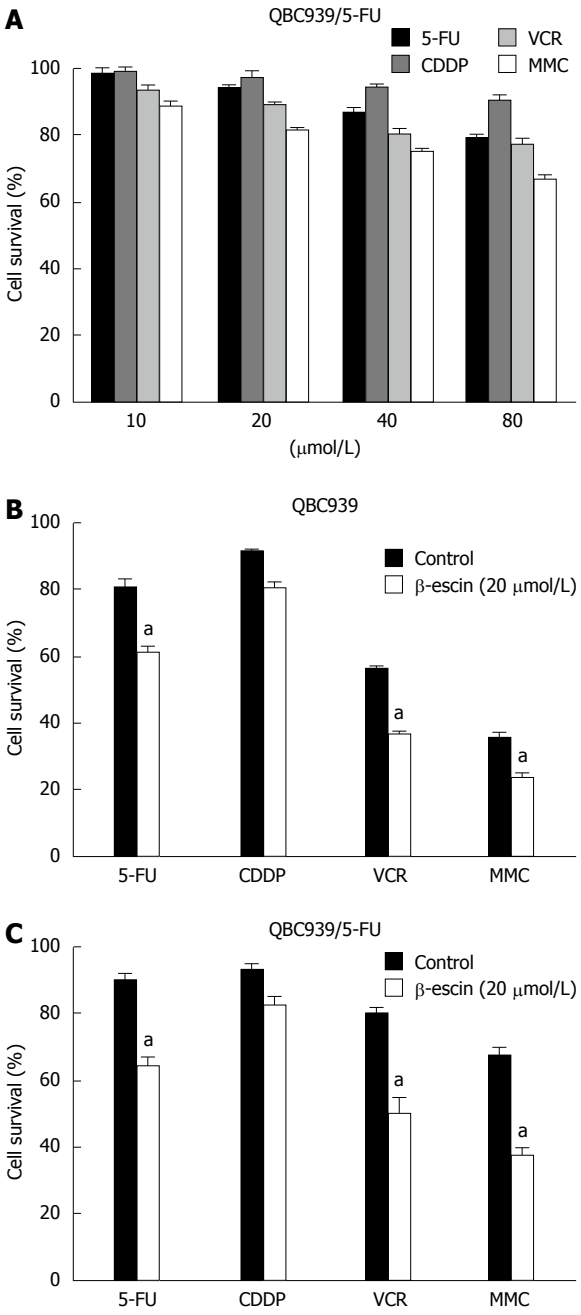

Figure 1 β-escin enhanced the sensitivity of cholangiocarcinoma cells to chemotherapeutic drugs. A: MDR cell line QBC939/5-FU cells were treated with different concentrations of 5-FU, CDDP, VCR, and MMC for 24 h. Cell viability measured by MTT assays; B-C: Effects of β-escin (20 μmol/L) in combination with chemotherapeutics (40 μmol/L) on QBC939 cells and QBC939/5-FU cells. Cell viability measured by MTT assays after treatment for 24 h. $^{a}P < 0.05$ *vs* control. MDR: Multidrug resistance; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; 5-FU: 5-fluorouracil; VCR: Vincristine sulfate; MMC: Mitomycin C; CDDP: Cisplatin.

Statistical analysis

SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, United States) was used for statistical analysis. The data were expressed as the mean ± SEM for samples and evaluated by one-way analysis of variance or Kruskal-Wallis tests to compare mean values. Each assay was repeated in triplicate. The level of significance was set at $P < 0.05$.

RESULTS

Effect of β-escin in combination with chemotherapeutics on CCA cells

To explore the mechanisms of MDR in CCA, we established a human MDR CCA cell line QBC939/5-FU[22]. The MTT assay was performed after exposure of QBC939/5-FU cells to a series of concentrations of 5-FU, CDDP, VCR, and MMC. MDR tumor cells QBC939/5-FU presented resistant to all chemotherapeutics (Figure 1A). To identify effective treatment for human CCA, our findings showed that β-escin at a concentration of 20 μmol/L showed a significant synergistic effect in combination with 5-FU, VCR, and MMC on CCA cells ($P < 0.05$) (Figures 1B and C). The effects of β-escin in combination with 5-FU and VCR on QBC939 and QBC939/5-FU cells are further detailed in Tables 1 and 2. The 50% inhibition concentrations (IC50) of QBC939 cells to 5-FU in combination with β-escin (0, 5, 10, and 20 μmol/L) were 114 ± 2.17, 101 ± 1.14, 96 ± 1.54, and 31 ± 1.89 μmol/L, respectively (Table 1). Similarly, the IC50 of QBC939/5-FU cells to 5-FU were 135 ± 2.59, 130 ± 2.75, 99 ± 2.96, and 37 ± 1.32 μmol/L, respectively (Table 1). Significantly, the IC50 of 5-FU in QBC939 and QBC939/5-FU cells was decreased, especially when combined with β-escin (20 μmol/L). Similar results for VCR were showed in Table 2. The IC50 of QBC939 and QBC939/5-FU cells to VCR were 102 ± 1.83, 88 ± 2.47, 72 ± 1.53, 18 ± 1.25 μmol/L, 125 ± 2.37, 104 ± 1.59, 82 ± 2.57, and 35 ± 1.36 μmol/L, respectively. These results indicate that drug sensitivity of QBC939 and QBC939/5-FU cells to 5-FU and VCR was enhanced by β-escin.

CI analysis was performed to determine the effect of β-escin in combination with 5-FU or VCR. The results of β-escin in combination with 5-FU or VCR on QBC939 and QBC939/5-FU cells are showed in Tables 1 and 2. The CIs of β-escin (20 μmol/L) in combination with 5-FU (20, 40, 80 μmol/L) on QBC939 and QBC939/5-FU cells were 0.992, 0.907, 0.768, 0.793, 0.693, and 0.507, respectively, indicating synergistic interactions ($0.7 < CI < 1$) and significantly synergistic interactions ($CI < 0.7$). However, the CIs of β-escin (5, 10 μmol/L) were mostly greater than 1, which showed an antagonistic interaction (Table 1). Likewise, the effects of β-escin (20 μmol/L) in combination with VCR were also synergistic interactions and significantly synergistic interactions, which were better than that of β-escin (5, 10 μmol/L) (Table 2). Together, these results demonstrated that a combination of β-escin with chemotherapeutics might be an effective therapy for CCA.

Down-regulation of P-gp mRNA and protein expression induced by β-escin

To investigate the mechanisms that cause β-escin

Table 1 Effect of β-escin in combination with 5-fluorouracil on QBC939 and QBC939/5-fluorouracil cells

β-escin (μmol/L)	5-FU (μmol/L)	QBC939			QBC939/5-FU		
		A490	CI	IC50	A490	CI	IC50
0	0	1.058 ± 0.073			0.998 ± 0.063		
5	0	1.003 ± 0.112			0.992 ± 0.091		
10	0	0.943 ± 0.088			0.894 ± 0.072		
20	0	0.658 ± 0.033			0.653 ± 0.027		
0	20	0.964 ± 0.023			0.997 ± 0.051		
0	40	0.872 ± 0.067		114 ± 2.17	0.952 ± 0.071		135 ± 2.59
0	80	0.812 ± 0.089			0.882 ± 0.099		
5	20	0.932 ± 0.014	1.019[1]		0.989 ± 0.031	1.000[2]	
5	40	0.852 ± 0.082	1.031[1]	101 ± 1.14	0.901 ± 0.062	0.952[3]	130 ± 2.75
5	80	0.798 ± 0.041	1.037[1]		0.797 ± 0.026	0.909[3]	
10	20	0.892 ± 0.066	1.038[1]		0.851 ± 0.045	0.953[3]	
10	40	0.802 ± 0.072	1.031[1]	96 ± 1.54	0.747 ± 0.036	0.876[3]	99 ± 2.96
10	80	0.724 ± 0.018	1.000[2]		0.657 ± 0.031	0.832[3]	
20	20	0.595 ± 0.062	0.992[3]		0.517 ± 0.041	0.793[3]	
20	40	0.492 ± 0.041	0.907[3]	31 ± 1.89	0.452 ± 0.020	0.693[4]	37 ± 1.32
20	80	0.388 ± 0.024	0.768[3]		0.331 ± 0.015	0.507[4]	

[1]Antagonism, CI > 1; [2]Additivity, CI = 1; [3]Synergy, CI < 1; [4]Significant synergy, CI < 0.7. 5-FU: 5-fluorouraci.

Table 2 Effect of β-escin in combination with vincristine sulfate on QBC939 and QBC939/5-fluorouracil cells

β-escin (μmol/L)	VCR (μmol/L)	QBC939			QBC939/5-FU		
		A490	CI	IC50	A490	CI	IC50
0	0	1.112 ± 0.093			1.005 ± 0.042		
5	0	0.989 ± 0.089			0.988 ± 0.086		
10	0	0.902 ± 0.064			0.933 ± 0.067		
20	0	0.674 ± 0.044			0.712 ± 0.044		
0	20	0.922 ± 0.071			0.992 ± 0.052		
0	40	0.752 ± 0.053		102 ± 1.83	0.892 ± 0.035		125 ± 2.37
0	80	0.689 ± 0.032			0.743 ± 0.028		
5	20	0.901 ± 0.045	1.098[1]		0.985 ± 0.035	1.010[1]	
5	40	0.687 ± 0.052	1.027[1]	88 ± 2.47	0.851 ± 0.022	0.961[3]	104 ± 1.59
5	80	0.614 ± 0.042	1.002[1]		0.698 ± 0.017	0.946[3]	
10	20	0.815 ± 0.036	1.089[1]		0.901 ± 0.043	0.978[3]	
10	40	0.631 ± 0.026	1.034[1]	72 ± 1.53	0.784 ± 0.035	0.946[3]	82 ± 2.57
10	80	0.556 ± 0.024	0.995[3]		0.602 ± 0.011	0.872[3]	
20	20	0.559 ± 0.025	1.000[2]		0.605 ± 0.021	0.861[3]	
20	40	0.368 ± 0.011	0.807[3]	18 ± 1.25	0.499 ± 0.009	0.789[3]	35 ± 1.36
20	80	0.289 ± 0.008	0.692[4]		0.326 ± 0.010	0.619[4]	

[1]Antagonism, CI > 1; [2]Additivity, CI = 1; [3]Synergy, CI < 1; [4]Significant synergy, CI < 0.7. VCR: Vincristine sulfate.

to enhance the drug sensitivity of CCA cells to common chemotherapeutics, real-time PCR, immunocytochemistry (ICC), and western blot were performed to assess the expression of MDR-associated genes in QBC939 and QBC939/5-FU cells after treatment with various concentrations of β-escin (0, 5, 10, and 20 μmol/L) for 24 h. As shown in Figure 2A, there was no significant change in MRP1, MRP2, or MRP3 mRNA expression after β-escin treatment, but a significant decrease was found in P-gp mRNA expression (Figure 2B). In addition, results of ICC show that β-escin reduced the expression of P-gp in a dose-dependent manner (Figure 2C) consistent with the suppressed protein expression detected by western blot (Figure 2D). These results indicated that β-escin could down-regulate the expression of P-gp in QBC939 and QBC939/5-FU cells.

Down-regulation of P-gp induced by β-escin via inhibition of GSK3β/β-catenin pathway

We recently reported that P-gp might be a downstream target gene of the Wnt/β-catenin pathway in CCA cells[23], so we evaluated whether down-regulation of P-gp induced by β-escin was associated with the inhibition of said pathway. Luciferase reporter assays showed that activation of the Wnt/β-catenin pathway was remarkably enhanced by Wnt3a treatment and significantly reduced by β-escin on both QBC939 and QBC939/5-FU cells. Moreover, Wnt3a-induced activation of the Wnt/β-catenin pathway was almost completely prevented by β-escin (Figure 3A). In an effort to study the precise regulation of β-escin on Wnt/β-catenin signaling, we

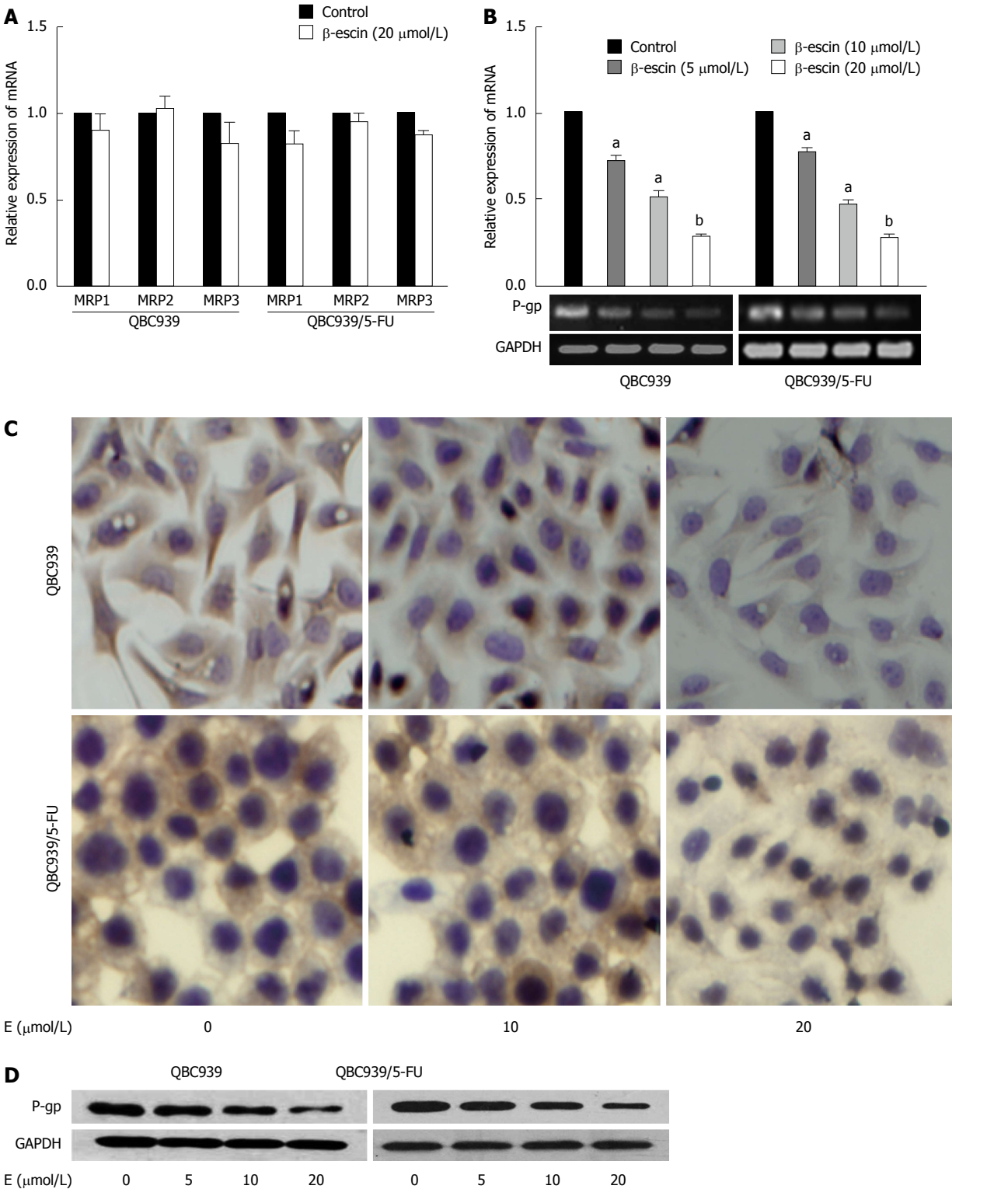

Figure 2 Expression of P-gp down-regulated by β-escin. A: Expression of MRP mRNA analyzed using real-time PCR after treatment with β-escin for 24 h; B: Expression of MDR mRNA analyzed using real-time PCR (upper panel) and agarose gel electrophoresis analysis (lower panel) after treatment with β-escin for 24 h. Data were normalized to GAPDH; C: Expression of P-gp protein detected by ICC. Zoom: × 400; D: Expression of P-gp protein detected by western blot. β-Actin used as loading control. E: β-escin. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. PCR: Polymerase chain reaction; MDR: Multidrug resistance; MRP: MDR-related protein; P-gp: P-glycoprotein; ICC: Immunocytochemistry.

examined the expression levels of related proteins in this pathway. As shown in Figure 3B, the levels of β-catenin phosphorylation and degradation were increased after β-escin treatment. In addition, the level of phosphorylation at Ser-9 was decreased and dephosphorylation at Tyr-216 was increased, indicating that β-escin inhibited the activation of the GSK3β/β-catenin pathway in both QBC939 and

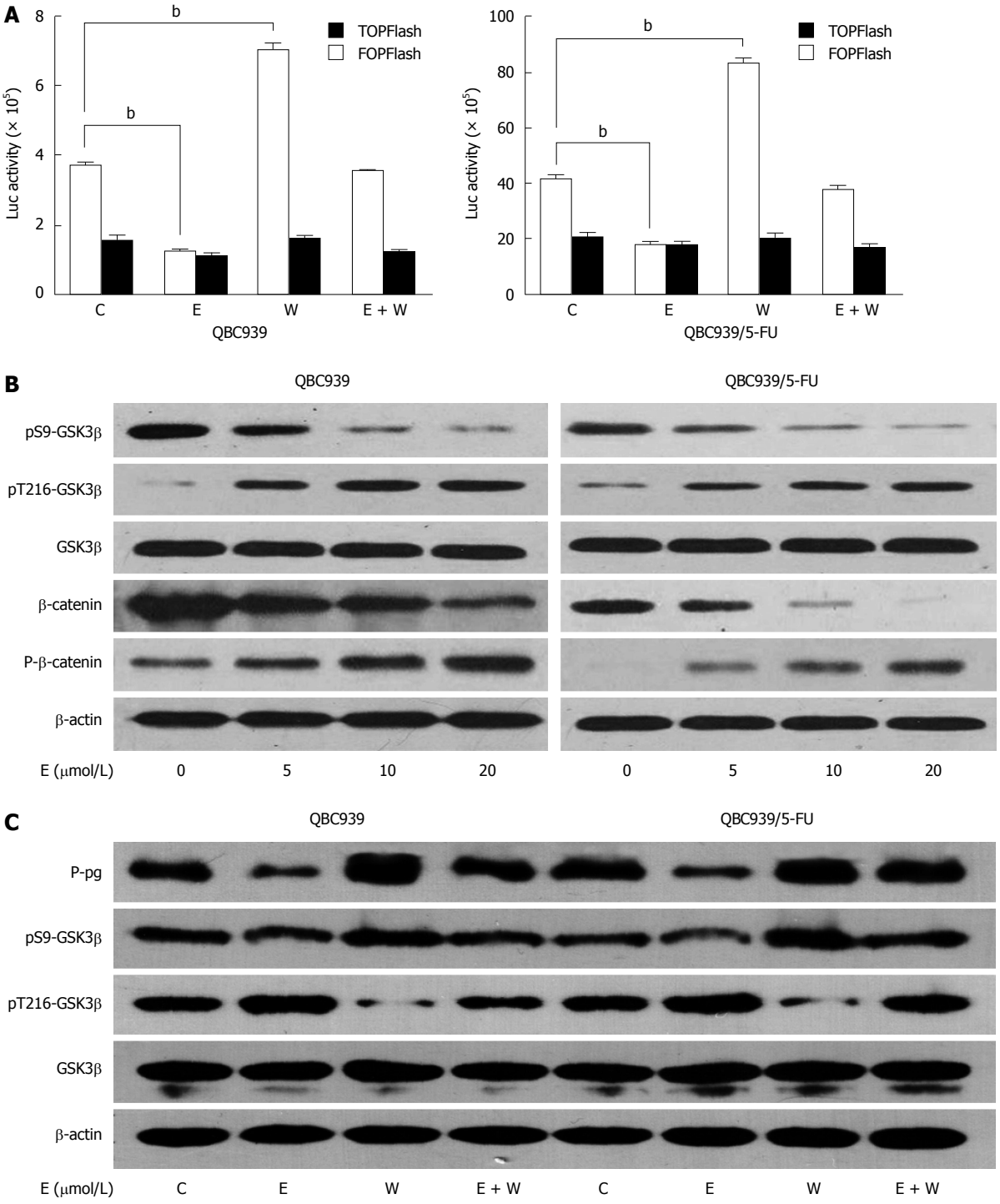

Figure 3 Down-regulation of P-glycoprotein induced by β-escin *via* inhibiting the activation of the GSK3β/β-catenin pathway. A: The activation of the Wnt/β-catenin pathway assessed by TCF/LEF responsive luciferase reporter activity in QBC939 and QBC939/5-FU cells after treatment with β-escin. Data were shown as the mean ± SD of three independent experiments; B: Western blot analysis of GSK3β and β-catenin expression in QBC939 and QBC939/5-FU cells treated with a series of concentrations of β-escin; C: Western blot analysis of GSK3β and P-gp in QBC939 and QBC939/5-FU cells after different treatments for 24 h. β-actin used as loading control. E: β-escin (20 μmol/L); W: Wnt3a (50 μg/L); C: Control (PBS). $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* control. TCF: T-cell factor; LEF: Lymphoid enhancer factor.

QBC939/5-FU cells.

We further studied whether β-escin-induced P-gp down-regulation was GSK3β dependent. Western blot analysis showed that β-escin led to phosphorylation in Tyr-216 of GSK3β and dephosphorylation in Ser-9 of GSK3β, as well as down-regulation of P-gp (Figure 3C). In contrast, Wnt3a led to phosphorylation in Ser-9, dephosphorylation in Tyr-216 of GSK3β, and up-regulation of P-gp. Interestingly, co-treatment of cells with β-escin almost completely suppressed the increase of P-gp induced by Wnt3a. Similar changes were seen in the protein expression of pS9-GSK3β with β-escin and Wnt3a treatment. These data suggested that β-escin-induced P-gp down-

regulation was GSK3β dependent.

DISCUSSION

An increase in basic research on CCA has been seen in recent years. However, there is still a notable lack of an effective targeted therapy for CCA patients because of MDR[7,22,24]. Overexpression of ATP-binding cassette transporters of the MDR protein and MDR-related protein (MRP) family in cancer cells is a major cause of multidrug resistance[24]. P-gp, intrinsically over-expressed in many neoplasms, including the majority of carcinomas arising in the colon, rectum, pancreas, liver, kidneys, and bile duct[22,24], is known to play a pivotal role in the development of MDR, which leads to the failure of chemotherapy in numerous cancers, including CCA[10]. Due to the complex pathological mechanisms of CCA, drug combination treatments have received extensive attention. In an effort to research a series of potent and efficacious P-gp-dependent MDR reversers, we have undertaken the screening of common chemotherapeutics, and found that β-escin combined with 5-FU, VCR, or MMC produced remarkable inhibitory effects in CCA cells. The IC50 of 5-FU and VCR in combination with β-escin were reduced significantly, showing a synergistic effect. Due to the combination of β-escin and other drugs (such as 5-FU and VCR) being particularly efficacious on CCA cells, we speculated that β-escin might decrease the expression of the tumor's drug-resistant protein P-gp, thus allowing another kind of drug to enter the cell and prevent the tumor from adapting its microenvironment. Furthermore, in agreement with our previous studies that P-gp expression was up-regulated in MDR CCA cell line QBC939/5-FU[22], we demonstrated that β-escin reversed MDR of human CCA cell line QBC939 and QBC939/5-FU at least partly *via* the down-regulation of P-gp.

Overcoming MDR is broadly known to be effective in cancer therapy. β-escin, obtained *via* its isolation from the seed of the horse chestnut[25], played an important role in circumventing drug resistance in drug combination on CCA cells. However, the molecular mechanisms of β-escin on down-regulating P-gp expression remain unclear. It has been confirmed that the PI3K/Akt, NF-κB, Wnt/β-catenin, and ERK signal pathways are associated with P-gp expression in cancer[26,27]. In our previous study, we found that the role of the Wnt/β-catenin pathway has been implicated in chemoresistance in CCA[22]. An important finding of the present study is that the Wnt/β-catenin pathway was involved in the β-escin-induced down-regulation of P-gp on CCA cells. Aberrant regulation of Wnt/β-catenin signaling has been shown to cause a wide spectrum of diseases, and especially tumors[28-31]. It is very clear that the activation of β-catenin through the canonical Wnt pathway plays a role in a number of human cancers, including CCA[23]. When stable, non-phosphorylated β-catenin migrates from the cytoplasm to the nucleus, where it interacts with T-cell factor/lymphoid enhancer factor that binds to the promoters of downstream target genes, recruiting transcriptional activators[30,32,33]. Our results showed that β-escin suppressed Wnt3a-induced β-catenin-mediated expression of P-gp. Therefore, β-escin might represent a lead for classes of anticancer agents targeting the Wnt/β-catenin pathway for CCA therapy.

GSK3β, a "destruction complex"[34] and key mediator of the Wnt/β-catenin pathway, suppresses tumor progression by down-regulating the Wnt survival pathway through the phosphorylation and degradation of β-catenin[35]. The phosphorylation in Ser-9 of GSK3β inhibits its activity[36]. In contrast to ser-9 phosphorylation, phosphorylation of GSK3β on Tyr-216 increases its activity[37]. Activated GSK3β phosphorylates and degrades β-catenin[35]. We found that β-escin activated GSK3β *via* phosphorylation of Tyr-216 and simultaneous dephosphorylation of Ser-9 and, paralleled with a decreasing accumulation of free cytoplasmic β-catenin, consequently led to the down-regulation of P-gp expression. Additionally, expression of P-gp was up-regulated after Wnt3a treatment, which is an activator of the GSK3β/β-catenin pathway. Conversely, down-regulation of P-gp expression induced by β-escin was consistent with the inhibition of the GSK3β/β-catenin pathway. Upregulation of P-gp expression induced by Wnt3a could be suppressed by β-escin, providing insight into the mechanism of GSK3β-mediated P-gp regulation.

Taken together, our study has demonstrated that β-escin can sensitize CCA cells to common chemotherapeutics, and also clarified that β-escin could inhibit P-gp expression through the inhibition of the GSK3β/β-catenin pathways. This study might offer a possible molecular basis for the further development of combinations of β-escin and common agents as a novel therapeutic approach for CCA patients.

COMMENTS

Background

Cholangiocarcinoma (CCA), a chemoresistant bile duct carcinoma which results in a poor prognosis, is resistant to all currently available chemotherapeutics because of its multidrug resistance (MDR). A safe and effective agent for CCA chemotherapy is therefore urgently needed.

Research frontiers

β-escin could enhance the drug sensitivity of cholangiocarcinoma cells to common chemotherapeutics. Furthermore, β-escin could down-regulate P-gp expression *via* inhibiting the activation of GSK3β/β-catenin pathways. The results may provide a reference for additional insight into the potential synergistic effects of β-escin in combination with chemotherapeutics on CCA cells.

Innovations and breakthroughs

Overcoming MDR is broadly known to be effective in cancer therapy. β-escin, isolated from the seed of the horse chestnut, plays an important

role in circumventing drug resistance in drug combination on CCA cells. An important finding of the present study is that the Wnt/β-catenin pathway was involved in the β-escin-induced down-regulation of P-gp on CCA cells. This study has demonstrated that β-escin can sensitize CCA cells to common chemotherapeutics, and also clarified that β-escin could inhibit P-gp expression through the inhibition of the GSK3β/β-catenin pathways.

Applications

β-escin was a potent reverser of P-gp-dependent MDR, and this effect was likely through the inhibition of the GSK3β/β-catenin pathway, suggesting a promising strategy of developing combination drugs for CCA.

Terminology

β-escin, the major active compound in extracts of the horse chestnut (*Aesculus hippocastanum*) seed, has shown clinically significant anti-inflammatory activity, as well as inhibitory effects on colon cancer. MDR belonging to a superfamily of ATP-dependent transporters, is the major obstacle to a successful cancer therapy.

Peer review

This is a comprehensive manuscript with fine structure which adequately highlights the role of β-escin in cholangiocarcinoma. To date, there is no sufficiently adequate study concerning this subject. Usually this disease is already at the advanced stage by the time of diagnosis, and thus responds poorly to chemotherapy. Genetic study of the tumor and research into inhibitors of pathways make up the future of targeted therapy.

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ORIGINAL ARTICLE

Case Control Study

Factors associated with significant liver fibrosis assessed using transient elastography in general population

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Abstract

AIM: To investigate the prevalence of significant liver fibrosis assessed using transient elastography (TE) and its predictors in asymptomatic general population.

METHODS: A total of 159 subjects without chronic viral hepatitis who underwent comprehensive medical health check-up between January 2012 and July 2012 were prospectively recruited. Significant liver fibrosis was defined as liver stiffness value > 7.0 kPa.

RESULTS: The mean age and body mass index (BMI) of the study population (men 54.7%) was 56.0 years and 24.3 kg/m^2. Among the study subjects, 11 (6.9%) showed significant liver fibrosis. On univariate analysis, BMI, alanine aminotransferase (ALT), homeostasis model assessment of insulin resistance, carotid intimal media thickness (IMT), number of calcified plaques on carotid ultrasound, and visceral fat area on computed tomography were significantly higher in subjects with significant liver fibrosis than in those without (all $P < 0.05$). However, on multivariate analysis, BMI [odds ratio (OR) =1.487; $P = 0.045$], ALT (OR = 1.078; $P = 0.014$), carotid IMT (OR = 3.244; $P = 0.027$), and the number of calcified carotid plaques (OR = 1.787; $P = 0.031$) were independent predictors of significant liver fibrosis.

CONCLUSION: The prevalence of significant liver fibrosis assessed using TE was 6.9% in apparently healthy subjects. High BMI, high ALT, thicker carotid IMT, and higher numbers of calcified carotid plaques

were independently associated with the presence of significant liver fibrosis.

Key words: Transient elastography; Healthy subjects; Fibroscan; Liver fibrosis; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Body mass index; Alanine aminotransferase; Carotid intimal medial thickness; Carotid artery plaque

Core tip: This is the first study which investigated the prevalence of significant liver fibrosis assessed using transient elastography and the correlation between comprehensive clinical metabolic parameters (body weight, visceral adiposity, insulin resistance, hepatic steatosis, and atherosclerosis) and the presence of significant liver fibrosis in asymptomatic general subjects. Finally, we found that the prevalence of significant liver fibrosis was fairly high (6.9%) and several factors including higher body mass index, higher alanine aminotransferase, thicker carotid intimal media thickness, and higher numbers of calcified carotid plaques on carotid sonography were significantly correlated to the risk of significant liver fibrosis.

You SC, Kim KJ, Kim SU, Kim BK, Park JY, Kim DY, Ahn SH, Lee WJ, Han KH. Factors associated with significant liver fibrosis assessed using transient elastography in general population. *World J Gastroenterol* 2015; 21(4): 1158-1166 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1158.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1158

INTRODUCTION

Along with tremendous progress in antiviral agents and treatment strategies, a vigorous national vaccination program has resulted in a gradually decreasing prevalence of chronic viral hepatitis. Despite this, the socioeconomic cost for managing chronic liver diseases (CLDs) is continuously increasing every year in South Korea[1]. This phenomenon is attributed to other CLDs, particularly non-alcoholic fatty liver disease (NAFLD), which has an estimated prevalence of up to 45% in the Asian population[2].

The prognosis of CLDs, including chronic viral hepatitis and NAFLD, generally depends on the severity of liver fibrosis. However, a non-negligible fraction of patients with CLDs, especially NAFLD, remain asymptomatic and even show normal liver function tests[3,4]. This makes it difficult to prevent the progression of liver fibrosis to liver cirrhosis and hepatocellular carcinoma. Thus, accurate and timely determination of significant liver fibrosis is important to prevent progression of liver fibrosis in patients with CLDs and this effective screening will eventually reduce medical costs and improve prognosis.

Although liver biopsy has been regarded as the gold standard for detecting liver fibrosis, it is innately invasive and can lead to mortality, misdiagnosis due to interpretational variability, and sampling errors due to uneven fibrosis distribution within the liver parenchyma[5]. Recently, liver stiffness (LS) measurement using transient elastography (TE) has emerged as a promising noninvasive tool for assessing the degree of liver fibrosis. This technique is non-invasive, accurate, reproducible, convenient, and useful for serial measurement in various CLDs[6-9]. However, few studies have investigated TE for use identifying asymptomatic subjects with significant liver fibrosis by screening the population without overt liver diseases[10,11].

Hence, this study investigated whether TE can screen asymptomatic general population without history of chronic viral hepatitis who received comprehensive medical health check-ups and identify subjects with significant liver fibrosis. In addition, we examined which factors other than chronic viral hepatitis are associated with the presence of significant liver fibrosis in this study population.

MATERIALS AND METHODS

Patients

This study prospectively and consecutively included 190 apparently healthy subjects who underwent a comprehensive medical health check-up including TE examination in Severance Hospital, Yonsei University College of Medicine, Seoul, Korea, between January 2012 and July 2012.

According to the exclusion criteria (Figure 1), 31 patients were excluded; the remaining 159 patients were selected for statistical analysis. Five patients were excluded due to LS measurement failure (no valid shot) or unreliable LS value. An additional 26 patients were excluded due to (1) chronic hepatitis B or C (n = 4); (2) missing laboratory test data (n = 7); (3) missing carotid sonography data (n = 5); (4) missing fat computed tomography data (CT) (n = 8); (6) right-sided heart failure (n = 0); or (7) lack of informed consent (n = 2). Baseline characteristics of the 31 excluded subjects were not statistically different from the 159 subjects included in this study (all $P > 0.05$, data not shown).

The study was performed in accordance with the ethical guidelines of the 1975 Declaration of Helsinki. Written informed consent was obtained from each participant or a responsible family member. This study was approved by the Institutional Review Board of Severance Hospital.

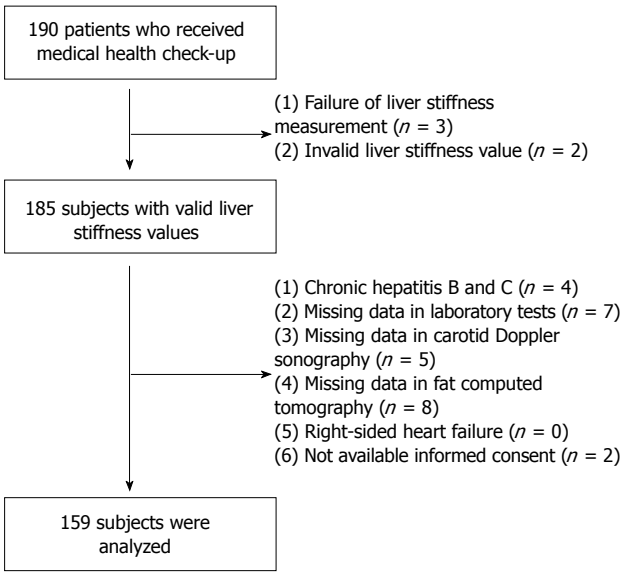

Figure 1 Recruitment algorithm. A total of 190 consecutive subjects who received full medical health check-ups were recruited. Five patients were excluded due to inappropriate liver stiffness measurements. Of 185 subjects with valid liver stiffness values, 26 were excluded based on exclusion criteria. A total of 159 subjects were selected for final statistical analysis.

Laboratory and imaging studies

In addition to demographic data, a comprehensive medical health check-up with laboratory and imaging studies was performed on the same day of LS measurement using TE. Aspartate aminotransferase (AST) level, alanine aminotransferase (ALT) level, platelet count, fasting plasma glucose (FPG), Homeostatsis Model Assessment of Insulin Resistance (HOMA-IR), and HbA1c were measured. HOMA-IR was calculated using the following formula: [(Fasting plasma insulin (μU/mL)*FPG (mg/dL))/405][12]. Imaging studies including fat CT and carotid sonography. Liver ultrasonography measured degree of hepatic steatosis as described previously[13].

Measurement of LS and controlled attenuation parameter

The principles of LS and controlled attenuation parameter (CAP) measurement using TE have been described previously[14,15]. TE was performed by one experienced technician (> 10000 examinations) blind to clinical subject data. TE results are expressed as kilopascals (kPa) for LS and dB/m for CAP. The interquartile range (IQR) was defined as an index of the intrinsic variability of LS and CAP values corresponding to the interval of LS and CAP results containing 50% of the valid measurements between the 25th and 75th percentiles. The median value of successful measurements was selected as representative of the LS and CAP values of a given patient. As an indicator of variability, the ratio of the IQR of LS and CAP values to the median values (IQR/M and IQR/M$_{CAP}$, respectively) was calculated. CAP was only calculated when the LS measurement was valid for the same signals, ensuring that liver ultrasonic attenuation was achieved simultaneously and in the same volume of liver parenchyma as the LS measurement.

In this study, only procedures with at least ten valid measurements, a success rate of at least 60%, and an IQR/M of LS value < 0.3 were considered reliable and used for statistical analysis. Because the influence of IQR/M$_{CAP}$ on the accuracy of CAP has not been fully validated, IQR/M$_{CAP}$ was not adopted as a determinant for invalid CAP values.

Definition of significant liver fibrosis

In this study, a cut-off LS value of 7.0 kPa was defined to identify significant liver fibrosis. This value is based on a previous study from South Korea that proposed a normal LS range of 3.7-7.0 kPa in men and 3.3-6.8 kPa in women[16] and a study from Hong Kong that proposed the optimal cut-off LS value as 7.0 kPa to diagnose significant liver fibrosis (≥ F2) in patients with NAFLD[17].

Statistical analysis

Results were expressed as mean ± SD, median (range), or *n* (%), as appropriate. The means or percentages of baseline characteristics between patients with and without significant fibrosis were compared using independent Student *t*-tests or Mann-Whitney *U* tests for continuous variables and χ^2 tests or Fisher's exact tests for categorical variables. Univariate and multivariate logistic regression analyses were performed for variables that were significantly different between groups with and without significant fibrosis. All statistical analyses were performed with standard procedures (SAS, version 18). Statistical significance was considered at $P < 0.05$.

RESULTS

Baseline characteristics

Baseline characteristics of the 159 study subjects are shown in Table 1. The mean age and body mass index (BMI) of the study population (87 men and 72 women) was 56.0 years and 24.3 kg/m^2, respectively. The prevalence of obesity, defined as BMI > 25 kg/m^2, was 41.5% (*n* = 66). Mean AST and ALT levels were 23.3 and 23.1 IU/L, respectively. On carotid sonography, mean intimal media thickness (IMT) was 0.75 mm. A carotid plaque was noted in 34 (21.4%) subjects, and the mean number of calcified carotid plaques was 0.48. Mean CAP and LS values were 248.3 dB/m and 4.7 kPa, respectively.

Distribution of LS values

LS values were statistically similar between genders (4.5 kPa in men and 4.8 kPa in women, $P = 0.360$).

Table 1 Baseline characteristics of study population (n = 159)

Variables	Values
Demographic variables	
Age, yr	56.0 ± 10.6
Male gender	87 (54.7)
Body mass index, kg/m^2	24.3 ± 3.1
Diabetes mellitus	19 (11.9)
Hypertension	40 (25.2)
Daily alcohol intake, mg	15.4 ± 30.5
Laboratory variables	
Aspartate aminotransferase, IU/L	23.3 ± 8.5
Alanine aminotransferase, IU/L	23.1 ± 12.2
Platelet count, 10^9/L	235.5 ± 57.9
Fasting plasma glucose, mg/dL	100.5 ± 24.5
HOMA-IR	1.84 ± 1.35
HbA1c, %	6.0 ± 0.7
Imaging variables	
Severe fatty liver on sonography	30 (18.9)
Carotid sonography	
Intimal media thickness, mm	0.75 ± 0.57
Presence of carotid plaque	34 (21.4)
Calcified carotid plaque, n	0.48 ± 1.13
Computed tomography	
Visceral fat area, cm^2	112.4 ± 54.8
Transient elastography	
Controlled attenuation parameter, dB/m	248.3 ± 44.4
Interquartile range$_{CAP}$, dB/m	35.91 ± 13.56
Interquartile range/median$_{CAP}$	0.16 ± 0.06
Liver stiffness value, kPa	4.7 ± 2.2
Interquartile range$_{LS}$, kPa	0.52 ± 0.21
Interquartile range/median$_{LS}$	0.13 ± 0.06

Values are expressed as the mean ± SD (range) or n (%). HOMA-IR: Homeostasis model assessment of insulin resistance; HbA1c: Glycated hemoglobin; CAP: Controlled attenuation parameter; LS: Liver stiffness.

Among the study subjects, most subjects (n = 60, 37.7%) had LS values of 4.0-4.9 kPa, whereas 11 (6.9%) subjects had LS values higher than 7 kPa, indicating the presence of significant liver fibrosis (Figure 2). Characteristics of patients with significant fibrosis are shown in Table 2, published online.

Factors associated with significant liver fibrosis

Various characteristics were compared between subjects with and without significant liver fibrosis (Table 3). BMI, ALT, HOMA-IR, carotid IMT, number of calcified carotid plaques on carotid sonography, and visceral fat area on CT were significantly higher in subjects with significant liver fibrosis than in those without (27.5 *vs* 24.1 kg/m^2; 32.3 *vs* 22.2 IU/L; 2.9 *vs* 1.7; 1.4 *vs* 0.7 mm; 1.5 *vs* 0.4, and 171.5 *vs* 107.2 cm^2, respectively; all P < 0.05). CAP value was elevated in patients with significant liver fibrosis but failed to show significant differences between the groups.

Independent factors associated with significant liver fibrosis

On multivariate logistic regression analysis using variables that were significant in univariate analysis (age, BMI, ALT, HOMA-IR, visceral fat area on CT, number of calcified carotid plaques, and carotid IMT), BMI [odds ratio (OR) = 1.487; 95% confidence interval (CI): 1.009-2.193; P = 0.045], ALT (OR = 1.078, 95%CI: 1.015-1.145; P = 0.014), carotid IMT (OR = 3.244, 95%CI: 1.140-9.234; P = 0.027), and number of calcified carotid plaques (OR = 1.787, 95%CI: 1.055-3.026; P = 0.031) were selected as independent predictors of significant liver fibrosis based on positive correlations (Table 4).

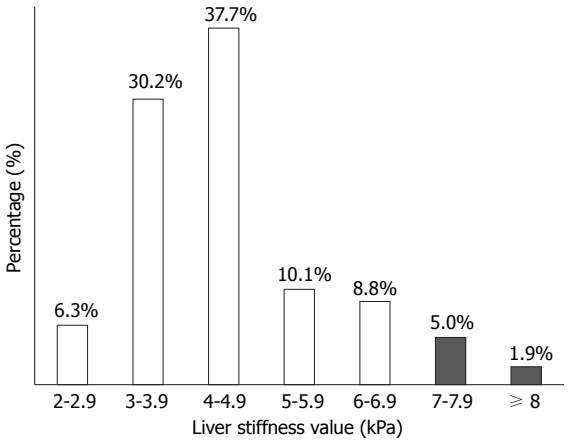

Figure 2 Distribution of liver stiffness values. Among study participants, significant liver fibrosis (> 7 kPa) was observed in 11 (6.9%).

Relative risk according to independent predictors

The study population was divided according to the medians of four independent factors (24.2 kg/m^2 for BMI, 19 IU/L for ALT, 0.68 mm for carotid IMT, and one or more calcified carotid plaques) to calculate the relative risks between groups (Figure 3). Significant liver fibrosis was observed in ten of 79 (12.7%) subjects with BMI ⩾ 24.2 kg/m^2, nine of 78 (11.5%) subjects with ALT ⩾ 19 IU/L, and ten of 79 (12.7%) subjects with carotid IMT ⩾ 0.68 mm. In contrast, significant liver fibrosis was noted in one of 80 (1.3%) subjects with BMI < 24.2 kg/m^2 (P = 0.005; OR = 11.4, 95%CI: 1.2-91.7), two of 81 (2.5%) subjects with ALT < 19 IU/L (P = 0.030; OR = 5.2, 95%CI: 1.1-24.7), and one of 80 (1.3%) subjects with carotid IMT < 0.68 mm (P = 0.005; OR = 11.4, 95%CI: 1.4-91.7). In addition, the prevalence of significant liver fibrosis tended to be higher in subjects with calcified carotid plaques than in those without [5/35 (14.3%) *vs* 6/124 (4.8%); P = 0.065].

DISCUSSION

Despite the increasing popularity and reliability of LS measurement using TE to assess the degree of liver fibrosis in subjects with CLDs, only a few Western studies have shown the applicability of screening the general population[10,11]. Thus, we

Table 2 Characteristics of patients with significant fibrosis

No.	Age (yr)	Gender	BMI (kg/m^2)	Alcohol intake (g/d)	ALT (IU/L)	CAP (dB/m)	Degree of fatty liver on US	LS value (kPa)
1	47	F	24.7	0	11.0	259	Normal	7.1
2	69	F	28.1	0	46.0	348	Moderate	7.1
3	52	F	25.9	23	43.0	212	Mild	7.2
4	77	M	27.9	0	13.0	185	Normal	7.6
5	53	M	30.2	29	70.0	324	Moderate	7.6
6	68	M	26.5	38	42.0	274	Mild	7.8
7	77	M	33.9	0	35.0	281	Moderate	7.8
8	56	M	23.1	11	22.0	258	Mild	7.9
9	52	M	30.3	5	41.0	287	Mild	8.0
10	67	M	25.7	0	24.0	259	Mild	14.3
11	77	F	26.6	0	44.0	247	Mild	25.7

BMI: Body mass index; ALT: Alanine aminotransferase; CAP: Controlled attenuation parameter; US: Ultrasound; LS: Liver stiffness.

Table 3 Comparison between patients with and without significant liver fibrosis (> 7 kPa)

Variables	Patients without significant fibrosis [*n* = 148 (93.1%), LS value ≤ 7 kPa]	Patients with significant fibrosis [*n* = 11 (6.9%), LS value > 7 kPa]	*P* value
Age, yr	55.5 ± 10.4	63.2 ± 11.5	NS
Male gender	80 (54.1)	7 (63.6)	NS
Body mass index, kg/m^2	24.1 ± 3.0	27.5 ± 3.0	0.001
Diabetes mellitus	17 (11.5)	2 (18.2)	NS
Hypertension	38 (25.7)	2 (18.2)	NS
Daily alcohol intake, mg	16.2 ± 31.7	11.0 ± 16.4	NS
Alanine aminotransferase, IU/L	22.2 ± 11.4	32.3 ± 17.1	0.024
Platelet count, 10^9/L	235.6 ± 55.5	234.2 ± 92.0	NS
Fasting plasma glucose, mg/dL	99.7 ± 24.3	106.1 ± 24.9	NS
HOMA-IR	1.7 ± 1.2	2.9 ± 2.0	0.034
HbA1c, %	6.0 ± 0.7	6.2 ± 0.6	NS
Presence of fatty liver on Sonography, *n*	53 (35.8%)	9 (81.8%)	0.004
Intimal media thickness, mm	0.7 ± 0.2	1.4 ± 2.1	0.011
Presence of carotid plaque	31 (20.9)	3 (27.3)	NS
Calcified carotid plaque, *n*	0.4 ± 1.0	1.5 ± 1.8	0.019
Visceral fat area, cm^2	107.2 ± 47.7	171.5 ± 93.0	0.002
Controlled attenuation parameter, dB/m	246.4 ± 43.8	266.7 ± 45.6	NS

Values are expressed as the mean ± SD (range), median (range), or *n* (%). NS: Not significant; LS: Liver stiffness; HOMA-IR: Homeostasis model assessment of insulin resistance; HbA1c: Glycated hemoglobin.

Table 4 Factors associated with significant liver fibrosis (> 7 kPa)

Variable	Univariate	Multivariate		
	P value	Odds ratio	95%CI	*P* value
Age, yr	0.021	1.065	0.981-1.156	NS
Body mass index, kg/m^2	0.001	1.487	1.009-2.193	0.045
Alanine aminotransferase, IU/L	0.010	1.078	1.015-1.145	0.014
HOMA-IR	0.013	0.950	0.555-1.626	NS
Visceral fat area, cm^2	0.004	0.995	0.979-1.011	NS
Intimal media thickness, mm	0.195	3.244	1.140-9.234	0.027
Calcified carotid plaque, *n*	0.008	1.787	1.055-3.026	0.031

HOMA-IR: Homeostasis model assessment of insulin resistance; NS: Not significant.

tried to demonstrate the applicability of TE as a screening tool to identify subjects with potential significant liver fibrosis. For this aim, we included a cohort of apparently healthy native Korean subjects without history of chronic viral hepatitis who underwent a comprehensive medical health check-up. The prevalence of significant liver fibrosis was fairly high (6.9%) in this cohort. In addition, the associations between significant liver fibrosis and other clinical factors were investigated. BMI, ALT, carotid IMT, and number of calcified carotid plaques were identified as independent predictors of significant liver fibrosis. Although an exact comparison is not feasible due to differences in ethnicity and BMI, lack of histological information, and potential bias caused by reasons for the medical health check-up, the prevalence of significant liver fibrosis in this study was similar to that of a previous French study (6.9% and 7.5%, respectively) that adopted 8 kPa as the cutoff value for screening the general population[11].

Regardless of etiology, significant liver fibrosis is associated with a risk of fibrosis progression

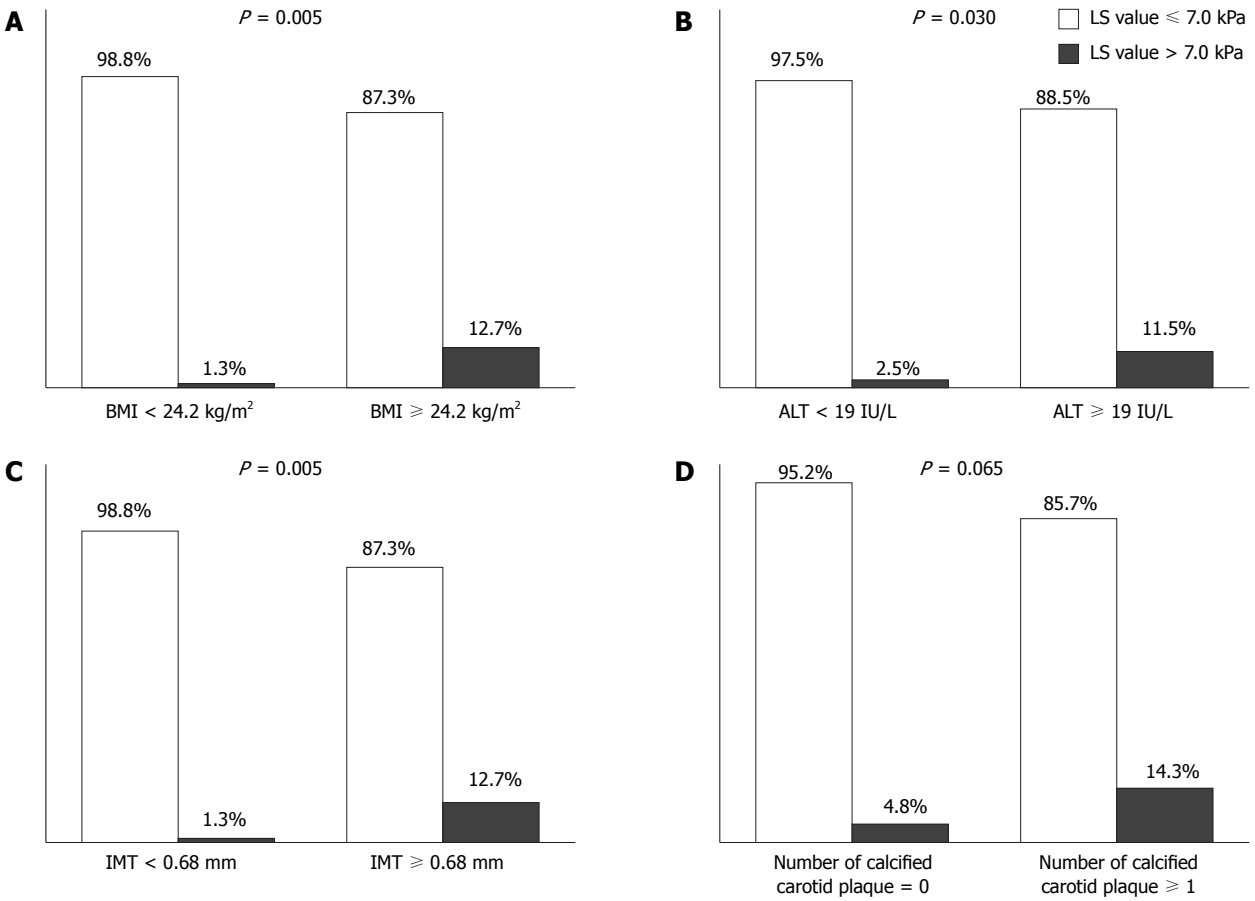

Figure 3 Proportion of subjects with significant liver fibrosis (> 7 kPa) according to four independent predictors. A: Body mass index; B: Alanine aminotransferase; C: Calcified carotid plaque; D: Number of calcified carotid plaques. The proportion of significant liver fibrosis was higher in subjects with high body mass index (⩾ 24.2 kg/m^2), high alanine aminotransferase (⩾ 19 IU/L), and thicker IMT (⩾ 0.68 mm) than in their counterparts (12.7% *vs* 1.3%, 11.5% *vs* 2.5%, 12.7 *vs* 1.3%, respectively; All $P < 0.05$). Subjects with higher numbers of calcified carotid plaques (⩾ 1) were prone to have significant liver fibrosis with borderline statistical significance (14.3% *vs* 4.8%, $P = 0.065$).

and poor prognosis[18,19]. Thus, it is of paramount importance to establish a screening strategy using non-invasive tools such as TE to detect potential underlying significant liver fibrosis in the asymptomatic general population. This will eventually reduce the social burden of liver fibrosis while improving patients' quality of life and survival by preventing disease progression. BMI, ALT, carotid IMT, and number of calcified carotid plaques, four independent predictors of significant liver fibrosis in this study, are generally known to have significant correlation to NAFLD[20-22], which is the leading cause of CLDs in the general population, with no evidence of chronic viral hepatitis worldwide[23-25]. This indicates that most subjects with significant liver fibrosis in the current study cohort may have NAFLD. Indeed, most patients with significant fibrosis (n = 9, 81.8%) showed more than mild fatty liver on ultrasonography. Of these, 6 patients showed elevated ALT level.

To evaluate the relative risks of significant liver fibrosis, the study population was divided into two groups by BMI, ALT, carotid IMT, and number of calcified carotid plaques. Subjects with ALT higher than a cutoff value of 19 IU/L had significantly increased risk of significant liver fibrosis. This finding that subjects with normal ALT level are not completely free from the risk of significant liver fibrosis is consistent with the results of previous studies. Elevated ALT level, even within the current normal range (⩽ 35-40 IU/L), has been associated with a risk of metabolic syndrome[26], CLDs[27], NASH[28], and mortality in the Korean population[29,30].

Subjects with BMI > 24.2 kg/m^2 showed a higher prevalence of significant liver fibrosis. Weight gain has been shown to be closely related to liver fibrosis progression[19,31]. Although the exact mechanism for fibrotic progression in patients with NAFLD has not been fully recognized, obesity with a combination of insulin resistance and visceral adiposity causes hepatic lipid accumulation with various free fatty acid influx into the liver, up-regulation of hepatic lipogenic transcription factors, and inhibition of free fatty acid oxidation[32].

Interestingly, liver fibrosis had a significant relationship with carotid IMT and the number of calcified carotid plaques in this study. In a previous study, the close association between the degree of NAFLD-related liver fibrosis and increased carotid

IMT independent of metabolic syndrome and insulin resistance was reported[33]. This suggests that atherosclerosis can independently influence risk of liver fibrosis progression. Further studies are required to reveal how cardio- or cerebrovascular diseases due to atherosclerosis can be affected by liver fibrosis in subjects with NAFLD[19,24]. In addition, studies are needed to determine whether TE can be incorporated into screening protocols to identify high-risk subjects with cardio- or cerebrovascular diseases.

Age and prevalence of diabetes mellitus, which may have significant correlation with NAFLD or liver fibrosis[28], were higher in subjects with significant liver fibrosis, but the results were not statistically significant. These negative findings should be interpreted with caution due to a relatively small sample size. Unexpectedly, CAP values were not significantly different between groups, although we assumed that most subjects with significant liver fibrosis suffered from NAFLD and would have increased CAP values. This can be explained by that CAP, similar to other imaging modalities[15], cannot generally differentiate NASH which is related to fibrosis progression from simple steatosis, although CAP does accurately reflect the amount of steatosis[34].

Our study still involves several unresolved issues. First, although previous studies[10,11] and our current study have demonstrated the applicability of TE as a screening tool for diagnosing significant liver fibrosis in the general population, insufficient histological examinations for subjects assumed to have significant liver fibrosis can be a major limitation. Since significant hepatic dysfunction is rarely observed in the asymptomatic general population, liver biopsies are not usually justified to confirm the histological diagnosis of liver disease. Thus, well-designed prospective studies that include a sufficient number of subjects with high LS value and histological evaluation are required. Second, we cannot be sure that the study participants are representative of the general population due to potential selection bias caused by recruiting subjects from health check-ups. However, because most baseline characteristics of the study subjects were similar to those in the general population, including daily alcohol intake[35], prevalence of hypertension and DM[36], carotid IMT thickness[37], and LS value[16], the results were not significantly influenced. Third, because this study was cross-sectional, further studies are needed to trace dynamic changes in LS values according to treatment interventions and to investigate the influence of LS changes on long-term clinical outcomes.

In conclusion, the prevalence of significant liver fibrosis assessed using TE was high in asymptomatic Korean general population, despite no evidence of underlying CLDs. High BMI, high ALT, thicker carotid IMT, and higher numbers of calcified carotid plaques were independently associated with the presence of significant liver fibrosis. Our results are helpful for identifying subjects who are at risk of significant asymptomatic liver fibrosis and for developing guidelines regarding the optimal utilization of TE in the general population.

COMMENTS

Background

The prevention from progression of mild liver fibrosis to liver cirrhosis and hepatocellular carcinoma is essential in management of chronic liver diseases (CLD). However, a considerable number of patients with CLD remains asymptomatic and even show normal liver function tests.

Research frontiers

Recently, liver stiffness measurement assessed using transient elastography (TE) has emerged as a promising noninvasive tool for serial measurement of liver fibrosis in various chronic liver diseases. However, few studies have investigated TE for identifying asymptomatic subjects with significant liver fibrosis by screening apparently healthy population.

Innovations and breakthroughs

The prevalence of significant liver fibrosis was 6.9% in apparently healthy Korean population. Body mass index, alanine aminotransferase, carotid intima media thickness, and number of calcified carotid plaques were identified as independent predictors of significant liver fibrosis in this study.

Applications

Significant liver fibrosis in apparently healthy subjects raises necessity of rigorous screening strategy for CLDs in general Korean population. Predictors of significant fibrosis demonstrated in this study can help to identify subjects who are at risk.

Terminology

The TE is the machine using a non-invasive probe, similar to an ultrasound probe. Ultrasound transducer of TE generates elastic shear wave propagating through the liver and measures its velocity. Since elastic shear wave runs faster in stiffer tissue, the velocity of shear wave is proportional to liver stiffness. Many previous studies reported that liver stiffness measurement assessed using TE accurately reflects degree of fibrosis in liver.

Peer review

This is a good cross-sectional study. The authors investigated the prevalence and predictors of liver fibrosis assessed by non-invasive TE in Korean general population. This study will give us useful information regarding optimal selection of patients who require monitoring for chronic liver disease.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Long-term outcome and quality of life after transoral stapling for Zenker diverticulum

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Abstract

AIM: To investigate long-term results and quality of life after transoral stapling of Zenker diverticulum.

METHODS: The data of all patients admitted to our institution for the surgical treatment of Zenker diverticulum were entered into a prospective database. Demographics, symptoms, intraoperative and post-operative data, morbidity, time to oral feeding, and length of hospital stay were recorded. All patients underwent upper gastrointestinal endoscopy and a barium swallow study to measure the length of the diverticulum from the apex of the septum to the bottom of the pouch. Transoral stapling was performed using a Weerda diverticuloscope under general anesthesia. Over time, the technique was modified by applying traction sutures to ease engagement of the common septum inside the stapler jaws. Perioperative variables, symptoms, long-term outcome, and quality of life were analyzed. The operation was considered successful if the patient reported complete remission (grade 1) or marked improvement (grade 2) of dysphagia, regurgitation, and respiratory symptoms. Statistical analysis was performed using Statistical Package for Social Science (SPSS, Version 15, SPSS, Inc., Chicago, IL).

RESULTS: Between 2001 and 2013, the transoral approach was successfully completed in 100 patients with a median age of 75 years. Patients with a larger ($\geqslant$ 3 cm) diverticulum were older than those with a smaller pouch ($P < 0.038$). Complications occurred in 4% of the patients but there was no mortality. A statistically significant improvement of dysphagia and regurgitation scores ($P < 0.001$) was recorded over a median follow-up of 63 mo. Similarly, a significant decrease in the median number of pneumonia episodes per year ($P < 0.001$) was recorded after surgery. The overall long-term success rate of the procedure was 76%. The success rate of the operation was greater in patients of 70 years of age or older compared to younger individuals ($P = 0.038$). Use of traction sutures on the septum was associated with an improved success rate compared with the standard procedure ($P = 0.04$). All items of the health related quality of life questionnaire were significantly higher compared to baseline ($P < 0.05$).

CONCLUSION: Transoral stapling is safe and effective. The operation significantly improves patients' quality of life. It appears that elderly patients with large diverticula significantly benefit from the procedure and that the modified surgical technique including traction sutures can further improve the success rate.

Key words: Zenker diverticulum; Dysphagia; Aspiration pneumonia; Cricopharyngeal myotomy; Diverticulectomy; Transoral stapling; Flexible endoscopy; Short-form health survey questionnaire

Core tip: Transoral stapling was introduced 20 years ago as an alternative to standard open cricopharyngeal myotomy and diverticulectomy for the treatment of Zenker diverticulum. Long-term results after this operation have seldom been reported and quality of life data are lacking. Between 2001 and 2013, 100 patients underwent transoral stapling under general anesthesia. Perioperative variables, symptoms, long-term outcome, and quality of life were analyzed. The median follow-up was longer than 5 years. The success rate of the operation was significantly greater in patients of 70 years of age or older compared to younger individuals. All items of the health related quality of life questionnaire were significantly higher compared to baseline.

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INTRODUCTION

Zenker diverticulum is a relatively rare disease, with an annual incidence of about 2 cases per 100000, and occurs most often in elderly men[1]. The transoral approach to the pharyngoesophageal diverticulum was first described almost a century ago by Mosher *et al*[2] who performed an endoscopic diverticulotomy with the blade of a knife. Electrocautery or laser have been used later on, but failed to gain consensus because of the risk of perforation[3]. The transoral approach using a stapler to divide the septum between the esophagus and the diverticulum was first described by Collard *et al*[4], Martin-Hirsch *et al*[5], and Narne *et al*[6] in 1993 to overcome the limitations and increase safety of the endoscopic approach. Subsequent studies have confirmed the safety and effectiveness of this operation[7-17]. The goal of this report was to review the long-term results and the quality of life of patients with Zenker diverticulum who have been treated with the transoral stapling operation in a single center.

MATERIALS AND METHODS

The data of all patients admitted to our institution for the surgical treatment of Zenker diverticulum were entered into a prospective database. Demographics, symptoms, intraoperative and postoperative variables, morbidity, time to oral feeding, and length of hospital stay were recorded. An upper gastrointestinal endoscopy and a barium swallow study were routinely performed to measure the length of the diverticulum from the apex of the septum to the bottom of the pouch and to exclude the presence of concomitant esophagogastric disorders. A questionnaire on symptoms was administered to grade the severity of dysphagia on a scale of 1 (absent) to 4 (liquid diet only). Regurgitation and respiratory symptoms such as cough, hoarseness, and episodes of pneumonia were recorded as to their frequency on a scale of 1 (absent) to 4 (daily). Preoperative quality of life was evaluated according to the short-form health survey questionnaire (SF-36), an instrument designed to characterize a person's view of health and quality of life[18,19]. Evaluation of the results was done by attributing scores to each question. A change of more than five scale points on any of the eight domains of the SF-36 is considered to be clinically relevant[20]. Each dimension was analyzed separately. The body mass index, the Mallampati and Cormack scores, the distance (in mm) between upper and lower incisors, the thyromental distance, and the distance from incisors to the upper esophageal sphincter were recorded. The Mallampati score is determined by the direct visibility of tonsils, uvula, and soft palate[21]. The Cormack score is based on the laryngeal view during direct laryngoscopy[22]. Postoperatively, an office visit was scheduled within 6 mo after surgery. Patients were then interviewed yearly by letter or by phone. A barium swallow study and an upper gastrointestinal endoscopy were performed at 1 year and during the follow-up whenever dysphagia, regurgitation, or other symptoms occurred. At the last follow-up, postoperative residual symptoms were assessed and quality of life was compared to preoperative values. The degree of postoperative satisfaction was also evaluated using a scale of 1 to 4. The operation was considered successful if the patient reported complete remission (grade 1) or marked improvement (grade 2) of dysphagia, regurgitation, and respiratory symptoms. The Statistical Package for Social Science (SPSS, Version 15, SPSS, Inc., Chicago, IL) was used for data analysis. Quantitative variables were expressed as mean ± SD or median (range). Fisher's exact test was performed for categorical data. Ordinal and quantitative variables

Table 1 Comparison of rate of patients with preoperative and postoperative grade 3-4 symptoms

Symptoms	Pre	Post	*P*
Dysphagia	75.6%	8.3%	0.001
Regurgitation	59.1%	7.5%	0.001
Respiratory symptoms	38.1%	7.8%	0.001

were analyzed by Wilcoxon test. The Kaplan Meier method was used to calculate the recurrence rate. $P < 0.05$ was considered significant.

Statistical analysis

The operation was performed under general anesthesia with orotracheal intubation and with the patient in the supine position. The technical details of the procedure have been described elsewhere[23]. Briefly, a bivalved Weerda diverticuloscope (Karl Storz, Tuttlingen, Germany) was introduced in the cervical esophagus and then withdrawn until the common septum was identified. The septum was divided using a linear endostapler (ETS 35 mm, Ethicon Endosurgery, Cincinnati, OH). Over the past five years, one or two full-thickness traction sutures were routinely applied at the apex of the common septum using a laparoscopic suturing device (Endostitch, Covidien, Norwalk, CT) to improve visualization and purchase of the septum within the stapler jaws.

RESULTS

Between July 2001 and April 2013, 116 patients with Zenker diverticulum were admitted to our Department. Three of these patients with a very small pouch (1-2 cm) underwent primary cricopharyngeal myotomy and are excluded from this study. The other patients were all considered eligible for transoral stapling. In 13 (11.5%) of the 113 patients, who are also excluded from the present study, the procedure required conversion to an open diverticulectomy combined with cricopharyngeal myotomy. Patients undergoing uneventful transoral approach had a larger mouth opening as assessed by the incisor distance (45 ± 7 mm *vs* 34 ± 6 mm, $P = 0.001$) and a longer thyromental distance (96 ± 9 mm *vs* 85 ± 15 mm, $P = 0.02$) compared to individuals requiring conversion to open surgery. The BMI, the Mallampati and Cormack scores, and the distance between incisors and the upper esophageal sphincter were similar in both groups.

Overall, 100 patients (57 males, 43 females) with a median age of 75 years (range 24-90 years) successfully underwent transoral stapling. The median diverticulum length was 3.5 cm (range 2-6 cm). Patients with a larger (≥ 3 cm) diverticulum were older than those with a smaller (< 3 cm) pouch (63.5 ± 14 years *vs* 78.8 ± 7.6 years, $P = 0.038$).

Table 2 Outcome of the operation according to clinical and operative variables *n* (%)

	Success rate	*P*
Age groups (yr)		
< 70	35 (65.7)	0.038
> 70	65 (84.6)	
Diverticulum size (cm)		
< 3	31 (54.9)	0.033
> 3	69 (88.4)	
Endostitch use		
No	49 (68)	0.04
Yes	51 (85)	

Intraoperative complications consisted of two dental lesions and one minor lip laceration. There were two postoperative complications (2%), atrial fibrillation in one patient and mediastinal abscess necessitating trans-cervical drainage in the other. Most patients started eating on the first postoperative day and were discharged on the second postoperative day.

At the latest follow-up, which was completed in 94 of the 100 patients, a significant decrease in the rates of grade 3-4 symptoms was reported (Table 1). Furthermore, in twelve octogenarian patients who presented preoperatively with recurrent episodes of pneumonia as the predominant symptom, a decrease in the median number of pneumonia episodes per year from 4.3 to 1.1 was recorded ($P < 0.001$). Overall, 84% of the patients were highly satisfied with the outcome (grade 1-2), whereas 10 (10.6%) were unsatisfied (grade 4). A further 5 (5.4%) patients, who were scored as grade 3, admitted that despite some residual or recurrent symptoms they would give consent to the operation if the decision had to be made again. Over a median follow-up time of 63 mo (range 12-139 mo), the long-term success rate of the operation was 76%. Patients' age, size of the diverticulum, and use of traction sutures significantly affected the outcome of the operation (Table 2).

Twenty-four patients complained of recurrent symptoms after a median follow-up time of 13 mo (range 2-60 mo). Among these patients, 14 refused further treatment due to mild symptoms, 3 required open diverticulectomy and cricopharyngeal myotomy, and 5 successfully underwent redo stapling without morbidity. The recurrence rate was higher in patients with a diverticulum < 3 cm (32% *vs* 11%, $P = 0.04$). Interestingly, these patients were significantly younger than patients with larger diverticula. The recurrence rate was lower in patients who underwent the modified surgical technique with the use of traction sutures (15% *vs* 32%, $P = 0.008$). Similarly, in the subgroup of patients with a diverticulum < 3 cm, there was a trend toward a lower recurrence rate when the traction suture was used (24% *vs* 38.9%, P = NS).

Assessment of quality of life parameters according

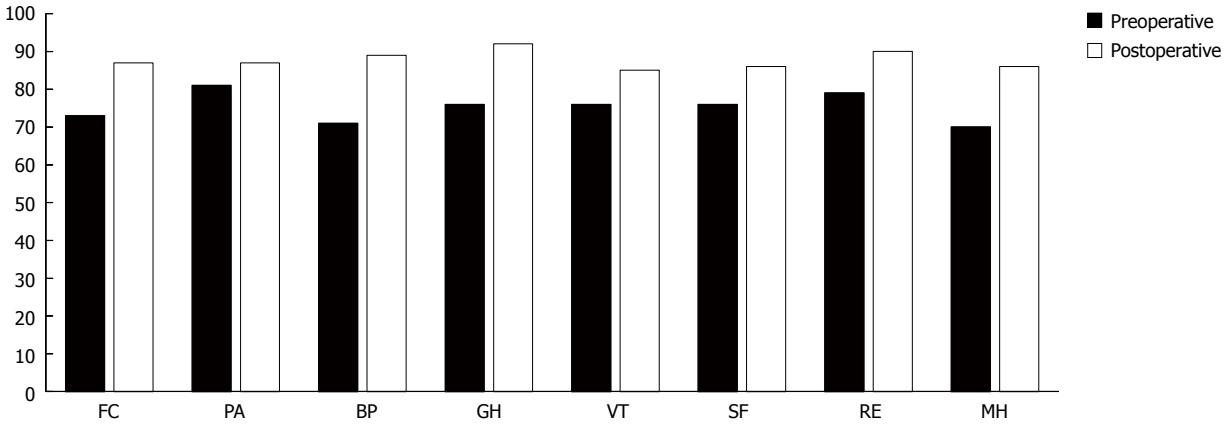

Figure 1 Preoperative and postoperative quality of life according to the 36-item short-form health survey health questionnaire. All items were significantly higher compared to baseline ($P < 0.05$ *vs* baseline). FC: Functional capacity; PA: Physical aspect; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social function; RE: Role emotional; MH: Mental health.

to the eight domains of the SF-36 showed a statistically significant effect of the operation on the items functional capacity, physical aspects, pain, general health, vitality, social aspects, emotional aspects, and mental health. All scores were significantly higher at the last patient follow-up compared to preoperative assessment (Figure 1).

DISCUSSION

This study confirms that transoral stapling is a safe, effective and repeatable procedure for Zenker diverticulum[24]. Manometric and scintigraphic studies from our group had shown that transoral stapling of the septum can restore pharyngoesophageal physiology by decreasing hypopharyngeal intrabolus pressure[25]. Potential advantages of the transoral approach include low morbidity, short length of hospital stay, and similar medium-term outcome compared with open diverticulectomy and cricopharyngeal myotomy. The higher long-term recurrence rate is offset by the lower morbidity and the ease by which the procedure can be successfully repeated. However, no prospective clinical trials are available and clear evidence to prove which surgical method is more effective is still lacking[26]. A recent retrospective review showed that the results of transoral stapling may decline over time and the outcome may be worse in patients with small diverticula in whom a complete cricopharyngeal myotomy is unlikely to be performed[27]. In some patients, an unfavorable anatomy and/or technical difficulties in positioning the diverticuloscope preclude optimal visualization and engagement of the septum in the stapler. The conversion rate in our series was 11.5%, and we found that only the degree of mouth opening and the thyromental distance were significantly associated with conversion of the procedure to open surgery through a left cervical incision.

The long-term outcome analysis in our patients showed an overall success rate of 76%. This is comparable to other smaller series with shorter follow-up time[28]. Over the past five years we have modified our transoral technique by applying to the septum one or two full-thickness traction sutures. The average gain in length of stapled tissue provided by the traction sutures was 1 cm. This suggests that the transoral approach is suitable also for patients with a diverticulum of 2-3 cm as measured intraoperatively. The use of traction sutures was safe, did not increase operative time, and was associated with a significant reduction of the symptom recurrence rate compared with the standard technique.

In the present study we have also evaluated the effect of transoral stapling on patients' quality of life using the SF-36 questionnaire. A significant improvement of all 8 items was recorded. To our knowledge, objective quality of life assessment before and after transoral stapling for Zenker diverticulum has not been previously reported in the literature.

In the future, transoral stapling should be prospectively compared with interventional flexible endoscopy, especially in the management of patients with small (< 3 cm) diverticula. At present, both techniques appear safe and effective, but long-term results of the flexible endoscopic approach are still lacking[29-33].

In conclusion, first-line minimally invasive treatment of Zenker diverticulum by means of transoral stapling appears to be safe and provides symptom control and good quality of life in the long-term follow up. The low morbidity associated with the operation makes this procedure suitable and of special interest for the elderly patient population.

COMMENTS

Background

Transoral stapling was introduced 20 years ago as an alternative to standard open cricopharyngeal myotomy and diverticulectomy for the treatment of Zenker diverticulum. Long-term results after this operation have seldom been reported and quality of life data are lacking.

Research frontiers

The research hotspot is to investigate a minimally invasive method to effectively relieve dysphagia and prevent aspiration in this patient population.

Innovations and breakthroughs

Use of traction sutures during the transoral procedure allowed the improvement of engagement of the septum in the stapler and increased the success rate of the operation especially in patients with a small pouch. The study also shows that quality of life was significantly increased in these patients after operation.

Applications

The transoral stapling procedure is an effective up-front therapy for Zenker diverticulum and provides symptom control and good quality of life in the long-term follow up. The low morbidity associated with the operation makes this procedure suitable and of special interest in the elderly patient population.

Terminology

Zenker diverticulum is an outpouching of the pharyngo-esophageal junction commonly diagnosed in elderly patients. It causes dysphagia, regurgitation, rumination, halitosis, and aspiration. Transoral stapling is a safe and effective treatment, and consists of sectioning the septum interposed between the esophagus and the diverticulum.

Peer review

The authors report on the long-term outcome of 100 patients with Zenker diverticulum treated over 12 years by transoral stapling. In the course of the recruiting period, a technical modification was introduced and traction sutures were used to improve engagement of the septum in the stapler. The message is certainly important and interesting for the medical community. Indeed, the results of the procedure appear to be better in elderly patients with larger diverticula. It remains to be seen in a prospective study whether transoral stapling with a rigid scope and interventional flexible endoscopy will show a similar outcome.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Improvement of diabetes and hypertension after gastrectomy: A nationwide cohort study

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Abstract

AIM: To evaluate the effect of gastrectomy on diabetes mellitus (DM) and hypertension (HTN) in non-obese gastric cancer patients.

METHODS: A total of 100000 patients, diagnosed with either type 2 DM or HTN, were randomly selected from the 2004 Korean National Health Insurance System claims. Among them, 360 diabetes and 351 hypertensive patients with gastric cancer who had been regularly treated without chemotherapy from January 2005 to December 2010 were selected. They were divided into three groups according to their treatment methods: total gastrectomy (TG), subtotal gastrectomy (STG) and endoscopic resection (ER).

RESULTS: The drug discontinuation rate of anti-diabetic and anti-hypertensive agents after gastric cancer treatment was 9.7% and 11.1% respectively. DM appeared to be improved more frequently (22.8%) and earlier (mean ± SE 28.6 ± 1.8 mo) in TG group than in the two other groups [improved in 9.5% of ER group (37.4 ± 1.1 mo) and 6.4% of STG group (47.0 ± 0.8 mo)]. The proportion of patients treated with multiple drugs decreased more notably in TG group compared to others (P = 0.001 in DM, and P = 0.035 in HTN). In TG group, adjusted hazard ratio for the

improvement of DM was 2.87 (95%CI: 1.15-7.17) in a multi-variate analysis and better control of DM was observed with survival analysis ($P < 0.001$).

CONCLUSION: TG was found to decrease the need for anti-diabetic medications which can be reflective of improved glycemic control, to a greater extent than either ER or STG in non-obese diabetic patients.

Key words: Diabetes; Hypertension; Gastrectomy; Gastric cancer; National cohort

Core tip: By following the long term outcome of diabetes and hypertension after gastrectomy, we have discovered that total gastrectomy has a profound impact on the improvement of both diabetes and hypertension compared with endoscopic resection in non-obese population.

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INTRODUCTION

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death in the world[1] with the highest mortality rates reported in Eastern Asia. Although the worldwide incidence of gastric cancer has been declining, its prevalence still remains high in South Korea[2] with the proportion of early gastric cancer (EGC) reported to have increased[3]. The 5-year survival rate of EGC has reached 90% or higher, resulting in the increase of long-term survivors. Thus, the quality of life after cancer treatment has emerged as an important health factor for gastric cancer patients, especially for those suffering with additional chronic diseases such as diabetes mellitus (DM) and hypertension (HTN).

DM and HTN are two of the most common chronic diseases, which increase the risk of vascular complications such as stroke, myocardial infarction, and renal failure. There is a growing concern about striking increase of these two diseases. The Asian population is not exceptional[4]; the age-standardized prevalence of DM was 9.7% in China[5] and 9.1% in South Korea[6], while the age-standardized prevalence of HTN was 25.3% in Korean adults 20 years or older[7]. The prevalence of DM in patients with gastric cancer was not different from that in general population; Ogle *et al*[8] reported comorbid DM was 8% of patients with gastric cancer using data from National Cancer Institute Surveillance, Epidemiology, and End Results program and Sarfati *et al*[9] reported DM with complication occurred in 10% of gastric cancer patients using administrative data from New Zealand Cancer Registry.

Obesity is one of the most important factors commonly contributing to an increased risk of DM and HTN. Thus, obesity has become a major public health concern and incurred enormous socioeconomic costs. Fortunately, several studies suggest that bariatric surgery results in a remarkable improvement for the obesity epidemic and it has been recently recommended that surgery (preferably the Roux-en-Y gastric bypass procedure) should be considered as an appropriate treatment for obese diabetic patients[10]. However, there are only a few large population-based studies conducted on non-obese population[11]. In addition, most of the studies compared metabolic changes before and after bariatric surgery, lacking an appropriate control enough to exclude the effect of dietary-habit changes. In this work, we assessed the effect of gastrectomy on DM and HTN compared to the control group of endoscopic resection using a nationwide data.

MATERIALS AND METHODS

Database

The Korean National Health Insurance System (KNHI) program is a mandatory social insurance, covering approximately 97% of the Korean population. The database includes all information for the purpose of reimbursement, such as sex, age, residential area, diseases, prescription history and survival outcome. The KNHI claims database has been analyzed for epidemiological[12,13] and health policy studies[14].

From the KNHI claims database, we identified 1314168 type 2 DM and 2667621 HTN patients who had been treated with either condition between the dates of January 1, 2004, through December 31, 2004. The data from January 1, 2005 through December 31, 2010 were collected including patient sociodemographic information, specific target diseases (DM, HTN, and gastric cancer), surgical procedures (total gastrectomy, subtotal gastrectomy and endoscopic resection), prescriptions (anti-hypertensive, anti-diabetics and chemotherapy drugs), and deaths. Patients of both DM and HTN were analyzed separately for each disease. Patients were grouped into one of the three categories by residential area (rural, urban, and metropolitan) according to their Korean ZIP code, since regional difference could affect medical behavior.

We extensively reviewed prescription history including the duration and the types of drugs; all

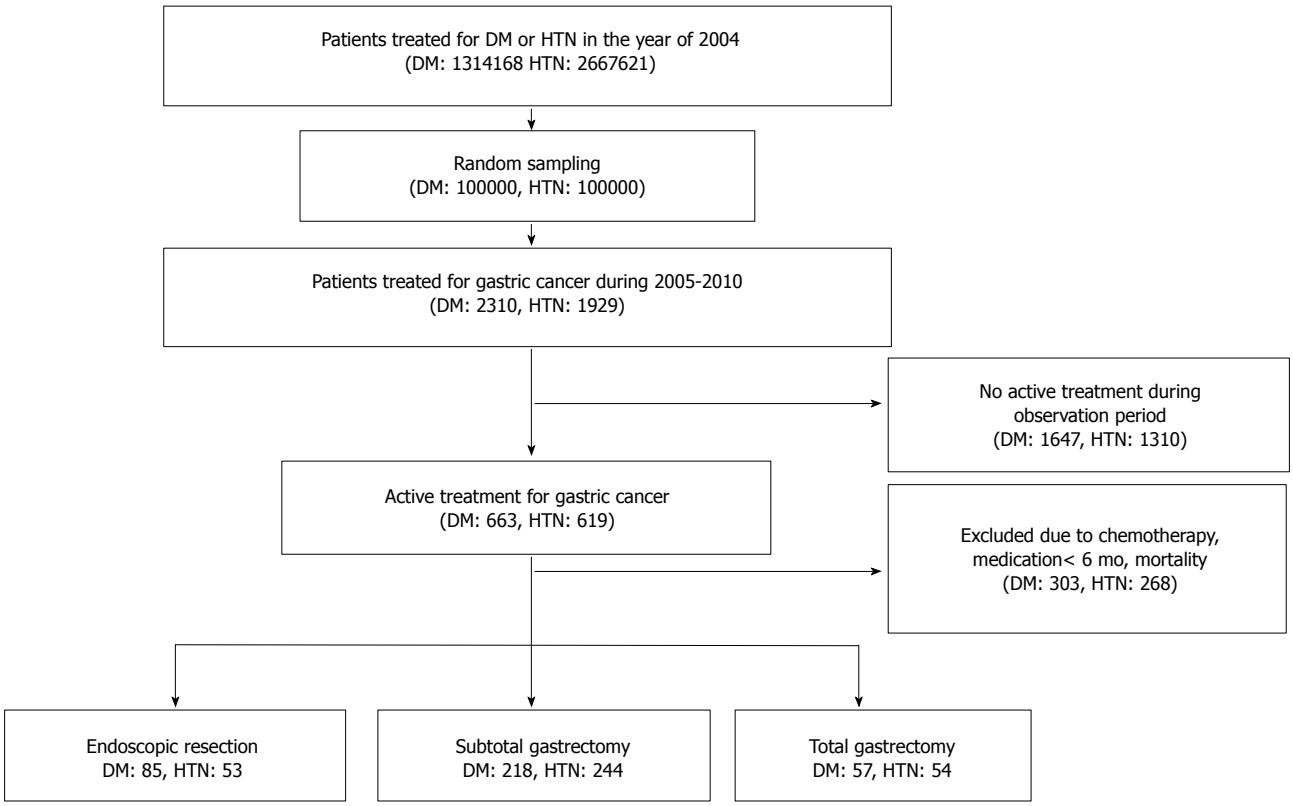


Figure 1 Flow chart of study population. No treatment during the follow-up period due to precedent operation, patient's condition, patients' refusal, *etc*. DM: Diabetes mellitus; HTN: Hypertension.

drugs used for treatment of DM were classified into one of the following groups: sulfonylurea, metformin, thiazolidinedione, α-glucosidase inhibitor and insulin, while drugs used for treatment of HTN were classified into the groups as "calcium channel blocker, diuretics, beta-blocker, angiotensin converting enzyme inhibitor, angiotensin receptor blocker". This study was approved by the Institutional Review Board of the National Cancer Center, South Korea (IRB No. NCCNCS-12-563). Informed consent was waived since the study was based on routinely collected administrative data.

Study population

Once DM and HTN patients were identified from the KNHI claims database, we randomly sampled 100000 subjects for each disease (Figure 1). After sampling, we selected the patients with gastric cancer (code C16), according to the International Classification of Diseases, Tenth Edition, Clinical Modification (ICD-10-CM) and excluded patients who did not undergo active gastric cancer treatment during the follow-up period. In addition, to confine study population to the patients with early stage of gastric cancer and to be unable to assess nutritional status and effects of chemotherapy on DM or HTN, subjects who were treated with chemotherapeutic agents were excluded. Mortality cases in the observational periods were also excluded.

Finally, the remaining patients were divided into three groups: total gastrectomy (TG), subtotal gastrectomy (STG) and endoscopic resection (ER). The endoscopic resection procedure included both endoscopic mucosal resection and endoscopic submucosal dissection. Patients were considered to have shown an improvement of DM or HTN if anti-diabetic or anti-hypertensive drugs had not been prescribed for a minimum of 6 mo. Relapses were considered to have occurred if anti-hypertensive or anti-diabetic drugs were resumed for three months or more after remission.

Statistical analysis

Descriptive analyses were performed to identify patient demographics and procedural distributions. Continuous variables were analyzed by using Student's unpaired two-sided *t*-test, whereas χ^2 approximations were performed for discrete variables such as differences in patient characteristics and remission rates in each group. Categorical variables were analyzed by Fisher's exact test. Survival analysis was used to determine the time from cancer treatment to discontinuation of drugs for DM or HTN. The cumulative probabilities of remission and the survival curves of disease relapse were obtained with Kaplan-Meier estimates. The Wilcoxon two-sample test was also used to assess whether the effect of each procedure on remission was stronger

Table 1 Baseline characteristics of diabetic patients *n* (%)

	Endoscopic resection (*n* = 85)	Subtotal gastrectomy (*n* = 218)	Total gastrectomy (*n* = 57)	*P*
Male	39 (45.9)	142 (65.1)	40 (70.2)	0.003[1]
Age at treatment (yr, mean ± SE)	64.9 ± 7.4	65.1 ± 8.7	64.1 ± 8.5	0.74
Comorbid HTN	57 (67.1)	120 (55.0)	29 (50.9)	0.09
Diabetes medication				
Sulfonylurea (S)	72 (84.7)	178 (81.7)	47 (82.5)	0.82
Metformin (M)	42 (49.4)	126 (57.8)	30 (52.6)	0.39
α-glucosidase inhibitor	20 (23.5)	45 (20.6)	19 (33.3)	0.13
Thiazolidinedione (T)	8 (9.4)	16 (7.3)	1 (1.8)	0.20
Insulin	54 (63.5)	181 (83.0)	47 (82.5)	0.001[1]
Combination (M, S)	3 (3.5)	5 (2.3)	2 (3.5)	0.73
Combination (M, T)	1 (1.2)	2 (0.9)	0 (0.0)	1.00
Others	1 (1.2)	9 (4.1)	1 (1.8)	0.48
Diabetes medications (*n*)				
1	12 (14.1)	22 (10.1)	6 (10.5)	0.24
2	38 (44.7)	79 (36.2)	23 (40.4)	
3	25 (29.4)	85 (39.0)	15 (26.3)	
⩾ 4	10 (11.8)	32 (14.7)	13 (22.8)	

[1]*P*-value by χ^2 test. HTN: Hypertension.

Table 2 Baseline characteristics of hypertensive patients *n* (%)

	Endoscopic resection (*n* = 53)	Subtotal gastrectomy (*n* = 244)	Total gastrectomy (*n* = 54)	*P*
Male	36 (67.9)	153 (62.7)	41 (75.9)	0.17
Age at treatment (yr, mean ± SE)	65.2 ± 8.1	65.8 ± 8.5	66.3 ± 9.3	0.79
Comorbid DM	5 (9.4)	46 (18.9)	13 (24.1)	0.13
Cardiovascular medication				
Lipid lowering agent	8 (15.1)	52 (21.3)	13 (24.1)	0.49
Antithrombotic agent	22 (41.5)	93 (38.1)	22 (40.7)	0.87
Other	1 (1.9)	5 (2.0)	2 (3.7)	0.85
Antihypertensive agent				
Calcium channel blocker (C)	41 (77.4)	166 (68.0)	33 (61.1)	0.19
Diuretics (D)	19 (35.8)	92 (37.7)	21 (38.9)	0.95
Beta-blocker (B)	19 (35.8)	76 (31.1)	17 (31.5)	0.80
ACEi or ARB (A)	15 (28.3)	82 (33.6)	18 (33.3)	0.75
Combination (A, D)	9 (17.0)	41 (16.8)	5 (9.3)	0.37
Combination (B, D)	0 (0.0)	9 (3.7)	1 (1.9)	0.54
Combination (A, C)	0 (0.0)	6 (2.5)	3 (5.6)	0.19
Antihypertensive medications (*n*)				
1	18 (33.3)	73 (29.9)	16 (30.2)	0.70
2	25 (46.3)	97 (39.8)	22 (41.5)	
⩾ 3	11 (20.4)	74 (30.3)	15 (28.3)	

ACEi: Angiotensin-converting enzyme inhibitor; ARB: Angiotensin receptor blocker; DM: Diabetes mellitus.

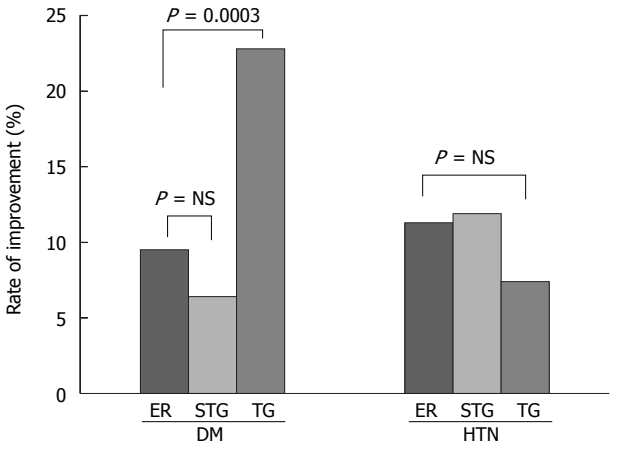
in the earlier phases of administration or became less effective over time[15].

After adjusting for age, sex and coexisting disease (DM or HTN), the effects of each procedure were evaluated using Cox's proportional hazard regression analyses. Proportionality assumption was examined by log-log plots of the hazard estimates against follow-up time and was considered satisfactory. Schoenfeld residuals for each adjusted co-variate indicated that the proportional-hazard assumption was met ($P > 0.05$). The proportional changes of multiple drug medication after treatment were compared using the generalized linear mixed model. Statistical analyses were performed by SAS (version 9.2; SAS Institute Inc, Cary, North Carolina), with the predetermined upper limit of probability set at $P < 0.05$.

RESULTS

Subject characteristics

Among 100000 randomly sampled patients, 2310 diabetics and 1929 hypertensive patients who were treated for gastric cancer from January 2005 to December 2010 were identified. Patients who did not undergo active treatment during observation period (1647 diabetics and 1310 hypertensive patients) and who were dead, were ever prescribed chemotherapeutic agents, or treated with anti-diabetic or anti-hypertensive agents for less than 6 mo (303 diabetics and 268 hypertensive patients) were excluded. Finally, 360 diabetics and 351 hypertensive



Time to improvement	Endoscopic resection (ER)	Subtotal gastrectomy (STG)	Total gastrectomy (TG)
DM, mo	37.4 ± 1.1	47.0 ± 0.8	28.6 ± 1.8
HTN, mo	35.7 ± 1.3	49.7 ± 1.1	10.8 ± 0.4

Figure 2 Comparison of improvement of diabetes mellitus or hypertension among endoscopic resection, subtotal gastrectomy and total gastrectomy. DM: Diabetes mellitus; HTN: Hypertension; TG: Total gastrectomy; STG: Subtotal gastrectomy; ER: Endoscopic resection.

patients were included in this study (Figure 1). For the diabetic patients, TG was performed in 57, STG in 218, and ER in 85. In the case of HTN, TG was performed in 54 patients, STG in 244 patients, and ER in 53 patients. In comparison among three treatment groups, no difference was found for age, residential area, or the rate of coexisting DM or HTN; only the sex ratio differed between the treatment groups of the diabetic patients (Tables 1 and 2). There were also no differences in the number or class of anti-hypertensive drug at the time of gastric cancer treatment; for the diabetic patients, only the rate of patients on insulin differed between the treatment groups.

Metabolic improvement after gastric cancer treatment

During follow-up period of median 36.7 mo in DM and 36.8 mo in HTN, approximately 10% of patients discontinued the anti-diabetics or anti-hypertensive drugs (9.7% in DM and 11.1% in HTN). Patients in TG group discontinued anti-diabetic drugs more often (ER 9.5%, STG 6.4% and TG 22.8%; P = 0.0003) and earlier [time to discontinue (means ± SE); 37.4 ± 1.1 mo in ER, 47.0 ± 0.8 mo in STG, and 28.6 ± 1.8 mo in TG] than patients in STG or ER groups (Figure 2). However, there was no difference in the ratio of patients showing improvement of HTN among the three groups (ER 11.3%, STG 11.9% and TG 7.4%).

Probability of metabolic improvement and its contributing factors

With survival analysis, the probability of improvement of DM was greater in TG group (Figure 3A) and the proportion of patients who were prescribed two or more anti-diabetic drugs was decreased from 89% to 30% after TG, from 90% to 56% after STG and from 86% to 57% after ER, which were all statistically significant (P = 0.001) (Figure 3B). The probabilities of improvement of HTN were not different among three groups with survival analysis (Figure 3C). However, the proportion of patients treated with two or more anti-hypertensive drugs was lowered in TG group than other two groups (P = 0.035) (Figure 3D).

Cox-proportional multi-variate regression analysis, adjusted for age, sex and comorbid HTN, indicated that total gastrectomy was significantly related to improvement of DM, when compared to ER group [control; adjusted hazard ratio (aHR), 2.87, 95%CI: 1.15-7.17] (Table 3). In contrast, the extent of surgical procedure was not related to improvement of HTN.

Resumption of medications for diabetes or hypertension

The resumption rate of anti-diabetic drug was significantly lower (P = 0.001) in TG group (69.2%) than in either STG (92.9%) or ER groups (75.0%). However, hypertensive patients relapsed in similar patterns among all three groups [proportion of relapse, 66.7% in ER, 75.9% in STG, and 100% in TG; and time to relapse (mean ± SE), 18.2 ± 4.5 mo in ER, 19.7 ± 3.2 mo in STG, and 26.6 ± 7.3 mo in TG]. According to Cox-proportional regression analysis, diabetic medications were re-prescribed more frequently after STG compared to ER (aHR for relapse, 5.21, 95%CI: 1.64-16.60), but the risk of relapse in HTN was not different among three treatment methods.

DISCUSSION

As indicated by the cessation of anti-diabetic drugs, this study showed that total gastrectomy has enduring effects on improving DM in gastric cancer patients. This study was the first report to evaluate the metabolic improvement of gastrectomy using a nationwide retrospective cohort study of South Korea. It has been reported that gastrectomy improves glycemic control in morbidly obese population, even independent of weight loss. However, the effect of gastrectomy in non-obese diabetic patients was controversial. In Asia including South Korea, obesity rates of general population are relatively low. The prevalence of obesity 25 kg/m^2 above body mass index (BMI) was 27% in South Korea[16] or 29% in China[17], compared to 34% in the United States[18]. Thus, most Koreans (about 73%) are non-obese, which is closely related with early insulin secretary defects, a unique feature of Korean diabetes[19]. Our study population was randomly sampled from all diabetic or hypertensive patients in South Korea, which suggests the average BMI of study population would reflect that of the Korean population. Our study

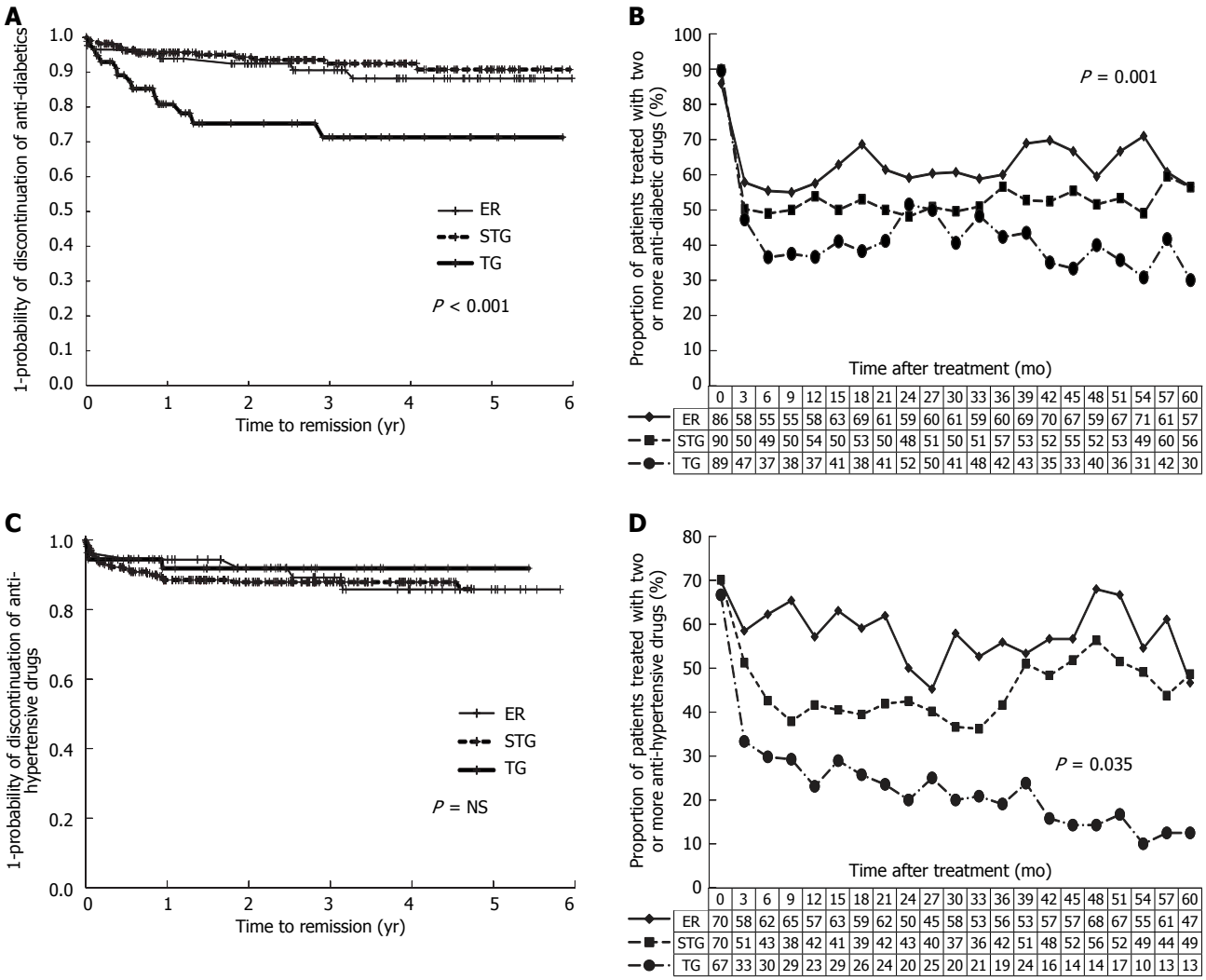


Figure 3 Time response of diabetes mellitus or hypertension after treatment of gastric cancer. A: Kaplan-Meier estimate of probability of improvement of diabetes mellitus (DM) after treatment; B: Proportion of patients used two or more anti-diabetic drugs after treatment; C: Kaplan-Meier estimate of probability of improvement of hypertension (HTN) after treatment; D: Proportion of patients used two or more anti-hypertensive drugs after treatment. TG: Total gastrectomy; STG: Subtotal gastrectomy; ER: Endoscopic resection.

observed that about two times of number of patients in TG group could discontinue anti-diabetics compared to STG or ER group. This observation suggested that TG might be beneficial for glycemic control in even non-obese diabetic patients, who randomly sampled from general population.

Many investigators have investigated metabolic improvement after bariatric surgery compared to after medical intervention or efficacy of various surgical strategies such as laparoscopic gastric banding, sleeve-gastrectomy, gastric-bypass, or biliopancreatic diversion[20,21]. We compared the outcomes in three groups of TG, STG and ER to evaluate the effects depending on surgical extent. As a result, only TG was effective in the improvement of DM. One reason for the difference of two surgical methods (STG and TG) in the metabolic effect may be due to the size of the remnant stomach. Intake is larger in STG than TG, because the remnant stomach plays the role of a reservoir to store food. There were no differences found in daily food intakes from preoperative to 1 year after subtotal gastrectomy[22].

A second reason could be due to the effect of gut hormones such as ghrelin, an orexigenic and pro-diabetic foregut hormone secreted from the fundus. Serum ghrelin levels were decreased by Roux-en-Y gastric bypass surgery due to the ghrelin-producing cells in the gastric fundus being unable to contact ingested nutrients, leading to profound weight loss[23-25]. Recently, one randomized prospective trial comparing Roux-en-Y gastric bypass to sleeve gastrectomy reported that the decrement of the hindgut hormones, glucagonlike peptide 1 and peptide YY after surgery, are also responsible for improved glucose homeostasis[26]. Yet another study suggested that metabolic markers show a similar improvement in both Roux-en-Y gastric bypass and sleeve gastrectomy[21]. However, it remains unclear whether the changes of gut hormones also occur in a non-

Table 3 Age and sex-adjusted hazard ratios for discontinuation and resumption of anti-diabetics or anti-hypertensive by treatment methods

Prescription history	Diabetic patients		Hypertensive patients	
	HR	95%CI	HR	95%CI
Discontinuation				
Endoscopic resection	1.00		1.00	
Subtotal gastrectomy	0.70	0.29-1.71	1.01	0.41-2.46
Total gastrectomy	2.87[a]	1.15-7.17	0.63	0.18-2.27
Resumption				
Endoscopic resection	1.00		1.00	
Subtotal gastrectomy	5.21[a]	1.64-16.60	2.19	0.65-7.39
Total gastrectomy	0.93	0.31-2.79	1.10	0.23-5.17

[a]$P < 0.05$ *vs* other groups. Adjusted by sex, age, coexistence of each disease.

obese population; therefore, a prospective study in a non-obese population is needed.

In our study, drug discontinuation rate of DM and HTN after gastric surgery were relatively lower than previous studies (9.7% in DM and 11.1% in HTN). The remission rate of DM after bariatric surgery has been reported to range between 55 to 95% in previous studies[11,20,21,27-29]. One randomized prospective trial of obese DM patients compared bariatric surgery to intensive medical therapy and found a remarkable reduction in mean glycated hemoglobin, the use of glucose lowering drugs, lipid and blood pressure level[21]. A meta-analysis including 136 observational studies and 22094 patients of morbid obesity (mean BMI of 46.85 kg/m^2) showed that resolution occurred in 76.8% of DM patients and 61.7% of HTN patients after bariatric surgery[30]. Recently, the rate of glycemic improvement after bariatric surgery reported 38% in gastric-bypass group and 24% in sleeve-gastrectomy group[31], which was lower than other short-term trials. The only study including diabetic patients with mild obesity (BMI, 30-35 kg/m^2) suggested that remission of DM occurred in 88% of patients[32]. Our study analyzed the effect of gastrectomy on DM in a non-obese population with gastric cancer, which was the reason for relatively lower remission rate than those of other studies. In addition, the definition of improvement was so tight (cessation of the drugs for six or more subsequent months) that the discontinuation rate might be lower than expected.

The improvement of DM after the treatment of gastric cancer has been reported in non-obese patients of mean BMI 23.4-24.9 kg/m^2. The researchers evaluated the efficacy of three reconstruction methods (Roux-en-Y, Billroth Ⅰ and Billroth Ⅱ). However, the outcome was not compared with "non-reconstructed" control. We discovered an interesting phenomenon that remission of DM or HTN also occurred even in endoscopic treatment, a control group. We suspect this is due to a change in food intake style. After the diagnosis of gastric cancer, many Koreans tend to change their style of food intake to avoid salty food, reduce the amount of carbohydrates, and consume fresh vegetables[33]. This kind of nutritional change (low carbohydrate and salt) could temporarily improve DM and HTN. For this reason, an investigation of the metabolic effects of gastrectomy on DM or HTN should be compared to that of endoscopic resection, rather than the general population. In this study, we confined the study population in a single disease entity of gastric cancer, and compared the outcome of gastrectomy with endoscopic resection, not with medical intervention.

One of the limitations of this study is that data on the reconstruction methods used after gastrectomy were not available. However, most Korean gastric surgeons generally perform gastroduodenostomy (Billroth Ⅰ) or loop gastrojejunostomy (Billroth Ⅱ) after distal gastrectomy and Roux-en Y esophagojejunostomy after TG[3]. The effect on DM and HTN after TG might be caused by either gastric resection or Roux-en Y bypass but this could not reach to conclusion due to limited information. A larger population is required to obtain more detailed information.

Another limitation was that the database did not contain the patient's body mass index, glycated hemoglobin or blood pressure information. Thus, the improvement of disease control could only be assessed from the cessation of medication (anti-diabetic or anti-hypertensive). Due to the limitation of the KNHI data, we did not adopt the concept of disease improvement, but simply discontinuation of therapeutic agents. In spite of lack of biochemical data, cessation of drugs for 6 or more months might be a powerful surrogate marker, because all prescription history have been collected in KNHI database under precise electronic medical system in South Korea. In addition, it could be supportive information that the sequential change of proportion of multiple drug prescription showed similar trends with survival curve for improvement of DM or HTN and best outcome also occurred in TG group.

In conclusion, TG showed decreased requirement of antidiabetic medications than STG or ER in the Korean general population who affected by gastric cancer. In addition, a transient improvement and reduction of the number of drugs were seen in hypertensive patients after gastrectomy.

COMMENTS

Background

Gastrectomy can induce improvement of diabetes or obesity, so it is recommended to treat diabetes in morbidly obese patients. But, there are no data about the long term effect of gastrectomy underwent in non-obese diabetic patients.

Research frontiers

Gastrectomy is also a standard method for the treatment of gastric cancer and numerous operations were applied to cure gastric cancer, especially in South

Korea, one of prevalent area of gastric cancer and of lower average body mass index than in other countries. In the scope of effect on public health, metabolic outcome of gastrectomy should be elucidated, but not yet. The research hotspots are how big impact of gastrectomy lied on metabolic disease such as diabetes and how long the metabolic effect of gastrectomy could be sustained.

Innovations and breakthroughs

In the previous publications, metabolic effect of gastrectomy was compared in individuals, before gastrectomy and after gastrectomy. The results can observe the degree of change of metabolic diseases; however, the effect of dietary habit change or life style modification was not excluded, which were one of important treatment modality of diabetes and hypertension. To overcome confounding effect of these factors, authors analyzed the metabolic change of patients with gastrectomy by comparing with patients with endoscopic intervention.

Applications

The study results suggest that total gastrectomy would induce the improvement of diabetes and hypertension, even in non-obese diabetic patients. Furthermore, clinicians should predict risk of hypoglycemia or symptomatic hypotension caused by metabolic improvement when they meet patients who underwent gastrectomy in clinic.

Terminology

Gastrectomy is a sort of surgical method to resect part of stomach. Gastrectomy is usually performed to treat gastric cancer or gastric perforation; Endoscopic resection is an alternative method to remove small size of gastric cancer or gastric polyp and a confirmative test for pathologic diagnosis of gastric disease.

Peer review

This is a good case-control study using nationwide administrative data in which the authors analyzed the beneficial effect of gastrectomy on diabetes and hypertension in non-obese patients. The results are interesting and suggest that total gastrectomy can be considered in morbidly obese gastric cancer patients with poorly controlled diabetes to expect their metabolic improvement with less number of medications.

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ORIGINAL ARTICLE

Retrospective Study

Optimizing perioperative Crohn's disease management: Role of coordinated medical and surgical care

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Abstract

AIM: To investigate rates of re-establishing gastroenterology care, colonoscopy, and/or initiating medical therapy after Crohn's disease (CD) surgery at a tertiary care referral center.

METHODS: CD patients having small bowel or ileocolonic resections with a primary anastomosis between 2009-2012 were identified from a tertiary academic referral center. CD-specific features, medications, and surgical outcomes were abstracted from the medical record. The primary outcome measure was compliance rates with medical follow-up within 4 wk of hospital discharge and surveillance colonoscopy within 12 mo of surgery.

RESULTS: Eighty-eight patients met study inclusion criteria with 92% (n = 81) of patients returning for surgical follow-up compared to only 41% (n = 36) of patients with documented gastroenterology follow-up within four-weeks of hospital discharge, $P < 0.05$. Factors associated with more timely postoperative medical follow-up included younger age, longer length of hospitalization, postoperative biologic use and academic center patients. In the study cohort, 75.0% of patients resumed medical therapy within 12 mo, whereas only 53.4% of patients underwent a colonoscopy within 12 mo of surgery.

CONCLUSION: Our study highlights the need for coordinated CD multidisciplinary clinics and structured handoffs among providers to improve of quality of care in the postoperative setting.

Key words: Coordinated care; Crohn's disease; Postoperative prophylaxis; Multidisciplinary clinics; Surgery

Core tip: Adherence to evidence based management of patients with Crohn's disease requires care coordination and communication between surgeons and

gastroenterologists. Surgeons need to facilitate return visits after surgery to the gastroenterologists.

Bennett JL, Ha CY, Efron JE, Gearhart SL, Lazarev MG, Wick EC. Optimizing perioperative Crohn's disease management: Role of coordinated medical and surgical care. *World J Gastroenterol* 2015; 21(4): 1182-1188 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1182.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1182

INTRODUCTION

Most patients with Crohn's disease will require surgical intervention during the course of their disease[1,2]. Despite the high lifetime risk of surgery, surgical resection is not curative and disease recurrence is relatively common. As a result, the decision to pursue surgical treatment for Crohn's disease is highly personalized. Historically, prophylactic medical therapy after surgery was not routinely recommended and instead, patients were managed expectantly, with initiation of treatment at the time of symptoms recurrence. Emerging evidence suggests that early postoperative consideration of medical therapy, especially for higher-risk patients, to prevent Crohn's disease recurrence may obviate the need for additional operations[3,4].

In Crohn's disease, postoperative disease recurrence is the norm. Within the first year of surgery, 70%-90% of patients develop endoscopic recurrence and within three years recurrence rates increase to 80%-100%[2,5,6]. Although clinical or symptomatic recurrence occurs in up to 30% of patients with a 10% increase each additional year, subjective manifestations of Crohn's disease may lag behind objective evidence of disease recurrence based on endoscopy[6,7]. Severity of early endoscopic lesions can predict the symptomatic course of disease after surgery. Therefore, postoperative surveillance evaluating for early endoscopic recurrence is helpful for identifying patients who will benefit from early, aggressive medical management[8]. As an example, early use of Infliximab after surgery has been demonstrated to significantly decrease the risk of endoscopic disease in patients with a history of multiple surgeries, stricturing, or penetrating disease[9]. Based on this and other emerging data on the benefits of aggressive postoperative medical management, recommendations for the postoperative management of Crohn's disease have been updated to include timely initiation of biologics or immunomodulators[10]. As Crohn's disease can recur starting almost immediately after surgical resection, particularly among higher-risk patients, recommendations are to initiate or resume medical therapy within the first 4 wk after ileal resection provided the postoperative recovery is unremarkable. Evaluation with repeat colonoscopy during the 6-12 mo after surgery to establish the presence of early disease recurrence is a cornerstone of this algorithm[10].

Little is known about the barriers of reestablishing care with gastroenterologists, early colonoscopy, and/or initiation of postoperative medical therapy after surgery for Crohn's disease in an academic surgery setting with referrals from both within the health system and from the community. We hypothesized that patient, provider, and system-related factors impact a patient's adherence to current postoperative medical management prophylaxis recommendations. Identification of these factors may guide quality improvement efforts aimed at improving the care of Crohn's patients and support the use of coordinated multidisciplinary care in this patient population.

MATERIALS AND METHODS

Patient population

We performed a single-center retrospective cohort study of Crohn's disease patients who underwent small bowel or ileocolonic resection surgery at the Johns Hopkins Hospital between January 2009 and January 2012. Patients who did not have a primary anastomosis at the time of operation were excluded, as surveillance recommendations do not apply to patients without an anastomosis. Additionally, patients with primary colon surgery (*e.g.,* colectomies) and incomplete postoperative data for review were excluded from evaluation. The Johns Hopkins University Institutional Review Board approved this study.

Patient and procedure-related data

Patient, procedure, and 30-d outcome information was abstracted from hospital's National Surgical Quality Improvement Program (NSQIP) database[11]. Specific information related to the patients gastroenterology care (provider location, office and colonoscopy visit dates and reports, medical therapy, providers, and past medical history including smoking history) were abstracted from the electronic health record by two clinicians (JB and CH). Colonoscopy reports were reviewed; preoperative Inflammatory Bowel Disease phenotypic information using the Montreal classification and Rutgeerts scores for postoperative surveillance colonoscopies were assigned (CH)[12,13]. Disease recurrence was classified as a Rutgeerts score of i2 or greater.

The majority of patients received their Crohn's disease care at two hospitals in the Johns Hopkins Health System (Johns Hopkins Hospital and Johns Hopkins Bayview Medical Center). For these patients, complete medical records were available

Table 1 Comparison of patients with > 4-wk, within 4-wk and no gastrointestinal follow-up *n* (%)

Demographics	4 wk follow-up with GI (*n* = 36)	> 4 wk follow-up with GI (*n* = 35)	No follow-up or lost to GI follow-up (*n* = 17)
Male	24 (67)	18 (51)	6 (35)
Receiving all care at Johns Hopkins Hospital	22 (61)[a]	22 (63)	4 (24)
Age at operation	33.6 +/- 10.8[a,c]	44.5 +/- 15.2	44.5 +/- 14.9
Laparoscopic Technique	20 (56)	21 (60)	5 (29)
Preoperative Admission within < 60 d	11 (31)	6 (17)	4 (24)
Current smoker	8 (22)	13 (37)	2 (12)
Disease phenotype			
B1	1 (2.8)	1 (2.9)	1 (5.9)
B2 Stricturing	19 (53)	24 (69)	12 (71)
B3 Penetrating	16 (44)	10 (29)	4 (24)
Perianal disease	4 (11)	6 (17)	4 (24)
Preoperative Therapy			
Preoperative antibiotics	16 (44)	10 (29)	4 (24)
Preoperative ASA	19 (53)	19 (54)	8 (47)
Preoperative Steroids	17 (47)	13 (37)	5 (29)
Preoperative IMM	9 (25)	5 (14)	5 (29)
Preoperative anti-TNF	20 (56)[c]	11 (31)	7 (41)
Preoperative TPN	9 (25)	3 (8.6)	3 (18)
Mean Length of Hospital Stay	8.5 +/- 5.5[c]	6.5 +/- 5.5	9.8 +/- 4.9
Postoperative Readmission within 30 d	6 (167)	4 (11)	4 (24)
Any Postoperative Complication	6 (17)	8 (23)	1 (5.9)
Postoperative Medical Complication	5 (14)	7 (20)	1 (5.9)
Postoperative Surgical Complication	2 (5.6)	3 (8.6)	0 (0)

[a]$P < 0.05$ *vs* > 4 wk follow-up with GI; [c]$P < 0.05$ *vs* no follow-up or lost to gastrointestinal follow-up. GI: Gastrointestinal; anti-TNF: Anti-tumor necrosis.

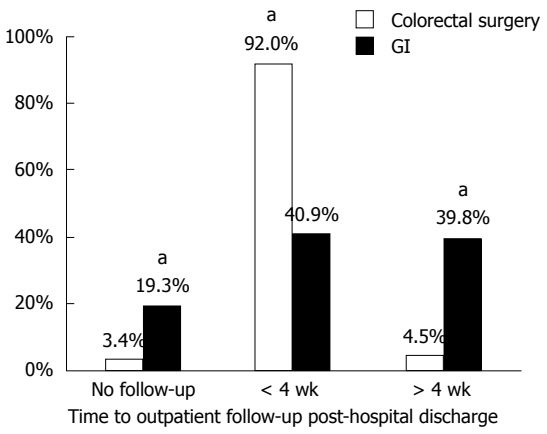

Figure 1 Comparison of postoperative follow-up to colorectal surgery and gastroenterology at 4 wk and greater than 4 wk. [a]$P < 0.05$ *vs* 4 wk or greater than 4 wk.

in the health system electronic medical records. For patients referred for surgical resection from non-Johns Hopkins gastroenterologists, written consent to review pre and postoperative gastroenterology office and colonoscopy records was obtained. Consent and medical records were obtained from 44.1% (15/34) of patients receiving care from non-Johns Hopkins gastroenterology practices. Records were reviewed from the 12 mo prior to and following the operation date.

Outcome

The primary goal of the study was to determine compliance with medical follow-up within 4 wk of hospital discharge and surveillance colonoscopy within 12 mo of surgery.

Statistical analysis

Descriptive statistics were reported as percentages, means, and standard deviations of the mean. All continuous variable comparisons were unpaired, and all tests of significance were two-tailed. Continuous variables were compared using the Student *t* test for normally distributed variables. The Fisher's exact test was used for comparison of categorical variable with a *P*-value $\leqslant 0.05$ considered as statistically significant. Statistical analyses were performed using SPSS version 20, IBM Corp.

RESULTS

We identified 88 patients with Crohn's disease who underwent resectional surgery with a primary anastomosis between January 2009 and January 2012. The average age at time of operation was 40.0 ± 14.4 years and at Crohn's disease diagnosis was 26.2 ± 12.5 years. Twenty two patients (26%) reported smoking cigarettes at the time of surgery and 11 patients (13%) were former smokers. Surgery was performed for non-stricturing non-penetrating disease (*n* = 3, 3.4%), stricturing disease (*n* = 55, 62.5%), and penetrating disease (*n* = 30, 34.0%). Forty percent (*n* = 38) of patients had prior intestinal resections for strictures or abdominal abscesses. Most patients were receiving medical therapy at the time of surgery: 43% (*n* =

Table 2 Comparison of postoperative recurrence risk subgroups: 4-wk follow up to gastrointestinal *vs* colorectal surgery and 12 mo care measures *n* (%)

	Postoperative follow-up at 4 wk			Postoperative 12 mo	
	With GI	With colorectal surgery	*P*-value	Colonoscopy	Medical therapy resumption
Overall (*n* = 88)	36 (40.9)	81 (92.0)	< 0.0001	47 (53.4)	66 (75.0)
Patient subgroups:					
Less than 10 yr since diagnosis or B2 disease (*n* = 35)	13 (37.1)	33 (94.3)	< 0.0001	20 (57.1)	18 (51.4)
2 or more surgeries (*n* = 38)	16 (42.1)	36 (94.7)	< 0.0001	21 (84.0)	25 (65.8)
B3 disease (*n* = 30)	16 (53.3)	28 (93.3)	0.0009	15 (50.0)	16 (53.3)
Current smokers (*n* = 23)	8 (34.8)	21 (91.3)	0.0001	14 (60.9)	17 (73.9)
Preoperative anti-TNF (*n* = 38)	20 (52.6)	35 (92.1)	0.0002	20 (52.6)	25 (65.8)
Preoperative Immunomodulator (*n* = 19)	9 (47.4)	19 (100.0)	0.0004	10 (52.6)	12 (63.2)
Preoperative steroids (*n* = 35)	17 (48.6)	33 (94.3)	< 0.0001	20 (57.1)	26 (74.3)

anti-TNF: Anti-tumor necrosis; GI: Gastrointestinal.

38) anti-tumor necrosis-alpha (anti-TNF) therapy, 22% immunomodulators (*n* = 19), and 40% steroids (*n* = 35). About a quarter (27%, *n* = 24) of patients were not on immunosuppression preoperatively and 17% (*n* = 15) were receiving total parenteral nutrition.

Postoperative follow-up

Almost all patients returned for surgical follow-up (*n* = 81, 92%) within a four-week window following hospital discharge. However, only 41% (*n* = 36) of patients returned to their medical provider during this interval (Figure 1). Patients who had established medical care at Johns Hopkins Hospital prior to surgery, were being treated with TNF-alpha inhibitors before surgery, were younger patients, and had a longer length of stay at the time of operation were more likely to re-establish medical care (Table 1).

A subgroup analysis of patients with Crohn's disease stratified by risk factors for postoperative recurrences is summarized in Table 2. All patient subgroups had lower rates of postoperative gastroenterology follow-up compared to colorectal surgery follow-up. In all patient subgroups, the rate of follow-up to colorectal surgery exceeded 90%, while rates of follow-up to gastroenterology varied among subgroups from only 34.8% to 53.3%. The subgroup with the lowest gastroenterology follow-up was current smokers. They also had the lowest rate of return to colorectal surgery.

Overall, about half of all patients (*n* = 47, 53.4%) received their postoperative surveillance colonoscopy within 12 mo. The subgroup of patients with two or more prior surgeries was most likely to have a postoperative colonoscopy (*n* = 21, 84%) compared to the remaining subgroups. Among patients whom underwent a postoperative colonoscopy by 12 mo, 27% had evidence of endoscopic recurrence with a Rutgeerts i2 score or higher.

Even though early postoperative gastroenterology follow-up was low among our study cohort, most of patients (*n* = 66, 75%) resumed or initiated at least one type of Crohn's disease medication by 12 mo: 48.9% (*n* = 43) on anti-TNFs, 13.6% (*n* = 12) on immunomodulators, and 21.6% (*n* = 19) on antibiotics. However, 8% (*n* = 7) were started on corticosteroids and 30.7% (*n* = 27) on 5-aminosalycilatesaminosalicylates, despite having a minimal role in postoperative Crohn's disease management.

DISCUSSION

Crohn's disease postoperative management is a complex interplay of coordination between patients, gastroenterologists, and surgeons. From the patient perspective, postoperative follow-up care is involved and the role of complementary providers can be confusing, particularly with respect to care coordination. In our study, we chose four-weeks as a primary endpoint for clinic follow-up based on the studies that investigated starting postoperative therapy within four-weeks of surgery[9]. The 12-mo cut-off for colonoscopy was based on the recommendation to undergo surveillance colonoscopy within 6-12 mo of surgery depending on disease behavior[14]. Our study demonstrates a substantial discrepancy in follow-up clinic visit rates to surgeons compared to gastroenterologists, driving the call to address gastroenterology follow-up for operative patients and motivating providers to redefine Crohn's disease perioperative management. The necessity of gastroenterology follow-up may be undermined by the overall sense of wellness Crohn's patients may feel following surgery, with substantially less disease burden following a surgically-induced "remission." It has been well documented that adherence to therapy and patients' perceptions regarding the need for continued therapy decreases during the maintenance of remission phase[15,16].

As we have discovered, the majority of patients returned to their surgeons for a postoperative ap-

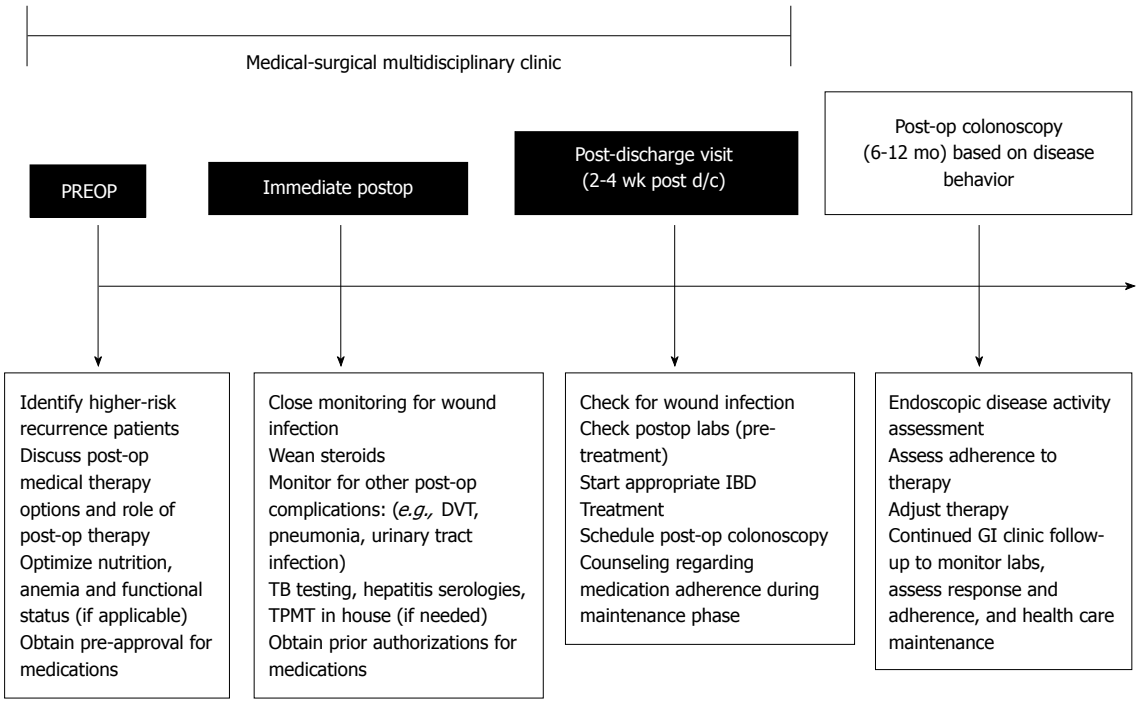

Figure 2 Proposed coordinated surgical and gastroenterology perioperative care plan for Crohn's patients. IBD: Inflammatory bowel diseases; GI: Gastrointestinal.

pointment. The decision for surgical intervention is best made with the gastroenterologist and surgeon in conjunction with the patient[17]. Thereby, the preoperative integration of surgery and gastroenterology should be continued into the postoperative period[17]. The need for surgical follow-up in the postoperative period may be more apparent to patients who develop postoperative complications, as they do not return to their baseline sense of wellness as rapidly as those who do not experience complications. Patients may be unaware of any disease recurrence until it progresses further as clinical symptoms of Crohn's disease are often absent with endoscopic recurrence[18]. The absence of symptoms underscores the importance of close gastroenterology follow-up, yet patients are less likely to accept treatment risks when their symptoms are minimal[19,20]. Patients need to be well-informed on their options for postoperative therapy as it tends to be a long-term commitment and patients dissatisfied with their medication may discontinue them or seek care from another provider[21]. Conversations and structured education prior to surgery about postoperative medical therapy is a prime opportunity to reiterate that surgery is not curative and restarting medical therapy postoperatively at some point should be an expectation.

In understanding the discrepancy in follow-up to surgeons compared to gastroenterologists in our data, we acknowledge the limitations in our ability to examine detailed reasons for the lack of gastroenterology follow-up. Patients may have been given follow-up appointments but may have cancelled or failed to appear. We also acknowledge that lack of a follow-up appointment does not inherently indicate that a patient is not receiving disease and medication counseling by their gastroenterologist as our data shows a sizable proportion of patients on postoperative medications by 12 mo. Furthermore, our study was not sufficiently powered to establish if medical follow-up/early resumption of medications impacted disease recurrence or Rutgeerts scores.

Despite our institution being a tertiary referral center, we feel our results are likely to reflect many institutions in the United States. Inefficiencies within the increasingly more complicated and disjointed healthcare system along with lack of coordination between healthcare providers has led to higher costs, errors and complications[22,23]. The Institute of Healthcare Improvement has suggested the creation of multidisciplinary teams along with effective teamwork and communication as means to decrease patient harm and mortality, improve patient satisfaction, ensure reliable evidence-based care is provided without gaps in care by race, ethnicity or language[24]. In other fields, the creation and implementation of multidisciplinary teams have improved the quality of care provided for patients and patient outcomes[22,25,26]. A Cochrane Review found evidence to support practice-based interprofessional collaborations can improve healthcare processes and outcomes[27]. The multidisciplinary clinic model should be considered as the new standard for perioperative Crohn's disease management as outlined in Figure 2. Although the ability to create a multidisciplinary clinic

may be influenced by the patient care setting and the resources available to fund a multidisciplinary clinic, the coordination of care is an important improvement for perioperative Crohn's management. Structured handoffs between providers should be considered for patients seeking care from multiple institutions. Other groups have also endorsed the effectiveness of multidisciplinary clinics and proposed multidisciplinary clinics as the new standard of care at academic centers for inflammatory bowel disease patients[28-31].

We view our data as an introduction into the issues and barriers surrounding the postoperative management of operative Crohn's disease patients. Effective multidisciplinary care will allow gastroenterology and surgery to take joint responsibility for the care of Crohn's patients and ultimately improve the quality of perioperative management and maintain care continuity.

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COMMENTS

Background

Emerging evidence suggests that early resumption of medical therapy after surgery for Crohn's disease (CD) can prevent recurrence. In order for patients to resume medical therapy, their care needs to be coordinated between their surgeon's and gastroenterologist's office.

Research frontiers

Guidelines suggest early colonoscopy and resumption of medical therapy can prevent CD recurrence after surgery. Multidisciplinary, team-based practices can facilitate care.

Innovations and breakthroughs

CD care is complex and without structured handoffs and communications between surgeons and gastroenterologists frequently gastroenterology followup and resumption of medical therapy is delayed.

Applications

Hospitals and practices should consider structured multidisciplinary care and handoff tools for optimal management of CD patients.

Peer review

This is honest assessment of CD care at a major academic medical center. It highlights areas that need to be targeted for improvement to adhere to new treatment guidelines.

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ORIGINAL ARTICLE

Retrospective Study

Characteristics of hepatocellular carcinoma in cirrhotic and non-cirrhotic non-alcoholic fatty liver disease

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Abstract

AIM: To determine characteristics and prognostic predictors of patients with hepatocellular carcinoma (HCC) in association with non-alcoholic fatty liver disease (NAFLD).

METHODS: We reviewed the records of all patients with NAFLD associated HCC between 2000 and 2012. Data collected included demographics; histology; presence or absence of cirrhosis, size and number of HCC, alpha-fetoprotein, body mass index (BMI), and the presence of diabetes, hypertension, or dyslipidaemia.

RESULTS: Fifty-four patients with NAFLD associated HCC were identified. Mean age was 64 years with 87% male. Fifteen percent (8/54) were not cirrhotic. 11%, 24% and 50% had a BMI of < 25 kg/m^2, 25-29 kg/m^2 and $\geqslant$ 30 kg/m^2 respectively. Fifty-nine percent were diabetic, 44% hypertensive and 26% hyperlipidaemic. Thirty-four percent of the patients had $\leqslant$ 1 of these risk factors. Non-cirrhotics had a significantly larger mean tumour diameter at diagnosis than cirrhotics (P = 0.041). Multivariate analysis did not identify any other patient characteristics that predicted the size or number of HCC.

CONCLUSION: HCC can develop in NAFLD without cirrhosis. At diagnosis such tumours are larger than those in cirrhotics, conferring a poorer prognosis.

Key words: Hepatocellular carcinoma; Non-alcoholic fatty liver disease; Cryptogenic cirrhosis; Metabolic syndrome; Screening

Core tip: Our study confirms that hepatocellular carcinoma can occur in non-cirrhotic non-alcoholic fatty liver disease, the incidence of which is rising worldwide. Moreover, these cancers were found to be significantly larger and more likely to be beyond Milan criteria for

liver transplantation than those occurring in cirrhotic patients. Further research is needed to identify clinical risk factor profiles predisposing to cancer development in patients with non-alcoholic fatty liver disease such that screening if implemented can be appropriately targeted.

Leung C, Yeoh SW, Patrick D, Ket S, Marion K, Gow P, Angus PW. Characteristics of hepatocellular carcinoma in cirrhotic and non-cirrhotic non-alcoholic fatty liver disease. *World J Gastroenterol* 2015; 21(4): 1189-1196 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1189.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1189

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) affects up to 30% of the population in industrialised countries and is becoming more prevalent in the developing world as living standards rise and dietary habits change[1,2]. Although most individuals with this condition do not develop serious liver disease, it is associated with an increased annual incidence of hepatocellular carcinoma (HCC) of 76-201 per 100000[3,4] compared to a background incidence of sporadic HCC of 4.9-16 per 100000[5,6]. Recent studies identified NAFLD or cryptogenic cirrhosis (which is thought to often represent end-stage NAFLD[7]) as the underlying cause in 13% to 38.2% of patients presenting with HCC[8-10].

Diabetes and obesity have been suggested as risk factors for HCC in large cohort and case-control studies[11-13] both with and without pre-existing NAFLD. A meta-analysis by Larsson *et al*[14] of 10 cohort studies found a relative risk of HCC of 1.89 in obese patients. Another meta-analysis of 17 case control studies and 32 cohort studies by Wang *et al*[15] found a relative risk of HCC of 2.31 in diabetics.

Whether other features of the metabolic syndrome such as hypertension and dyslipidaemia may also contribute to HCC risk is less well studied, though these conditions have been shown to independently correlate with NAFLD fibrotic severity which itself may increase HCC risk[16-18].

2012 American Association for the Study of Liver Diseases (AASLD) guidelines state that the risk of HCC in NAFLD is "likely limited to those with advanced fibrosis and cirrhosis"[19] and as such there are no established HCC screening guidelines for NAFLD patients in general[20]. However, recent international reports that HCC occurs in non cirrhotic patients with NAFLD[21] suggest that work is needed to identify factors other than severe fibrosis or cirrhosis that could be used to identify patients in whom HCC screening may be justified.

The aims of this study in a cohort of patients with NAFLD associated HCC were: (1) to describe the risk factor profile of these patients focussing on features of the metabolic syndrome; (2) to determine any demographic or risk factor profile differences between cirrhotics and non-cirrhotics; and (3) to determine if any risk factors correlated with poorer prognosis, in terms of HCC size and number.

MATERIALS AND METHODS

This retrospective study was conducted at the Victorian Liver Transplant Unit which provides all liver transplant services to the states of Victoria and Tasmania covering a population of approximately 5 million people. As the sole liver transplant referral centre for this population, it has become the major tertiary referral centre for patients with HCC. The records of patients with "hepatocellular carcinoma", "HCC", "liver cell cancer" or "hepatoma" and "NAFLD", "fatty liver", "NASH" or "cryptogenic cirrhosis" between 2000 and 2012 based on International Classification of Diseases 10 coding were audited, using hospital medical records and pathology department databases.

Patients were considered to have HCC if they had radiological and/or histological diagnoses according to AASLD guidelines[22]. Patients were included if they had characteristic radiological or histological features of NAFLD as recommended in Asia-Pacific Guidelines[23], or if the diagnosis of NAFLD or cryptogenic cirrhosis had previously been made in a patient with relevant risk factors and if concomitant causes of liver diseases including cleared or chronic hepatitis B (defined as having detectable hepatitis B core antibody, with or without positive surface antigen), chronic hepatitis C, Wilson's disease, haemochromatosis, autoimmune hepatitis, alpha1-antitrypsin deficiency, cystic fibrosis, primary biliary cirrhosis, primary sclerosing cholangitis and other chronic biliary tract diseases and other hepatic malignancies had been excluded by relevant blood tests and/or liver histology. Patients were excluded if they had an alcohol intake of over 140 g per week for men and 70 g per week for women. These diagnostic criteria broadly concur with those in international (Chinese, Italian, European and American) guidelines as summarised by Nascimbeni *et al*[24].

Information was collected from the time of HCC diagnosis including patient sex, age, radiological or histological evidence of cirrhosis, Child-Pugh scores, histological NAFLD grade and stage where available according to Brunt criteria[25], size and number of HCCs, alpha-fetoprotein (AFP); metabolic profile (presence of diabetes, hypertension, dyslipidaemia and obesity) and medications. Obesity was measured via body mass index (BMI) instead of waist circumference which was not recorded in a majority of patients. No patients were of Asian background (in whom altered BMI cut-offs for overweight or obesity

Table 1 Comparison between non-alcoholic fatty liver disease cirrhotics and cryptogenic cirrhosis in terms of demographics and hepatocellular carcinoma risk factors

	NAFLD cirrhotics (n = 31)	Cryptogenic cirrhotics (n = 15)	P value
Median age (yr)	65	65	0.680
Median BMI (kg/m^2)	31.35	28	0.407
Overweight/obesity	72%	73%	0.919
Diabetes	72%	40%	0.058
Hyperlipidaemia	29%	33%	0.952
Hypertension	48%	13%	0.025
Number of risk factors (n)			0.310
0	2	1	
1	6	6	
2	10	6	
3	9	2	
4	4	0	
Child-Pugh Score			0.880
A	12	6	
B	10	4	
C	8	5	

NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

would have otherwise applied).

Diabetes was defined as having had a previous diagnosis of the disorder and/or being on relevant medication. Hypertension was defined as having a systolic blood pressure of over 130 mmHg and/or being on relevant medication. Dyslipidaemia was defined as being on relevant medication, having serum triglyceride levels > 2.0 mmol/L and/or total serum cholesterol levels > 5.5 mmol/L.

Univariate analysis was performed using Kruskal-Wallis testing, Fisher's exact test and two-sample t testing to analyse for differences between cirrhotic and non-cirrhotic cohorts. Multivariate analysis was performed with Pearson correlation analysing for predictors of tumour size and number, and thus prognosis. Ethical approval was obtained by the Austin Hospital Human Research Ethics Committee.

RESULTS

Patients

Between 2000-2012, 39 (13%) patients with HCC had a primary diagnosis of NAFLD and 15 (5%) cryptogenic cirrhosis. Therefore 54 patients were included in our study, 47 (87%) were male and mean age was 64 years.

Liver fibrosis and cirrhosis: Thirty (57%) patients had diagnostic biopsies of non-HCC liver parenchyma available for review. Of these, 7 (23%), 11 (37%), 9 (30%) and 3 (10%) had NAFLD grade 0, 1, 2 and 3 respectively. Twenty four were cirrhotic (stage 4) on biopsy. Six were non-cirrhotic: 2 were stage 0 with NAFLD grade 1; and 4 were stage 1-2 with NAFLD grade 2. As such, all non-cirrhotic patients had some degree of inflammation. Notably, there were none

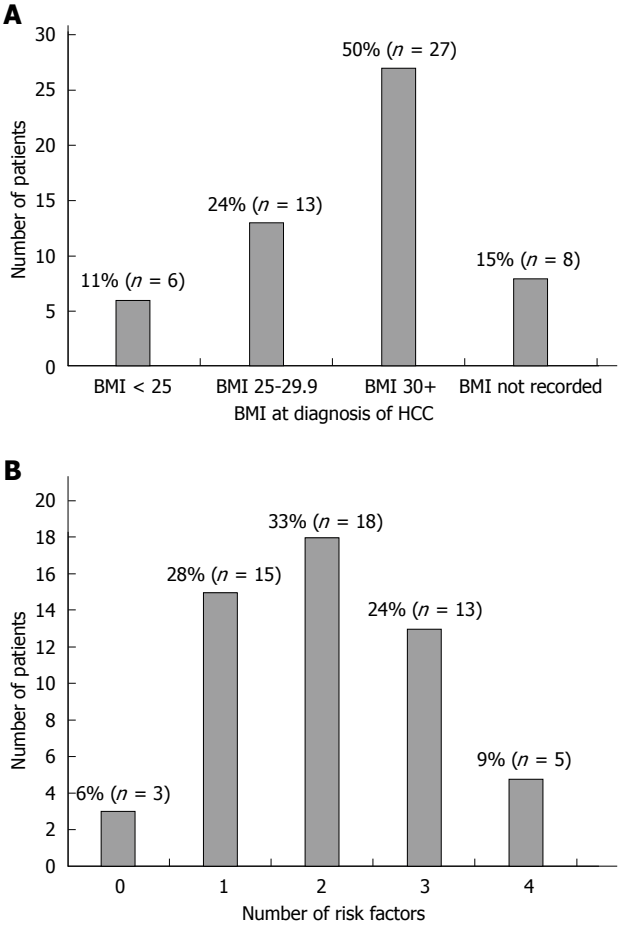

Figure 1 Body mass index at diagnosis of hepatocelullar carcinoma demonstrating that a large proportion of patients were overweight or obese (A) and prevalence of number of risk factors for hepatocelullar carcinoma - overweight or obesity, diabetes, hypertension and dyslipidaemia (B). Notably 34% of patients had less than 2 risk factors.

with just simple steatosis (grade 0) in our cohort.

Twenty four patients did not have biopsies or had missing biopsy information, despite a previous diagnosis of either NAFLD or cryptogenic cirrhosis. Twenty two of these had evidence of cirrhosis on CT or ultrasound scanning, while another two were non-cirrhotic on imaging.

In total, forty six of the fifty four patients had cirrhosis which was diagnosed either histologically in 24 (52%) patients or radiologically in 22 (48%) patients. Of these, 18 (40%), 14 (30%) and 13 (28%) had Child-Pugh scores of A, B and C respectively at time of HCC diagnosis. One patient had missing biochemical information and thus a Child-Pugh score could not be calculated. Demographic and risk factor profiles of patients with NAFLD associated cirrhosis and cryptogenic cirrhosis were not statistically different, except for a higher prevalence of hypertension in the former cohort (Table 1).

Other HCC risk factors: Thirteen patients (24%) were overweight at HCC diagnosis (BMI 25-29.9 kg/m^2) and 27 (50%) were obese (BMI equal to or

Table 2 Comparison between cirrhotics and non-cirrhotics in terms of demographics, non-alcoholic fatty liver disease grade and hepatocellular carcinoma characteristics

	Cirrhotics (*n* = 46)	Non-cirrhotics (*n* = 8)	*P* value
Median age (yr)	65	69	0.227
Median BMI (kg/m^2)	30	30	0.939
Median HCC diameter (cm)	3.2	4.7	0.041
Median number of HCCs	1	2	0.147
Number of risk factors (*n*)			0.969
0	3	0	
1	12	3	
2	16	2	
3	11	2	
4	4	1	
NAFLD grade (*n*)			0.188
0	8	0	
1	9	2	
2	5	4	
3	3	0	
Level of HCC differentation (*n*)			0.317
Well	10	4	
Moderate	7	1	
Poor	0	0	
Necrotic	1	0	

NAFLD: Non-alcoholic fatty liver disease; HCC: Hepatocellular carcinoma; BMI: Body mass index.

Table 3 Comparison between cirrhotics and non-cirrhotics in terms of hepatocelullar carcinoma risk factors

	Cirrhotics (*n* = 46)	Non-cirrhotics (*n* = 8)	*P* value
Overweight/obesity	74%	75%	0.660
Diabetes	61%	50%	0.410
Hyperlipidaemia	24%	37.50%	0.340
Hypertension	43%	50%	0.767

greater than 30 kg/m^2) according to World Health Organisation guidelines. Six (11%) had a BMI under 25 kg/m^2. Median BMI was 31 kg/m^2. In the remaining 8 patients, BMI was not recorded (Figure 1A).

Overall 40 (74%) were either overweight or obese, 32 (59%) were diabetic, 24 (44%) had hypertension and 14 (26%) had dyslipidaemia. Six percent, 28%, 33%, 24% and 9% had 0, 1, 2, 3, 4 of the above risk factors respectively (Figure 1B).

HCC characteristics: Twenty seven (60%) cirrhotic patients were diagnosed while asymptomatic by scheduled screening, with 9 (20%) diagnosed due to symptoms of hepatic decompensation and 10 (20%) diagnosed incidentally when being imaged for other reasons. Four (50%) non-cirrhotic patients were diagnosed due to symptoms and in 4 it was found incidentally. Eighteen (33%) patients had AFP levels in the normal range (< 5.8 kU/L). Median AFP was 12.2 kU/L. In twenty four patients (43%), HCC was already multifocal by time of diagnosis. The median maximum diameter was 3 cm. Twenty patients (37%) were already outside the Milan criteria[26] for transplantation at diagnosis.

Table 4 Correlations between patient characteristics and tumour size

	Pearson correlation	*P* value
Overweight/obesity	-0.256	0.093
Diabetes	0.038	0.791
Hypertension	0.106	0.470
Dyslipidaemia	0.146	0.510
Age at diagnosis	0.142	0.320
Gender	0.042	0.770
BMI	-0.026	0.867
Number of risk factors	-0.090	0.534
Stage of fibrosis	-0.308	0.030
Grade of NAFLD	0.243	0.196
Child-Pugh Score	-0.170	0.238

NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

Differences between non-cirrhotics and cirrhotics: Between non-cirrhotics and cirrhotics there were no statistically significant differences in BMI, age or level of HCC differentiation, number of risk factors, number of HCC's or NAFLD grade. However non-cirrhotic patients had a higher median HCC diameter than cirrhotics at diagnosis (4.7 cm *vs* 3.2 cm, *P* = 0.041). Similarly, a greater proportion of non-cirrhotics failed the Milan criteria for transplantation (87.5% *vs* 28.2%, *P* = 0.003) (Table 2).

There were no statistically significant differences between cirrhotics and non cirrhotics in terms of the prevalence of individual HCC risk factors (Table 3).

Predictors of tumour characteristics: Multivariate analysis was conducted to determine if patient characteristics apart from presence or absence of cirrhosis could predict the size or number of HCCs and thereby the prognosis. Characteristics including sex, presence of diabetes, hypertension, hyperlipidaemia and/or overweight/obesity, number of risk factors, Child-Pugh scores, NAFLD grade and fibrosis stage, age at diagnosis of HCC and BMI were analysed. Only fibrosis stage significantly correlated with tumour size, with non-cirrhotics more likely to have larger tumours than cirrhotics (*P* = 0.03) (Table 4). No characteristics significantly correlated with tumour number (Table 5).

DISCUSSION

This is the first cohort study to examine NAFLD-associated HCC in an Australian context, where the majority were obese and diabetic. Furthermore, it is the first, to our knowledge, to include patients with cryptogenic cirrhosis as a subset of NAFLD given evidence strongly linking the two conditions. Indeed, in our cohort, patients with NAFLD cirrhosis had

Table 5 Correlations between patient characteristics and tumour number

	Pearson correlation	*P* value
Overweight/obesity	0.174	0.248
Diabetes	-0.270	0.053
Hypertension	-0.040	0.779
Dyslipidaemia	0.080	0.715
Age at diagnosis	-0.041	0.771
Gender	-0.090	0.523
BMI	0.064	0.677
Number of risk factors	0.055	0.696
Stage of fibrosis	-0.058	0.682
Grade of NAFLD	-0.192	0.310
Child-Pugh Score	0.179	0.208

NAFLD: Non-alcoholic fatty liver disease; BMI: Body mass index.

similar demographic and risk factor profiles to those with cryptogenic cirrhosis. This concurs well with other studies which have found that patients with cryptogenic cirrhosis are more likely to be obese and up to four times as likely to have diabetes than other forms of cirrhosis[27]. Moreover, there is a high rate of NAFLD development after transplantation for cryptogenic cirrhosis[28].

Our study also adds to the increasing body of evidence that HCC can occur in patients with non-cirrhotic NAFLD, even in those who are non-obese. This concurs well with recent studies including two large Japanese studies of of 292[29] and 87 patients[30] with NAFLD and HCC that reported non-cirrhotic patients comprised 38% and 49% respectively of these patients. Duan *et al*[31], pooling 169 NAFLD patients from 25 smaller cohorts in the literature, found 40% were non-cirrhotic and a French series of 31 HCC patients with at least 2 features of the metabolic syndrome (25 of whom had histological evidence of hepatic steatosis) found 66% were non-cirrhotic[32].

There are multiple postulated mechanisms for HCC occurring in non-cirrhotic NAFLD. Hepatic steatosis and concomitant insulin resistance causes oxidative (*via* increased reactive oxygen species[33] and carcinogenic metabolites of lipid peroxidation such as trans-4-hydroxy-nonenal[34]), inflammatory (upregulation of tumour necrosis factor-α, interleukin-6 and nuclear factor kappa-light-chain-enhancer of activated B cells[35]), apoptotic and hormonal changes. Increased activity of c-Jun amino-terminal kinase 1 and consequent phosphorylation of insulin receptor substrate-1[36] increase hepatic inflammation and apoptosis through downstream modulation of such pathways as mitogen-active protein kinase and phosphatidylinositol-3 kinase[37]. Adiponectin levels are decreased in NAFLD with subsequent loss of its anti-angiogenic and anti-inflammatory effects[38]. There are also decreased levels of binding proteins of insulin-like growth factor-1, with its subsequent increased bioavailability promoting cellular proliferation[39]. More novel pathways being investigated include the phosphorylation of adenosine monophosphate-activated protein kinase and activation of target of rapamycin complex 1 which then inhibits hepatic autophagy and its "quality control" effects in the liver to remove carcinogenic material[40].

Our finding that HCCs in non-cirrhotic patients are significantly larger at diagnosis is concerning as tumour size correlates with worse prognosis according to the Barcelona Clinic Liver Cancer staging system[6] and likely reflects lead time bias due to a lack of screening. Also, importantly, none of these patients with HCC had cholangiocarcinoma. A similar result was found by Paradis *et al*[32] in a French audit of 31 patients in whom 20 (65%) were non-cirrhotic with F0-F2 fibrosis scores, with larger tumours than in cirrhotics (median diameter 10.1 cm *vs* 6.6 cm, $P = 0.05$). While this suggests that HCC screening programs in non-cirrhotic NAFLD patients may prevent significant morbidity, the high prevalence of NAFLD in the general population makes such screening difficult to justify in terms of cost-effectiveness.

Furthermore, the incidence of HCC development in non-cirrhotic NAFLD appears to be low. There is limited literature addressing this issue with many longitudinal cohort studies having either focussed on NAFLD patients who are cirrhotic at baseline, or are confounded by not analysing if non-cirrhotic NAFLD patients who develop HCC had also become cirrhotic in the interim. In 399 non-cirrhotic NAFLD patients followed up over a mean of 7.6 years who did not develop cirrhosis on serial biopsies, Adams *et al*[41] found no HCCs. Similarly, no HCCs were found in a study of 64 non-cirrhotic patients followed up over a mean of 13.7 years[42] and a cohort of 109 patients over 16.7 years[43]. In the largest cohort of NAFLD patients prospectively studied, Arase *et al*[3] found in 1600 ultrasound-diagnosed NAFLD patients followed up over a median of 8.2 years, a HCC incidence of 0.6% (10 patients) was found, which is equivalent to 0.08% per year. Out of these 10 patients, 7 had histology taken at the time of HCC diagnosis with 6 being non-cirrhotic (Stage 0-3). Unfortunately, this study did not specify the proportion of cirrhotics in the total cohort. These low rates correlate with the data from our large centre study which identified only 15 non-cirrhotic NAFLD patients in 13 years. Moreover, there is no way to establish a clear causal link between steatosis and carcinogenesis in all these patients. Some may have been patients who developed sporadic HCC in whom the presence of hepatic steatosis may have been coincidental. Others may have had HCC derived from a pre-existing adenoma, since there is increasing literature postulating that the metabolic syndrome may drive malignant transformation of adenomas[44].

If HCC screening of non-cirrhotic NAFLD patients

were to be viable, this would require narrowing the screened population by targeting those with specific risk factor profiles associated with HCC. Unfortunately we could not identify such a profile that defined our non-cirrhotic group. Comparisons of previous large cohort studies suggest that patients with NAFLD-associated HCC are more likely to be diabetic than the general NAFLD population (up to 70%[29] *vs* 46%[45], respectively). However, the proportion of non-cirrhotics who develop HCC in these studies was not clear and they were performed in Japanese cohorts making their findings potentially less generalisable to a Western population. Moreover, the high prevalence of diabetes in the NAFLD population means this cannot be used alone as an indication for cost-effective HCC screening. We have also shown that the cumulative number of HCC risk factors is not useful in determining HCC risk, as many of our non-cirrhotic cohort had less than 2 risk factors. Similarly we were unable to identify patient characteristics or risk factors correlating with poorer tumour prognosis that theoretically could guide targeted screening. Finally we have found AFP poorly sensitive for the presence of HCC, reflecting previous literature and 2011 AASLD guidelines that recommend against the use of AFP to guide screening[22].

In conclusion, this is the first Australian study describing the risk factor profile of patients with NAFLD-associated HCC. Even though we have a small study size and a significant bias toward males, we have shown that HCC occurs in non-cirrhotic NAFLD patients. However this is of uncertain clinical significance in the context of the burgeoning NAFLD epidemic as rates of this phenomenon still appear low. In such patients, tumours are larger than in cirrhotics which may be due to delayed diagnosis due to lack of screening. In regards to screening, we suggest, firstly, that novel biomarkers with more accuracy than AFP should be identified to better complement radiological screening. Secondly, of more importance, HCC screening in non-cirrhotic NAFLD cannot be deemed cost-effective without further studies identifying specific risk factor profiles that significantly restrict the potential target population.

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COMMENTS

Background

The prevalence of non-alcoholic fatty liver disease (NAFLD) is rising worldwide, especially in industrialised countries such as Australia. This condition can progress to cirrhosis and the development of hepatocellular carcinoma (HCC). Furthermore, the condition of "cryptogenic cirrhosis" is often thought to represent end stage-NAFLD.

Research frontiers

Basic science research has elucidated pro-carcinogenic mechanisms by which NAFLD could cause HCC in the absence of cirrhosis. At present, however, no guidelines recommend screening for HCC in non-cirrhotic NAFLD patients.

Innovations and breakthroughs

Of concern, there have been increasing reports of HCC occurring in non-cirrhotic NAFLD internationally over the last decade. This phenomenon, however, has not yet been described in an Australian cohort. Diabetes and obesity have been found to be independent risk factors for HCC development in NAFLD, but further research is needed to define such risk factors in specifically non-cirrhotic NAFLD cohorts that could guide cost-effective HCC screening.

Applications

This study reaffirms that HCC can develop in non-cirrhotic NAFLD, but could not identify particular risk factor profiles differentiating such patients from cirrhotic patients who develop HCC. Non-cirrhotic patients, however, had larger tumours at diagnosis than cirrhotic patients. This study thus underlines the need for further research into HCC risk factors amongst non-cirrhotic NAFLD patients, such that future HCC screening guidelines may take such patient groups into consideration in a cost-effective and targeted manner.

Peer review

In this paper, the authors examined clinicopathological features of cases of NAFLD that developed HCC. As a result, they confirmed that HCC could develop in NAFLD without cirrhosis, and non-cirrhotics had a significantly larger mean tumour diameter than cirrhotics. HCC cases originating from NAFLD have been increasing, and to elucidate clinicopathological features of these cases is important. The present study elucidates a clinical aspect of carcinogenesis in NAFLD, and its results are important in establishing the screening method for non-cirrhotic NAFLD patients.

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ORIGINAL ARTICLE

Retrospective Study

Inflammatory bowel disease in an underdeveloped region of Northeastern Brazil

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Abstract

AIM: To evaluate the demographic characteristics and clinical phenotypes of inflammatory bowel disease (IBD) in a geographic area in Northeastern Brazil.

METHODS: This retrospective study was conducted at the Hospital of the Federal University of Piauí in Northeastern Brazil. Demographic characteristics and clinical phenotypes of IBD were analyzed in relation to the time of diagnostic confirmation, which was defined as the date of disease onset. Data were collected between January 2011 and December 2012 and included all census patients 18 years of age or older during that period for whom there was diagnostic confirmation of Crohn's disease (CD), ulcerative colitis (UC), or unclassified colitis according to the Montreal criteria. We also analyzed the period of time between the onset of clinical manifestations and the diagnosis of IBD (delay in the diagnosis). Statistical analyses included means and standard deviations for numeric variables and the Pearson χ^2 adherence test for nominal variables. The annual index occurrence and overall prevalence of IBD at our institution were also calculated, with P values < 0.05 indicating statistical significance. This study was approved by the Institutional Ethics and Research Committee.

RESULTS: A total of 252 patients with IBD were included, including 152 (60.3%) UC patients and 100 (39.7%) CD patients. The clinical and demographic characteristics of all patients with IBD showed a female to male ratio of 1.3:1.0 and a mean age of

35.2 (SD = 14.5) years. In addition, the majority of patients were miscegenated (171, 67.9%), had received higher education (157, 62.4%), lived in urban areas (217, 86.1%), and were under the age of 40 years (97, 62.5%). For patients with CD, according to the Montreal classification, the predominant features present from the onset of disease were an age between 17 and 40 years (A2); colonic disease location (L2); and nonstricturing, nonfistulizing disease behavior (B1). However, approximately one-quarter of all CD patients demonstrated perineal involvement. We also observed considerable delay in the diagnosis of IBD throughout the entire study period (mean = 35.5 mo). In addition, the annual index occurrence rose from 0.08 to 1.53 cases/10^5 inhabitants/year during the study period, and the prevalence rate was 12.8 cases/10^5 inhabitants in 2012. Over the last two decades, there was a noted increase in the frequency of IBD in the study area.

CONCLUSION: In this study, there was a predominance of patients with UC, young people under 40 years of age, individuals with racial miscegenation, and low annual incomes.

Key words: Inflammatory bowel diseases; Crohn's disease; Ulcerative colitis; Epidemiology; Human Development Index

Core tip: This study addressed the demographic characteristics and clinical phenotypes of inflammatory bowel disease (IBD) patients in Northeastern Brazilian, where living conditions are poor and there is a lack of data on this subject. Over the last two decades, there was a noted increase in the frequency of IBD in the study area, although there was considerable delay in disease diagnosis throughout the study period. There was a predominance of patients with ulcerative colitis, but there was no difference between males and females in terms of disease frequency. Most individuals were aged below 40 years, had miscegenated ethnic characteristics, and received low annual incomes.

Parente JML, Coy CSR, Campelo V, Parente MPPD, Costa LA, da Silva RM, Stephan C, Zeitune JMR. Inflammatory bowel disease in an underdeveloped region of Northeastern Brazil. *World J Gastroenterol* 2015; 21(4): 1197-1206 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1197.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1197

INTRODUCTION

Inflammatory bowel disease (IBD) encompasses a group of chronic and idiopathic inflammatory diseases preferentially affecting the gastrointestinal tract (GIT). Two subcategories, Crohn's disease (CD) and ulcerative colitis (UC), have been highlighted[1]. CD is characterized by discontinuous and transmural inflammation that can involve any segment of the GIT, sometimes presenting stenotic or penetrating behavior with the formation of abscesses and fistulas[2]. UC is an inflammatory process confined to the mucosa and submucosa of the large intestine, with a characteristic gradient of greater to minor severity in the distal to proximal direction[3,4]. Unclassified colitis is defined when the disease involves only the large intestine and presents superimposed clinical and endoscopic characteristics of both CD and UC[5,6].

The incidence and prevalence of IBD are higher in countries with greater economic development, especially in the northern countries of Western Europe, Canada, the United States of America, Australia, and New Zealand[7,8]. In recent decades, there has also been an increase in these rates in countries of Southern and Eastern Europe and, to a lesser extent, the Middle East, North Africa, and some Asian countries[9-11]. In Latin America, there are few epidemiological studies of IBD, although some studies have reported growth in the frequencies of both CD and UC in this region, despite the low incidence of these diseases[12,13].

In Brazil, epidemiological studies of IBD are also very scarce, although increased frequencies of outpatient visits and hospitalizations in the major urban centers of Brazil have been observed[14-16]. However, no studies have been conducted with large Brazilian territorial coverage regarding the demographic and clinical aspects of IBD.

The purpose of this study was to address the lack of data on IBD in the state of Piauí, an area in the Brazilian Northeast, where living conditions are considered the worst (Figure 1). In recent decades, Brazil has experienced a continuous increase in the Human Development Index (HDI) from 0.590 in 1990 to 0.718 in 2010, although the country still stands at 84th in United Nations rankings. In Piauí, the HDI was reportedly lower (0.646 in 2010) than the HDI of southern and southeastern Brazilian states (HDIs between 0.731 and 0.783) and the federal capital (an HDI of 0.824)[17].

The main objective of this study was to identify the demographic characteristics and clinical phenotypes of IBD in a geographic area in Northeastern Brazil with a low HDI. In addition, we sought to calculate the annual index rate for the occurrence and prevalence of IBD at our institution.

MATERIALS AND METHODS

Study location

The study was conducted at the Hospital of the Federal University of Piauí (HU-UFPI), which is considered a reference center for the treatment of

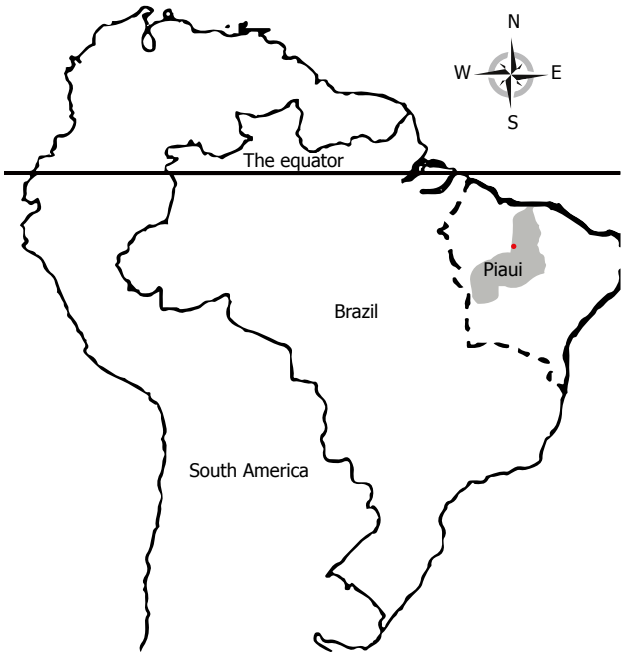

Figure 1 Study area (Piauí State), located in the tropical zone in the northeastern region of Brazil.

patients with IBD. The strategic location of this hospital in the capital of Piauí, Teresina, and its inclusion in a computerized public health network result in the referral of patients from all other hospitals and public health centers throughout the state. In the state of Piauí, approximately 85% of the population receives health care solely through the public system, and our institution has been the only public hospital in the state to care for patients above 15 years of age with IBD.

Diagnosis of IBD

The diagnosis of IBD was established according to previously developed criteria for CD and UC[1], including clinical, ileocolonoscopic, laboratory, and histopathological aspects as well as computed tomography (CT) or magnetic resonance imaging enterography studies of the small intestine. When necessary, we performed endoscopic examinations of the upper GIT to evaluate the esophagus, stomach, and duodenum. All patients underwent investigation for gastroenteritis (coproculture) and intestinal parasites (stool test). In view of the high prevalence of enteroparasitoses in the study region, all patients received antiparasitic treatment with albendazole, secnidazole, and ivermectin regardless of the outcome of the stool examinations. The differential diagnosis of intestinal tuberculosis was based on clinical data, chest radiography, Mantoux intradermal testing, and the histological results of biopsy specimens. Mansonic schistosomiasis is endemic in many areas of Northeastern Brazilian, although there were no outbreaks in the region covered by this study.

Study design

The study was designed to describe the demographic and clinical characteristics of patients with IBD at the time of diagnostic confirmation, which was defined as the date of disease onset. This retrospective study involved a cohort of patients who were in clinical follow-up at HU-UFPI. The subjects' demographic and clinical data were collected directly from the medical records of the Digestive System Unit of HU-UFPI and were supplemented with patient interviews during periodic outpatient clinical reviews.

The data were collected between January 2011 and December 2012 and included all census patients 18 years of age or over for whom there was diagnostic confirmation of CD, UC, or unclassified colitis. Individuals who received disease diagnoses during childhood or adolescence but who were at least 18 years of age at the time of data collection were also included.

The dependent variables included diagnosis (CD or UC), classification of CD and UC according to the Montreal criteria[6], and the period of time between the onset of clinical manifestations and the diagnosis of IBD. The independent demographic variables included age, gender, race, education, family income, and residence in an urban or rural area. The independent clinical variable was a family history of IBD. Patients who were diagnosed with unclassified colitis at the onset of the disease were included in the CD or UC groups, considering the subsequent diagnostic definition established during clinical follow-up of these individuals.

Statistical analysis

To perform statistical analyses, we first created a database using Microsoft Excel, the results of which are presented in tables and graphs. The following analyses were used: means and standard deviations for numeric variables and the Pearson χ^2 adherence test for nominal variables (gender, race, education, and income). These variables were compared with the respective census data for the population of the state of Piauí. The significance level used for all tests was 5%.

We also calculated the annual index occurrence and the prevalence rate of IBD in our hospital based on the annual frequency of IBD and annual population data from the state of Piaui (85% of people over 15 years of age, as explained in the "study location" section) for the period from 1988 to 2012, according to census data from the Brazilian government (Instituto Brasileiro de Geografia e Estatística)[18].

Ethical considerations

This study was approved by the Research Ethics Committee of our institution (CAAE: 0140.0.045.000-11), and ethical principles for medical research involving

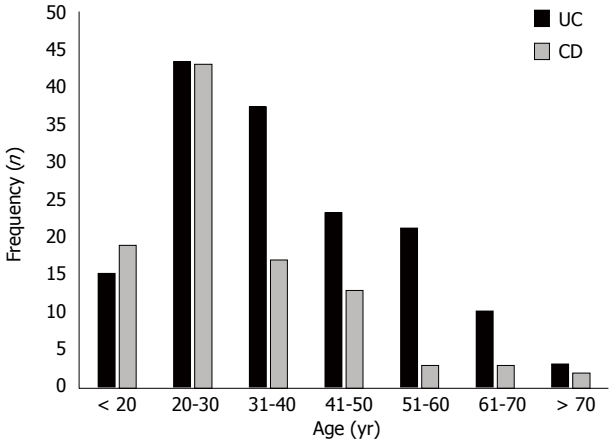

Figure 2 Distribution of patients with ulcerative colitis and Crohn's disease according to age group in Piauí State (Brazil), 1988-2012. UC: Ulcerative colitis; CD: Crohn's disease.

human subjects were observed during all stages, including ensuring the anonymity of patients. All participants were adequately informed about the study and signed an informed consent form authorizing their inclusion in the study.

RESULTS

The confirmation of IBD diagnosis for all patients included in this study occurred between 1988 and 2012. Two hundred fifty-two consecutive patients treated in the IBD outpatient unit were included, of which 152 (60.3%) had UC and 100 (39.7%) had CD. The age at disease onset ranged from 12 years to 82 years, with a mean of 35.2 (SD = 14.5) years. The mean ages for the onset of CD and UC were 32.9 (SD = 13.6) years and 36.8 (SD = 14.8) years, respectively. Figure 2 shows the frequency distribution of the ages of patients with CD and UC at the time of diagnosis.

Regarding gender, there was a male to female ratio of 1.2 to 1.0 in the group of patients with CD, but there was no significant association with gender upon statistical analysis (P = 0.32). Patients with UC were predominantly female, with a female to male ratio of 1.8 to 1.0; this association was statistically significant (P = 0.005). Table 1 shows the demographic aspects of the study subjects and the population characteristics of the state of Piauí for comparison and statistical analysis. The patients' clinical features according to the Montreal[6] classification are shown in Table 2 for CD and Table 3 for UC.

The annual rate of new IBD cases increased slowly between 1988 and 1998, corresponding to a rate of one to five new patients per year in that decade. In the last years of the twentieth century and the first decade of this century, significant increases in the gross annual frequencies of these diseases were observed, reaching a rate of 25 new cases of IBD (CD = 12, UC = 13) in 2012. In this context, given both the natural population growth of the state of Piauí and the gross annual rate of new cases identified in this study, the annual index occurrence of IBD at our institution was 0.08 cases/10^5 inhabitants/year in 1988, and this rate reached its peak in 2007 with 1.53 cases/10^5 inhabitants/year (Figure 3). In 2012, the prevalence of IBD at our institution was 12.8 cases/10^5 inhabitants.

Throughout the study period, there was considerable delay in the diagnosis of IBD. In particular, the mean time (in mo) between the onset of clinical manifestations and the diagnosis of IBD throughout the entire study period was 35.5 mo.

Regarding the etiopathogenesis of IBD, we analyzed two aspects: a family history of UC or CD and tobacco use. There was a history of IBD among first- and second-degree relatives in 29/252 (11.5%) cases, including 16/100 (16.0%) CD patients and 13/152 (8.6%) UC patients. A personal history of previous or current smoking was noted in 53/252 (21.0%) of all patients with IBD, including 21/100 (21.0%) with CD and 32/152 (21.1%) with UC.

DISCUSSION

Historical data for overall IBD geographic distribution worldwide have consistently shown higher rates of incidence and prevalence in more developed countries, the populations of which are predominantly Caucasian[12]. More recently, IBD has been detected with increasing frequency across all continents, including less developed countries, affecting people with different ethnic characteristics[9,10,19].

This study was conducted in a Brazilian region with the lowest socioeconomic human development indicators. In line with the low HDI, the average income per capita of the population of the state of Piauí (2965.00 USD per year) is well below the average per capita income of Brazil (4602.12 USD per year), while the average family income of participating patients in this research was higher [7084.80 (SD = 531.50) USD][18]. In this Brazilian region with poor living conditions, IBD is still a rare clinical condition compared to countries with high HDIs, where incidence rates are historically much higher, usually from 10.0 to 20.0 cases/10^5 inhabitants/year as well as higher than 20.0 cases/10^5 inhabitants/year[8]. However, in the 25 years of this study, we found that there was a gradual increase in the annual index occurrence at our hospital, reaching 1.53 cases/10^5 inhabitants/year and culminating in an intermediate prevalence rate corresponding to 12.8 cases/10^5 inhabitants in 2012. Our results were still much lower compared to those reported by Victoria *et al*[15] for the period of 1986 to 2005 in a more industrialized area of Southeastern Brazil, where

Table 1 Demographic characteristics of the population of the state of Piauí (Brazil) in 2010 and of patients with inflammatory bowel disease (total), Crohn's disease, and ulcerative colitis according to gender, race, education, family income, and residence (urban or rural) in Piauí (Brazil), 1988-2012

Demographic variables		General population of Piauí[1]: (*n* = 3118360)	IBD phenotype		
			CD (*n* = 100) *n* (%)	UC (*n* = 152) *n* (%)	Overall IBD (*n* = 252) *n* (%)
Gender	Male	49.0%	54 (54.0)	55 (36.2)	109 (43.3)
	Female	51.0%	46 (46.0)	97 (63.8)	143 (56.7)
	χ^2 test	-	*P* = 0.32	*P* = 0.00	*P* = 0.07
Race	Miscegenated	64.0%	64 (64.0)	107 (70.4)	171 (67.9)
	White	24.4%	26 (26.0)	34 (22.4)	60 (23.8)
	Black	9.4%	10 (10.0)	10 (6.6)	20 (7.9)
	Yellow	2.2%	0 (0)	1 (0.6)	1 (0.4)
	χ^2 test	-	*P* = 0.50	*P* = 0.24	*P* = 0.18
Education (yr of schooling)	Uneducated and < 9 yr	58.2%[2]	25 (25.0)	70 (46.0)	95 (37.8)
	⩾ 9 yr	41.8%	75 (75.0)	82 (53.9)	157 (62.4)
	χ^2 test	-	*P* = 0.00	*P* = 0.00	*P* = 0.00
Residence	Urban area	65.8%	93 (93.0)	124 (81.6)	217 (86.1)
	Rural area	34.2%	7 (7.0)	28 (18.4)	35 (13.9)
	χ^2 test	-	*P* = 0.00	*P* = 0.00	*P* = 0.00
Average income	Monthly[3]	247.00 USD	643.50 USD	555.40 USD	590.40 USD

[1]Source: Brazilian Institute of Geography and Statistics (Instituto Brasileiro de Geografia e Estatística - IBGE [database online], 2010); [2]Individuals aged > 18 yr; [3]State of Piauí = income per capita, study population: family income. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis.

Table 2 Clinical features of patients with Crohn's disease at diagnosis according to the Montreal classification6 in Piauí (Brazil), 1988-2012

Phenotypic elements		*n* (%)	Female, *n* (%)	Male, *n* (%)
Age at diagnosis (A)	A1: ⩽ 16 yr old	8 (8.0)	2 (25.0)	6 (75.0)
	A2: 17-40 yr old	71 (71.0)	30 (42.3)	41 (57.7)
	A3: > 40 yr old	21 (21.0)	14 (66.7)	7 (33.3)
Disease location (L)	L1: Terminal ileum	15 (15.0)	7 (46.7)	8 (53.3)
	L2: Colonic	36 (36.0)	17 (47.2)	19 (52.8)
	L3: Ileocolonic	17 (17.0)	7 (41.2)	10 (58.8)
	L4: Isolated upper disease	7 (7.0)	1 (14.3)	6 (85.7)
	L1, L2 or L3: Concomitant with L4	25 (25.0)	14 (56.0)	11 (44.0)
Disease behavior (B)	B1: Nonstricturing, nonfistulizing	69 (69.0)	33 (47.8)	36 (52.2)
	B1 + p (perianal disease modifier)	19 (27.0)	7 (36.8)	12 (63.2)
	B2: Stricturing	18 (18.0)	6 (33.3)	12 (66.7)
	B2 + p (perianal disease modifier)	3 (16.7)	2 (66.7)	1 (33.3)
	B3: Penetrating[1]	13 (13.0)	7 (53.8)	6 (46.2)
	B3 + p (perianal disease modifier)	5 (38.5)	2 (40.0)	3 (60.0)

[1]Rectovaginal fistula = 4; Entero-cutaneous fistula = 6; Entero-enteric fistula = 2; Acute perforated abdomen = 1.

the incidence rates rose from 1.0 to 8.0 cases/10^5 inhabitants/year and the prevalence increased from 1.2 to 20.5 cases/10^5 inhabitants in the same period. However, our results are consistent with the findings of researchers from other Brazilian regions[20,21] and South American countries[12,13], who have observed higher frequencies of CD and UC in hospitals based in South America. This finding suggests that IBD is also increasing in Latin America, even in regions with specific geographical, climatic, and socioeconomic characteristics that differ from those where IBD was commonly reported a few decades ago.

We assumed that the above data pertaining to the annual index occurrence and prevalence rates were not the true incidence and prevalence rates for IBD in the entire population of the state of Piauí but instead represent only an estimated statistical calculation, as this was not the main focus of the study design. In addition, other relevant factors in this regard should be emphasized, including the possibility that patients were diagnosed and treated without being referred to our institution and the lack of a state-wide registry of billing codes to verify that all IBD patients were identified and registered. Despite these potential biases, and considering that there are no epidemiological studies of IBD in this Brazilian region, we believe that the annual index occurrence and prevalence rates found in this study are representative of the true rates in the state of Piauí, which have yet to be properly calculated in future studies.

Table 3 Clinical aspects of patients with ulcerative colitis (*n* = 152) at the time of diagnosis according to the modified Montreal classification in Piauí (Brazil), 1988-2012

Phenotypic elements		*n* (%)	Female, *n* (%)	Male, *n* (%)
Age at diagnosis (A)[1]	A1: ⩽ 16 yr old	7 (4.6)	3 (42.9)	4 (57.1)
	A2: 17-40 yr old	88 (57.9)	56 (63.6)	32 (36.4)
	A3: > 40 yr old	57 (37.5)	38 (66.7)	19 (33.3)
Disease extent (E)	E1: Ulcerative proctitis	14 (9.2)	9 (64.3)	5 (35.7)
	E2: Keft-sided ulcerative colitis	93 (61.2)	63 (67.7)	30 (32.3)
	E3: Extensive ulcerative colitis (pancolitis)	45 (29.6)	25 (55.6)	20 (44.4)
Disease severity (S)	S1: Mild	41 (27.0)	28 (68.3)	13 (31.7)
	S2: Moderate	60 (39.5)	40 (66.7)	20 (33.3)
	S3: Severe	51 (33.5)	29 (56.9)	22 (43.1)

[1]Age at diagnosis is not a phenotypic element of the Montreal classification for ulcerative colitis.

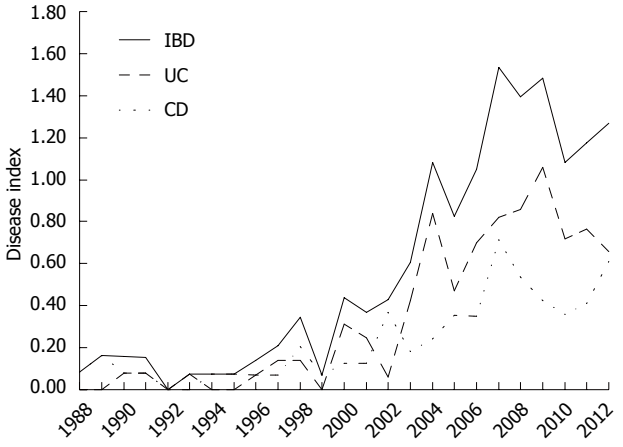

Figure 3 Annual index occurrence of inflammatory bowel disease (total), ulcerative colitis, and Crohn's disease in Piauí State (Brazil) in the period from 1988-2012. IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

The racial phenotype of Brazil's population is extremely heterogeneous. In particular, the population of Brazil has historically been influenced by individuals with European, African, Asian, and Amerindian ancestries, and there is significant miscegenation variability across geographical regions of the country. The general population of the state of Piauí, where the subjects of this study resided, predominately consists of individuals with miscegenated ethnic characteristics, with only a small portion of people with unique characteristics of white or black race and with little representation of Asian or purely indigenous individuals[18]. The subjects participating in our study also exhibited an ethnic profile similar to the general population where they reside; that is, the study showed no correlation between racial phenotype and the occurrence of IBD. Therefore, it appears that the ethnic characteristics of the study population differ from the pattern established in countries where there are higher traditional IBD incidence and prevalence rates, *i.e.*, countries with a predominance of Caucasian individuals[22]. In fact, this miscegenated aspect of IBD patients in this region of Brazil is in keeping with the results of other studies that characterized these diseases as clinical entities emerging in the most diverse latitudes and longitudes of the planet, which are gradually and increasingly affecting other races and ethnicities in addition to Caucasians[11,23-26].

The literature data show that there are usually similar prevalence rates of IBD in men and women, although some studies have reported a slight predominance in males[27,28]. Our study showed that for UC patients, there was a significantly greater prevalence of women with this disease. These results are consistent with those reported by Kleinumbing-Júnior *et al*[14] in Southern Brazil.

In relation to the patient's age at CD diagnosis, we observed disease occurrence in all age groups, although there was a predominant initial involvement in young individuals, with a well-pronounced peak incidence between 21 and 30 years of age. Our data are in agreement with the results reported by Thia *et al*[29] in a large population-based study, in which most patients were aged between 17 and 40 years (A2 in the Montreal classification). When segments of the GIT were considered individually, the topographic region most affected by CD in our study was the large intestine. However, the overall involvement of the small intestine above the distal ileum (L4 alone or associated with L1-L3) was well above that reported by other studies in the literature. Considering the behavior of CD, our results are similar to those reported by other researchers[14,27-30]. Approximately one-quarter of all CD patients demonstrated perineal involvement from the time of disease onset, which was more predominant in patients with involvement of the terminal ileum.

As observed in the group of patients with CD, the onset of UC occurred in groups of younger individuals. In UC, the highest peak incidence occurred in the age groups between 21 and 40 years, although this disease also achieved significant frequency in middle-aged individuals, with a second peak in the age groups between 41 and 60 years. As a result, IBD has strong social, educational, economic, and family impacts on

affected individuals, as the phases of disease activity coincide with the period of life when they are in full educational activity, starting their professional career, and forming their family bases. Our results are very similar to those reported by Manninen *et al*[31] in Finland and the multicenter study conducted by Tozun *et al*[32] in Turkey. However, studies of other populations, such as the multicenter study conducted by Ng *et al*[19] in Asia-Pacific countries, the review by Rocchi *et al*[33] on IBD in Canada, and the population-based study conducted by Vind *et al*[34] in Denmark, have indicated that UC can start at all ages, commencing with a peak incidence in the first decades of life but maintaining a plateau of new cases in all subsequent age groups, even after 60 years of age.

Regarding the extent of UC, there are variations in the results presented in various studies, but in general, there is a higher incidence of distal UC (proctitis) in the initial presentation of the disease, followed by left UC and, to a lesser degree, pancolitis[3]. Our series disagreed on this point, as we observed a higher frequency of involvement of left UC and a low frequency of involvement of the rectum (E2 > E3 > E1, according to the Montreal classification for UC). These results are similar to those of studies by Zeng *et al*[27] in China and Lakatos *et al*[28] in Hungary. Regarding the profile of clinical severity, there was a slight predominance of the moderate form of UC, followed by the mild and severe forms (S2 > S3 > S1).

Given the low frequency of IBD in Brazil until a few decades ago, it has been difficult for physicians to readily recognize these diseases as a result of various factors, including the lack of IBD-related knowledge among health professionals and the lack of adequate diagnostic resources. For these reasons, there has been a delay in making correct IBD diagnoses in Brazil. We observed that there was a noted reduction in the time interval between the onset of clinical manifestations and the diagnosis of IBD in the last three 5-yr periods; the mean delay in diagnosis was initially 67.5 mo, although this time decreased to 40.7 mo and more recently to 25.1 mo. We can likely ascribe this fact to the opening of specialized services for the treatment of IBD in HU-UFPI, with improvements in physical facilities and complementary examinations, in addition to better training of health care staff responsible for patient service. Currently, the time required for IBD diagnosis in the state of Piaui is still well above the few months of delay observed by Gower-Rousseau *et al*[30] in France and Vind *et al*[34] in Denmark, although similar to results obtained by Zeng *et al*[27] in China and Manninen *et al*[31] in Finland.

Our analysis of educational data indicated that patients with IBD had a higher level of education than the general population of the state where they reside, which signifies that they had fully completed primary education, had at least 9 years of schooling, and had attended or completed high school or higher education. The results of the demographic profile of the subjects of our research, including educational level and age at disease onset, were similar to the results reported in other epidemiological studies of IBD; this profile consisted of disease onset at any age, but affecting mainly young people and tending to occur in individuals with higher educational levels[28,30,35-37].

Family history is considered the main risk factor for the onset of IBD because of several studies that demonstrated the existence of familial aggregation, concordance between monozygotic twins, and a greater prevalence in Ashkenazi Jews. The involvement of CD or UC in a family member indicates a significant increase for the risk of a first-degree relative also having the same disease[2,3,38,39]. In this sense, our results revealed that 4.0% of patients with IBD also had first-degree relatives with one of these diseases, and this rate was increased to 11.5% when second-degree relatives were also considered. In this regard, there was a greater association with family history in the CD group (16.0%) than in the UC group (8.6%).

In addition to familial aggregation, other correlations between IBD and environmental factors have been considered. We analyzed some of the factors that may influence the pathogenesis of IBD in this group of patients, including the recent population migration to urban centers. In the last 50 years, there has been an acceleration of population migration from rural to urban areas in Brazil, although this phenomenon has become more significant only in the last 30 years in Northeastern Brazil. In the period from 1980 to 2010, the urban Brazilian population increased sharply from 67.6% to 84.4% in all regions of Brazil, from 50.5% to 73.2% in Northeastern Brazil, and from 42.0% to 65.8% in the state of Piaui[18]. The progressive increase in the annual index occurrence of IBD in this study (Figure 2) coincides precisely with the period in which the migratory wave of rural populations to urban areas was observed. In fact, most patients with IBD in our study resided in urban areas, while only 14% lived in the countryside when the disease was diagnosed. It is possible that the level of higher education among patients with IBD may be related to increased access to education in urban areas, in contrast to the lower education of the general population of the state of Piauí, rather than to an etiopathogenic association or an increased risk to develop these diseases. However, further studies need to be conducted in this developing and newly urbanized population group to assess the impact of social changes, including lifestyle, eating habits, types of occupation and other environmental factors, on the risk of IBD emergence. Such a study would make it possible to demonstrate whether there is in

fact a positive association between the environment in urban areas, possibly related to lifestyle and eating habits, and the onset of both CD and UC, as previously suggested in several studies[40-44].

Analysis of the association between prior or current tobacco use and the onset of IBD in our patients indicated that the frequency was similar in both groups with CD and UC. Therefore, no protective effects or increased susceptibility to CD were observed in relation to tobacco use, as previously suggested by some epidemiological[39,42,43] and experimental studies[45]. However, this issue was not analyzed in greater depth, and there may be other factors that could adequately explain the associations between onset, phenotypes, or IBD behavior and tobacco smoking.

In conclusion, the results of our study showed that there was a predominance of IBD patients with UC. In total, there was no difference between males and females in terms of disease frequency, although there was a significantly greater UC frequency in women. Most individuals were aged below 40 years, had miscegenated ethnic characteristics, and received low annual incomes. There was also a significant increase in the annual index occurrence of IBD at our institution. The IBD prevalence rate was found to be intermediate, lying between the high rates measured in more developed countries and the low prevalence rates in other areas. There was also considerable delay in the diagnosis of IBD, which, on average, was approximately two and a half years.

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COMMENTS

Background

Crohn's disease (CD) and ulcerative colitis (UC) are chronic inflammatory bowel diseases (IBD) of unknown etiology and multifactorial pathophysiology, which affect the gastrointestinal tract. Historically, the incidence and prevalence of IBD have been highest in economically developed nations with a predominantly Caucasian population, such as the northern countries of Western Europe, Canada, United States, Australia and New Zealand. Recent epidemiological studies have shown a gradual increase in the prevalence of CD and UC in different continents.

Research frontiers

In Brazil, there are still few epidemiological studies regarding the demographic and phenotypic characteristics of IBD. This study was carried out at a specialized university center for the care of IBD in Northeast Brazil, a region that is much less economically developed and has a lower Human Development Index (HDI) than the South and Southeast regions of the country.

Innovations and breakthroughs

The results of the study showed some similarities with other studies: most subjects lived in urban areas, were aged under 40 years, had a higher level of education and higher family income than the population of that region. On the other hand, this population presented some other characteristics: they were predominantly female, especially patients with UC and they had ethnic characteristics similar to those of the population of the studied region: predominantly with characteristics of racial miscegenation and less interaction between white and black people. Of considerable interest was the observation that there was a marked increase in the incidence of IBD in the studied region during recent decades.

Applications

The study results suggest that IBD have become more frequent in recent decades in this region of Brazil, and they affect populations with different racial and socioeconomic characteristics than those with a historically high prevalence and incidence of CD and UC.

Terminology

The HDI, the index adopted by the United Nations, classifies countries into: developed (HDI from 0.800 to 1.000, *i.e.*, very high development), developing (HDI from 0.700 to 0.799 and HDI from 0.600 to 0.699, *i.e.*, high and medium human development, respectively) and underdeveloped countries (HDI from 0.500 to 0.599 and HDI from 0.000 to 0.499, *i.e.*, low and very low human development, respectively). According to this classification, Brazil has a high human development (HDI = 0.730). However, the region studied has a medium human development (HDI = 0.646).

Peer review

This is an excellent descriptive study in which the authors analyzed the increased frequency of IBD in a region of Brazil that has a medium human development index. The study results showed that IBD are expanding to other parts of the world, and they also occur in populations other than those that have a higher prevalence of individuals with Caucasian ethnic characteristics.

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ORIGINAL ARTICLE

Retrospective Study

Optimal duration of the early and late recurrence of hepatocellular carcinoma after hepatectomy

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Abstract

AIM: To determine the best cut-off value between the early and late recurrence periods after the initial recurrence of hepatocellular carcinoma (HCC).

METHODS: The clinical records of 404 patients who underwent macroscopic curative hepatectomy for HCC between 1980 and 2010 were retrospectively examined. We divided the 252 patients experienced a recurrence of HCC into two groups, the early and late recurrence groups using the "minimum *P*-value" approach. Factors for early recurrence were investigated using all 404 patients, and factors related to late recurrence were investigated in the patients who were confirmed to be recurrence free at the end of the early recurrence period.

RESULTS: For the 252 patients who experienced a recurrence, the optimal cut-off value for differentiating early and late recurrence based on the overall survival after initial recurrence was 17 mo (5-year overall survival after initial recurrence: 15.4% *vs* 36.3%, $P = 0.000018$). Cox proportional hazard analysis identified early recurrence ($P = 0.003$) as one of the independent prognostic factors associated with overall survival after initial recurrence. A logistic regression model showed that an alpha-fetoprotein level > 100 ng/mL ($P < 0.001$), multiple HCC ($P < 0.001$), serosal invasion ($P = 0.031$), and microvascular invasion ($P = 0.012$) were independent factors associated with early recurrence, whereas the only independent factor related to late recurrence was liver cirrhosis ($P = 0.002$).

CONCLUSION: Seventeen months after hepatectomy is a useful cut-off value between early and late recurrence of HCC based on the prognosis and different etiologies.

Key words: Early recurrence; Late recurrence; Hepatocellular carcinoma; Hepatectomy; Minimum *P*-value approach

Core tip: The optimal cut-off value for differentiating early and late recurrence after hepatectomy for the hepatocellular carcinoma based on the overall survival after initial recurrence was 17 mo, and this cut-off may distinguish recurrences with different etiologies.

Yamamoto Y, Ikoma H, Morimura R, Konishi H, Murayama Y, Komatsu S, Shiozaki A, Kuriu Y, Kubota T, Nakanishi M, Ichikawa D, Fujiwara H, Okamoto K, Sakakura C, Ochiai T, Otsuji E. Optimal duration of the early and late recurrence of hepatocellular carcinoma after hepatectomy. *World J Gastroenterol* 2015; 21(4): 1207-1215 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1207.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1207

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, especially in East Asian countries[1]. Several studies have demonstrated that patients who underwent resection for HCC more recently had better survival results in the past decade[2,3]. Whereas, the time interval between resection for HCC and recurrence has been reported to influence the survival time after recurrence[4], and the early recurrence of HCC is now recognized as an important condition with a poor prognosis[4-8].

Recurrent HCC can originate from either metastases from the primary tumor or a multicentric occurrence. Several authors have stated that early recurrence might primarily represent metastasis from the primary tumor, whereas late recurrence is most likely due to a multicentric occurrence[9-11]. However, the term "early recurrence" has never been clearly defined. The period from 6 mo to 2 years after hepatectomy has been used as the early recurrence period in previous reports[6,10,12]. There is no consensus regarding the meaning of the terms "early recurrence" and "late recurrence" using an evidence-based cut-off value to provide the greatest difference in prognosis between the two groups.

The aim of this study was to determine the best cut-off value between the early and late recurrence periods based on the difference in the prognosis of the two groups after the initial recurrence of HCC. In addition, we investigated factors that may contribute individually to early and late recurrence and evaluated the differences in the prognostic factors for survival between the early and late recurrence groups.

MATERIALS AND METHODS

Patients

A total of 404 patients underwent macroscopic curative hepatectomy for HCC between 1980 and 2010 at the Department of Surgery, Division of Digestive Surgery, Kyoto Prefectural University of Medicine, and all of these patients were analyzed in this study. There were 316 men and 88 women included in this study. The mean ± SD age was 62.0 ± 9.7 years. Underlying liver diseases included cirrhosis in 210 patients (52.0%) and non-cirrhotic liver diseases in 194 patients (48.0%). A total of 90 patients were seropositive for hepatitis B surface antigen, and 186 were seropositive for antibodies to hepatitis C. One patient died within 30 d of the surgery as a result of acute renal failure. According to Child's classification system modified by Pugh *et al*[13], 393 patients (97.2%) were grouped in class A and 11 (2.8%) in class B. The mean ± SD tumor diameter was 4.1 ± 3.0 cm. Hepatectomies and the tumor location were defined according to Couinaud's definition of liver segmentation[14].

Treatment

The indications for hepatectomy and the type of surgical procedure used were usually determined based on the patients' liver function, which was primarily assessed using the Makuuchi criteria, which comprise preoperative measurements of ascites, the serum bilirubin level and the indocyanine green retention rate at 15 min (ICGR15)[15]. A total of 350 patients underwent anatomical resection, and 54 underwent non-anatomical resection. Anatomical hepatectomy was defined here as the removal of a Couinaud's hepatic segment (subsegment) or segments confined by tumor-bearing portal tributaries. Limited resection or enucleation smaller than Couinaud's segment (subsegment) was defined as non-anatomical resection. Adjuvant therapy was not administrated during the current study.

Pathological examination

All resected liver specimens were cut at a thickness of approximately 5 mm, and microscopic sections were viewed after staining with hematoxylin and eosin. The pathologic diagnosis and classification of the resected HCC tissues were performed according to The General Rules for the Clinical and Pathological Study of Primary Liver Cancer. Tumors were staged using the TNM classification scheme of the International Union Against Cancer[16]. Liver cirrhosis was defined as formation of regenerative nodules with surrounding fibrotic septa in liver parenchyma.

Follow-up

The follow-up evaluations of the patients involved hepatic ultrasonography, computed tomography, and

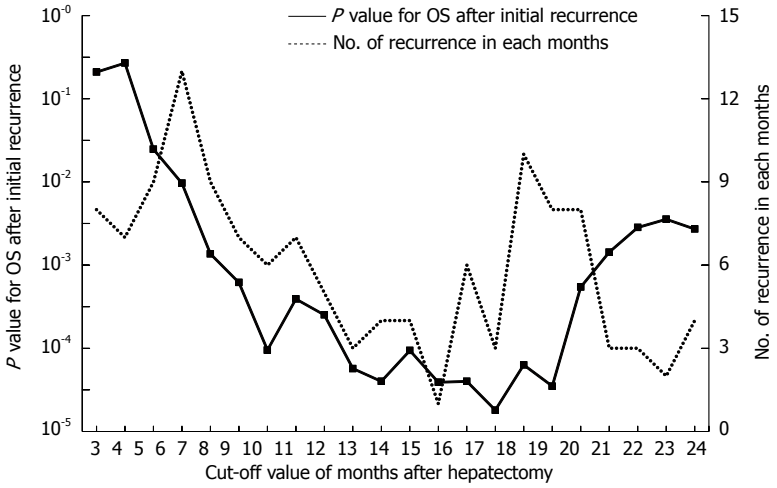

Figure 1 Kaplan-Meier analysis of the interval between the initial hepatectomy and recurrence. The minimum *P* value approach indicated that the most significant cut-off value for the interval prior to recurrence time was 17 mo. OS: Overall survival.

measurement of the serum alpha-fetoprotein (AFP) level and the serum protein induced by vitamin K absence Ⅱ level every 3-6 mo. Disease-free survival (DFS) was defined as the interval between surgery and the date of diagnosis of the first recurrence or the last follow-up. Overall survival (OS) was defined as the interval between surgery and the date of death caused by HCC recurrence or the last follow-up. The median follow-up duration was 44.6 mo.

Optimal cut-off value between early and late recurrence of HCC

Of the 404 patients, 252 patients (62.3%) experienced a recurrence of HCC. We divided the 252 patients experienced a recurrence of HCC into two groups, the early and late HCC recurrence after hepatectomy groups. The "minimum *P*-value" approach, which was performed using the log-rank test for the overall survival after the initial recurrence of HCC, was used to determine the best cut-off with which to divide up patients based on their overall survival after the initial recurrence of HCC[17,18]. The clinicopathological data were analyzed and compared between patients in the early and late HCC recurrence groups.

Treatment for the hepatic recurrence of HCC

Local treatment for the initial hepatic recurrence of HCC consisted of percutaneous ethanol injection therapy, radiofrequency ablation and repeat hepatectomy. Repeat hepatectomy was performed in 32 patients, and local ablation methods were used in 30 patients. Transarterial chemoembolization (TACE) was performed in 148 patients using the Seldinger technique[19] with iodized oil (Lipiodol) or gelatin sponge cubes as an embolus material and adriamycin (10-30 mg) and mitomycin C (10-20 mg) as anticancer drugs.

Statistical analysis

We performed univariate analyses of the clinical and pathologic factors that were potentially associated with overall survival. Survival was calculated using the Kaplan-Meier method and was compared between groups using the log-rank test. For the purpose to compare the prognostic value of the early recurrence to that of the late recurrence, Cox proportional hazard model was used in analysis of categorical variables influencing overall survival after initial recurrence using 252 patients who developed recurrence of HCC. Factors for early recurrence after the initial hepatectomy for HCC were investigated using all 404 patients who underwent hepatectomy for HCC. Factors related to late recurrence were investigated in the patients who were confirmed to be recurrence free at the end of the early recurrence period. All significant factors identified in the univariate analysis were entered into a multivariate regression analysis to identify independent factors. A P value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS for Windows 11.5 (SPSS, Chicago, IL).

RESULTS

The cumulative 5-year overall survival (5-year OS) and disease-free survival (5-year DFS) rates for all 404 patients together were 58.8% and 30.9%, respectively. In the 252 patients who experienced a recurrence of HCC, the optimal cut-off value between early and late recurrence for dividing patients into two groups based on the greatest difference in overall survival after initial recurrence was 17 mo (P = 0.000018) by using the minimum P value approach (Figure 1). The 107 patients who experienced an initial recurrence of HCC within 17 mo were defined as the early recurrence group, and the 145 patients who had an initial recurrence after more than 17 mo were defined as the late recurrence group. Figure 2 shows the comparison of the overall survival curves after the initial hepatic

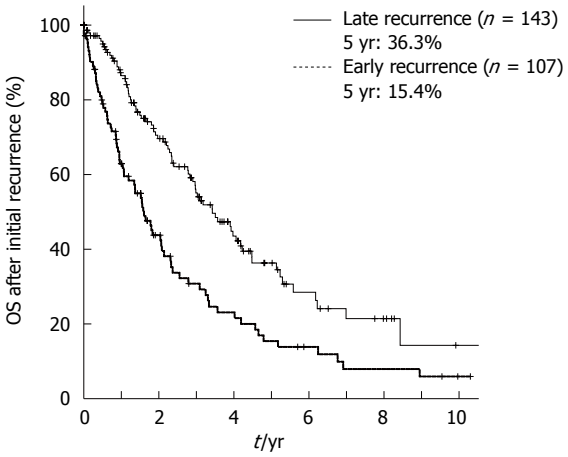

Figure 2 Comparison of the overall survival curves after initial hepatic recurrence for the early and late recurrence groups. The overall survival (OS) after initial recurrence in the late recurrence group was significantly better than that of the early recurrence group (5-year OS after initial recurrence: 36.3% *vs* 15.4%, $P = 0.000018$).

recurrence between the early and late recurrence groups. The OS after initial recurrence in the late recurrence group was significantly better than that of the early recurrence group (5-year OS after initial recurrence: 36.3% *vs* 15.4%, $P = 0.000018$).

Table 1 shows the results of the univariate and multivariate analyses of prognostic factors associated with overall survival after initial recurrence. Cox proportional hazard analysis identified an ICGR15 $\geqslant$ 10% ($P = 0.021$), liver cirrhosis ($P = 0.048$), multiple HCC ($P = 0.021$), infiltrating growth ($P = 0.008$), and early recurrence ($P = 0.001$) as independent prognostic factors associated with overall survival after initial recurrence.

Recurrence was observed in 107 patients within 17 mo after initial hepatectomy. Seven patients developed only distant metastatic disease (lung metastasis in two, bone metastasis in two, lymph node metastasis in two, and brain metastasis in one), 4 patients developed both intrahepatically and extrahepatically (lung metastasis in two and adrenal grand metastasis in two), and the other 96 patients developed intrahepatic recurrences. In addition, we examined the risk factors for early recurrence within 17 mo after the initial hepatectomy for HCC using all 404 patients who underwent hepatectomy for HCC. Table 2 shows the results of the χ^2 test and the logistic regression model used to analyze the 12 clinicopathological factors related to early recurrence. An AFP level $\geqslant$ 100 ng/mL ($P < 0.001$), multiple HCC ($P < 0.001$), serosal invasion ($P = 0.031$), and Microvascular invasion ($P = 0.012$) were identified as independent factors associated with early recurrence. Nine patients underwent repeat hepatectomy for the recurrence of HCC within 17 mo, and pathological data of recurrent HCC of 5 patients were available. All of them were same differentiation with primary tumor.

Recurrence was observed in 145 patients after more than 17 mo. Two patients developed distant metastatic disease (lung metastasis in one and brain metastasis in one), one patient developed both intrahepatically and extrahepatically (bone metastasis), and the other 142 patients developed intrahepatic recurrences. Factors related to late recurrence ($\geqslant$ 17 mo) were investigated in the 236 patients who were confirmed to be recurrence free at 17 mo after the initial hepatectomy for HCC. Table 3 shows the results of the univariate and multivariate analyses of the clinicopathologic factors contributing to late recurrence, independent factor related to late recurrence was only liver cirrhosis ($P = 0.002$). Twenty-three patients underwent repeat hepatectomy for the recurrence of HCC after more than 17 mo, and pathological data of recurrent HCC of 12 patients were available. Nine of them were same differentiation with primary tumor. On the other hand, 2 of them were well differentiated HCC, despite primary tumors were moderately differentiated HCC.

DISCUSSION

The early recurrence of HCC after the initial hepatectomy has been reported to be a prognostic factor for survival after recurrence[4-8]. However, in previous reports, the term "early recurrence" has not been defined based on the best cut-off value for prognosis between the early and late recurrence groups. Hayashi *et al*[5] and Shah *et al*[6] used 1 year as the period of early recurrence without any sufficient reasons. Park *et al*[7] and Lu *et al*[8] used 6 mo as the period of early recurrence because multinodular recurrence most often recurred within 6 mo after surgery. Imamura *et al*[11] used 2 years as the period of early recurrence after the initial hepatectomy because after the early peak for recurrence at approximately 1 year postoperatively, the recurrence rate decreased but persisted over a long period, resulting in a second peak at 4 years after surgery. We could not found any previous studies that classified patients into early and late recurrence groups based on the assessment of the best cut-off value to provide the largest difference in prognosis between the two groups. In this study, we analyzed the cut-off value between early and late recurrence based on minimum *P*-value approach, and we found that the best cut-off value was 17 mo after the initial hepatectomy. This is the first study to calculate the most significant cut-off value between early and late recurrence based on the prognosis after the initial recurrence of HCC. The minimum *P*-value approach has been proposed as a means of reducing the risk of missing a significant association[17,18]. However, this approach may give

Table 1 Results of the univariate and multivariate analyses of prognostic factors

	Total (*n*)	5-yr OS after initial recurrence	MST (mo)	Univariate analysis *P*	Multivariate analysis	
					Hazard ratio (95%CI)	*P*
Age (yr)				0.726		
< 60	115	29.9%	30.6			
⩾ 60	137	24.1%	32.3			
Sex				0.464		
Male	204	26.3%	30.6			
Female	48	32.7%	38.1			
Indocyanine green retention rate at 15 min				0.023		0.021
< 10%	58	47.4%	41.1		1	
⩾ 10%	194	21.3%	28.4		1.680 (1.081, 2.613)	
Underlying liver disease				0.017		0.048
Other	110	37.3%	39.7		1	
Cirrhosis	142	20.0%	27.4		1.422 (1.003, 2.017)	
Alpha-fetoprotein (ng/mL)				0.010		
< 100	163	29.4%	35.8			
⩾ 100	89	23.1%	21.3			
Type of resection				0.561		
Non-anatomical resection	52	17.5%	33.4			
Anatomical resection	200	28.5%	28.5			
Number of tumors				0.009		0.021
Single	177	31.7%	37.6		1	
Multiple	75	17.0%	21.6		1.530 (1.066, 2.196)	
Growth pattern				0.001		0.008
Expanding	229	28.8%	33.9		1	
Infiltrating	23	12.4%	14.3		1.963 (1.191, 3.234)	
Histological differentiation				0.001		
Poorly differentiated	27	10.6%	11.1			
Others	225	28.7%	35.7			
Capsule				0.242		
Absent	54	13.1%	25.4			
Present	198	31.5%	33.4			
Serosal invasion				0.905		
Negative	218	26.2%	33.3			
Positive	34	31.7%	21.6			
Microvascular invasion				0.012		
Absent	185	27.0%	35.7			
Present	67	26.8%	14.4			
Surgical margin				0.839		
Negative	232	26.7%	28.7			
Positive	20	34.3%	48.7			
Tumor size (mm)				0.182		
< 50	187	25.0%	26.9			
⩾ 50	65	20.3%	16.6			
Recurrence period				< 0.001		0.001
Early group (< 17 mo)	107	15.4%	19.2		1.735 (1.237, 2.432)	
Late group (⩾ 17 mo)	145	36.3%	41.1		1	

The results of the univariate and multivariate analyses of prognostic factors associated with overall survival after initial recurrence in the 252 patients who developed recurrence of hepatocellular carcinoma (HCC) after hepatectomy for HCC. MST: Median survival time.

false positive associations. The potential validity and use of such an approach is currently further tested using the clinical material of the present and other studies.

There are two possible causes of HCC recurrence, metastasis from primary tumor and metachronously multicentric occurrence. Both types of disease may have been present before hepatectomy in the early postoperative recurrent cases. Some authors have noted that early recurrence might represent primarily metastasis from primary tumor, whereas late recurrence might most likely be due to multicentric occurrence[8,9,20]. However, it is usually difficult to distinguish intrahepatic recurrences of different etiologies because histopathological analysis is not performed in clinical practice. Previously reported risk factors for early recurrence include PIVKA Ⅱ, AFP, Milan criteria status, nonanatomic resection, microscopic vascular invasion, intrahepatic metastasis, and positive surgical margins[5-8,11]. In this study, serosal invasion, multiple tumors, microvascular invasion, and an AFP ⩾ 100 ng/mL, which were only primary tumor factors, were associated with early recurrence after the initial hepatectomy. In contrast, the factors associated with late recurrence included only liver cirrhosis, which

Table 2 Results of the univariate and multivariate analyses of the clinicopathologic factors

	Total (*n*)	Early group (*n* = 107)	Others (*n* = 297)	Univariate analysis *P*	Multivariate analysis	
					RR (95%CI)	*P*
Age (yr)				0.290		
< 60	175	51	124			
⩾ 60	229	56	173			
Sex				0.933		
Male	316	84	232			
Female	88	23	65			
Indocyanine green retention rate at 15 min				0.193		
< 10%	110	24	86			
⩾ 10%	294	83	211			
Underlying liver disease				0.225		
Other	194	46	148			
Cirrhosis	210	61	149			
Alpha-fetoprotein (ng/mL)				< 0.001		< 0.001
< 100	280	52	228		1	
⩾ 100	124	55	69		2.939 (1.788, 4.830)	
Number of tumors				< 0.001		< 0.001
Single	306	63	243		1	
Multiple	98	44	54		2.633 (1.548, 4.445)	
Growth pattern				0.002		
Expanding	372	91	281			
Infiltrating	32	16	16			
Capsule				0.230		
Absent	82	26	56			
Present	322	81	241			
Serosal invasion				0.006		0.031
Negative	358	87	271		1	
Positive	46	20	26		2.110 (1.069, 4.164)	
Microvascular invasion				< 0.001		0.012
Absent	312	64	248		1	
Present	92	43	49		2.001 (1.163, 3.441)	
Surgical margin				0.081		
Negative	374	95	279			
Positive	30	12	18			
Tumor size (mm)				0.010		
< 50	312	73	239			
⩾ 50	92	34	58			

The results of the univariate and multivariate analyses of the clinicopathologic factors contributing to early recurrence after the initial hepatectomy for hepatocellular carcinoma.

are the host-related factor reflecting the increased carcinogenicity of the liver. Consistent with our results, Imamura *et al*[11] and Poon *et al*[10] reported that the grade of hepatitis activity and cirrhosis, respectively, are risk factors for late recurrence. These findings support the hypothesis that early recurrence is mainly influenced by factors related to the status of the primary tumor and late recurrence is chiefly caused by a second primary lesion and not by the initial tumor stage. Moreover, 17 mo after the initial hepatectomy may be a good cut-off value to distinguish recurrences of different etiologies, which strongly influence the prognosis after recurrence.

A multivariate analysis showed that liver cirrhosis, an ICGR15 ⩾ 10%, an infiltrating growth pattern, multiple HCC and early recurrence were independent prognostic factor associated with overall survival after initial recurrence. The four former parameters were related to liver function and HCC stage, which have been previously reported to be important prognostic factors[21-23]. It is not surprising that early recurrence was also an independent prognostic factor, because early recurrence represents mainly metastatic recurrence, and it may be associated with the presence of minimal metastasis at the time of the initial hepatectomy. In contrast, late recurrence represents mainly multicentric recurrence. To prevent early and late recurrence, different adjuvant therapeutic methods may be required.

In patients with a high risk of early recurrence after the initial hepatectomy for HCC, early detection through close postoperative surveillance is necessary. To date, various systemic adjuvant treatments, such as chemotherapy, immunotherapy, and TACE, have been tried[24,25]. There are several systematic reviews on the role of neoadjuvant/adjuvant therapy for HCC treated with hepatectomy[26-30]. A clinical trial to examine the recurrence-preventing effect of sorafenib when administered after curative treatments such as resection or ablation (STORM trial) is in progress[29]. Whereas, Lau *et al*[27] reported that adjuvant intra-arterial ^{131}I-lipiodol after curative

Table 3 Results of the univariate and multivariate analyses of the clinicopathologic factors

	Total (*n*)	Late group (*n* = 145)	Without recurrence (*n* = 91)	Univariate analysis *P*	Multivariate analysis	
					RR (95%CI)	*P*
Age (yr)				0.125		
< 60	95	64	31			
⩾ 60	141	81	60			
Sex				0.194		
Male	189	120	69			
Female	47	25	22			
Indocyanine green retention rate at 15 min				0.008		
< 10%	70	34	36			
⩾ 10%	166	111	55			
Underlying liver disease				0.002		0.002
Other	123	64	59		1	
Cirrhosis	113	81	32		2.333 (1.359, 4.008)	
Alpha-fetoprotein (ng/mL)				0.199		
< 100	187	111	76			
⩾ 100	49	34	15			
Number of tumors				0.069		
Single	194	114	80			
Multiple	42	31	11			
Growth pattern				0.878		
Expanding	225	138	87			
Infiltrating	11	7	4			
Capsule				0.905		
Absent	45	28	17			
Present	191	117	74			
Serosal invasion				0.411		
Negative	216	131	85			
Positive	20	14	6			
Microvascular invasion				0.641		
Absent	199	121	78			
Present	37	24	13			
Surgical margin				0.703		
Negative	224	137	87			
Positive	12	8	4			
Tumor size (mm)				0.113		
< 50	193	114	79			
⩾ 50	43	31	12			

The results of the univariate and multivariate analyses of the clinicopathologic factors contributing to late recurrence in the 236 patients who were confirmed to be recurrence-free at 17 mo after the initial hepatectomy for hepatocellular carcinoma.

liver resection provided a survival benefit in a randomized control trial. If these risk factors for early recurrence are found after resection for HCC, clinical trials involving adjuvant therapy should be performed.

Considering that the late recurrence was not related to primary tumor factors, but related only liver cirrhosis, late recurrence may be consider secondary primary tumors. The management of late recurrence should be similar to that of an initial diagnosis of HCC. Muto *et al*[31] mentioned that polyprenoic acid can prevent second primary tumors in patients who are clinically free of disease after their primary hepatomas are treated in a randomized controlled study, although the mechanism of action of polyprenoic acid is not fully revealed. On the other hand, Singal *et al*[32] reported that interferon treatment after curative resection or ablation of HCC in HCV-related cirrhotics prevents HCC recurrence and improves survival. To prevent late recurrence, the suppression of multicentric occurrence using polyprenoic acid or interferon treatment will be indicated in patients with cirrhotic livers.

In conclusion, this study demonstrates that 17 mo after hepatectomy is a good cut-off value between early and late recurrence based on the prognosis after the initial recurrence of HCC, and this cut-off may distinguish recurrences of different etiologies. The early recurrence was influenced mainly by the primary tumor stage, and late recurrence was influenced only by the liver function status. It is important to prevent early and late recurrences using different adjuvant therapeutic approaches.

COMMENTS

Background

The time interval between resection for hepatocellular carcinoma (HCC) and recurrence has been reported to influence the survival time after recurrence, and the early recurrence of HCC is now recognized as an important condition with a poor prognosis. However, there is no consensus regarding the meaning of the terms "early recurrence" and "late recurrence" using an evidence-based cut-off value to provide the greatest difference in prognosis between the two

groups.

Research frontiers

The period from 6 mo to 2 years after hepatectomy has been used as the early recurrence period in previous reports without any sufficient reasons. Authors could not found any previous studies that classified patients into early and late recurrence groups based on the assessment of the best cut-off value to provide the largest difference in prognosis between the two groups.

Innovations and breakthroughs

Authors firstly examined the best cut-off value between the early and late recurrence periods based on the difference in the prognosis of the two groups after the initial recurrence of HCC. In addition, they investigated factors that may contribute individually to early and late recurrence and evaluated the differences in the prognostic factors for survival between the early and late recurrence groups.

Applications

Authors divided the patients experienced a recurrence of HCC into two groups, the early and late HCC recurrence after hepatectomy groups. The "minimum *P*-value" approach, which was performed using the log-rank test for the overall survival after the initial recurrence of HCC, was used to determine the best cut-off with which to divide up patients based on their overall survival after the initial recurrence of HCC.

Terminology

HCC is one of the most common malignant tumors worldwide, especially in East Asian countries. Several studies have demonstrated that patients who underwent resection for HCC more recently had better survival results in the past decade. Recurrent HCC can originate from either metastases from the primary tumor or a multicentric occurrence. Several authors have stated that early recurrence might primarily represent metastasis from the primary tumor, whereas late recurrence is most likely due to a multicentric occurrence.

Peer review

The paper shows that 17 mo is the optimal cut-off value for differentiating early and late recurrence after hepatectomy for hepatocellular carcinoma based on the overall survival after initial recurrence. The approach to the problem that is the object of the study is quite original and the paper is well written; however, some questions should be answered.

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ORIGINAL ARTICLE

Retrospective Study

Preoperative CA 125 is significant indicator of curative resection in gastric cancer patients

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Abstract

AIM: To investigate the correlation among tumor markers, curative resection, and recurrence in gastric cancer.

METHODS: The patients with preoperative tumor makers [Carcinoembryonic antigen, Carbohydrate antigen (CA) 19-9, and CA 125] and elective gastrectomy between January 2000 and December 2009 at Chungbuk National University Hospital were enrolled in this study. We analyzed the relationship among the tumor makers, curative resection and recurrence, retrospectively.

RESULTS: Among the 679 patients with gastric cancer, curative resection was 93.6% (n = 636) and non-curative resection was 6.4% (n = 43). The independent risk factors for the non-curative resection were tumor location and the positivity of preoperative serum CA 19-9 and CA 125 levels. After curative resection, the independent prognostic risk factors for recurrence in curative resection were gender, stage, and preoperative increased serum CA 125 level (HR = 2.431, P =0.020), in a multivariate analysis.

CONCLUSION: Preoperative CA 125 is a useful predictive biomarker for curative resection and prognostic biomarker for recurrence in gastric cancer patients.

Key words: Gastric Cancer; Tumor Marker; Carcinoembryonic antigen; Carbohydrate antigen 19-9; Carbohydrate antigen 125

Core tip: Tumor marker such as carcinoembryonic antigen, Carbohydrate antigen (CA) 19-9, and CA 125 in gastric cancer are usual tools for predicting prognosis or monitoring. The aim of this study was to investigate the correlation among tumor markers, curative resection, and recurrence in gastric cancer. Our data showed that preoperative CA 19-9 and CA 125 are independent risk factor of non-curative operation. And preoperative CA 125 is independent risk factor for recurrence after curative operation. Preoperative CA 125 is considered useful marker for predicting curative operation and

prediction of recurrence after curative resection in gastric cancer patients.

Kim DH, Yun HY, Ryu DH, Han HS, Han JH, Yoon SM, Youn SJ. Preoperative CA 125 is significant indicator of curative resection in gastric cancer patients. *World J Gastroenterol* 2015; 21(4): 1216-1221 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1216.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1216

INTRODUCTION

Serum tumor marker is a simple and convenient study for predicting prognosis or monitoring, and it is widely being used in a gastrointestinal malignancy[1]. Carcinoembryonic antigen (CEA) is a glycoprotein that is often elevated in the serum of patients with variety malignancies such as gastric, pancreatic, colorectal, breast and lung cancer[2]. Carbohydrate antigen 19-9 (CA 19-9)is an incomplete glycolipid antigen of the Lewis blood group, and it can be increased in colorectal, liver, ovarian, bile duct and gastric cancer[3]. CEA and CA 19-9 are known prognostic risk factors in gastric cancer[4,5]. In American Joint Committee on Cancer (AJCC) 7th edition, CEA and CA 19-9 been recognized as prognostic factors, but Cancer antigen 125 (CA 125) is not[6]. CA 125 is a heterogeneous cell membrane glycoprotein and it is related with malignant conditions such as ovarian, uterine, lung, or pancreatic cancers[7]. CEA is related to liver metastasis, peritoneal metastasis, histologic type and CA 19-9 is related to T, N stage, and peritoneal dissemination[8]. CA 125 is related to peritoneal dissemination[9]. Peritoneal dissemination, after curative gastrectomy with extended lymphadectomy, is most common recurrence pattern in the east[10]. As diagnosis of peritoneal dissemination is not high as that of distant metastasis and direct invasion of adjacent organs in preoperative image studies[11], surgeons face to unforeseen non-curative operation. It has been reported that CA 125 is related with peritoneal dissemination in gastric cancer[9], but there were few or no studies concerning the correlation between CA 125 and prognosis.

This study was to clarify prognostic value of preoperative CA 125 for prognostic biomarker, and to investigate the correlation between tumor markers (CEA, CA 19-9 and CA 125) and prediction of curative resection in gastric cancer patients.

MATERIALS AND METHODS

A total of 679 gastric cancer patients admitted through the outpatient department of surgery from 2000 to 2009 were enrolled. All enrolled patients checked tumor marker before gastric operation and follow-up performed biannually after gastric operation. Preoperative measurement of CEA, CA19-9, and CA 125 were performed by radioimmunoassay. The normal ranges for CEA, CA 19-9, and CA 125 were: < 5 ng/mL, 25 U/mL, and 37 U/mL, respectively. We excluded remnant gastric cancer, synchronous primary malignancy, or gastric cancer with neoadjuvant therapy. Recurrence pattern was classified into four categories: loco-regional recurrence, peritoneal dissemination, hematogenous and distant lymph node. We performed gastric cancer operation according to Japanese gastric cancer treatment guidelines[12]. Pathologic staging was conducted after the operation according to the AJCC sixth edition[13]. Non-curative operations were defined as microscopically or macroscopically residual tumor after gastric operation.

We retrospectively analyzed the relationship among a non-curative operation, recurrence and tumor marker. We employed SPSS 20.0 for Windows for statistical analyses (SPSS, Inc., Chicago, IL, United States). The χ^2 and Fisher's exact tests were used to assess clinical and pathological characteristics for univariated analysis and logistic regression test for multivariate analsysis. The disease free survival was analyzed using the Kaplan-Meier method, and significance testing was performed with the log-rank test. The Cox proportional hazards model was used for multivariate analysis. Differences with *P* values less than 0.05 ($P < 0.05$) were considered statistically significant.

Table 1 Causes for a non-curative operations

	Peritoneal dissemination	Direct invasion	Distant metastasis	Incomplete resection	Total
Peritoneal dissemination	15	3	3		21
Direct invasion	3	13			16
Distant metastasis	3		3		6
Incomplete resection				6	6
Total	21	16	6	6	49

RESULTS

Of the 679 patients, 447 patients were male and 232 patients were female with a mean age of 60.7 ± 11.4 years, and the median follow-up period was 32.4 mo. Forty-three patients (6.3%) received non-curative operations. The main causes of a non-curative operations were peritoneal dissemination ($n = 21$) followed by direct invasion ($n = 16$) (Table 1). Risk factors for non-curative operation were tumor location, CEA, CA 19-9, and CA 125 in univariate analysis. In a multivariate analysis, location of tumor (HR = 21.303; $P < 0.001$), CA 19-9 positivity (HR = 5.883; $P < 0.001$), and CA 125 positivity (HR =

Table 2 Univariate and multivariate analysis of the risk factors for a non-curative operations *n* (%)

Variables	Univariate analysis			Multivariate analysis	
	Curative	Non-curative	*P* value	HR (95%CI)	*P* value
Age, yr			0.189		0.238
< 60	272 (95.1)	14 (4.9)			
⩾ 60	364 (92.6)	29 (7.4)		1.599 (0.733-3.487)	
Sex			0.574		0.642
Male	417 (93.3)	30 (6.7)			
Female	219 (94.4)	13 (5.6)		0.832 (0.384-1.803)	
Differentiation			0.080		0.082
Differentiated	382 (95.0)	20 (5.0)			
Undifferentiated	254 (91.7)	23 (8.3)		1.937 (0.919-4.085)	
Location			< 0.001		< 0.001
Lower 1/3	359 (93.2)	26 (6.8)			
Middle 1/3	212 (97.2)	6 (2.8)		0.334 (0.120-0.928)	0.036
Upper 1/3	60 (90.9)	6 (9.1)		1.223 (0.421-3.555)	0.711
Whole	5 (50.0)	5 (50.0)		21.303 (4.985-91.036)	< 0.001
CEA			0.038		
Negative	579 (94.3)	35 (5.7)			0.792
Positive	57 (87.7)	8 (12.3)		1.142 (0.427-3.056)	
CA 19-9			< 0.001		< 0.001
Negative	586 (95.6)	27 (4.4)			
Positive	50 (75.8)	16 (24.2)		5.883 (2.569-13.474)	
CA 125			< 0.001		< 0.001
Negative	616 (95.7)	28 (4.3)			
Positive	20 (57.1)	15 (42.9)		15.549 (6.473-37.352)	

CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen.

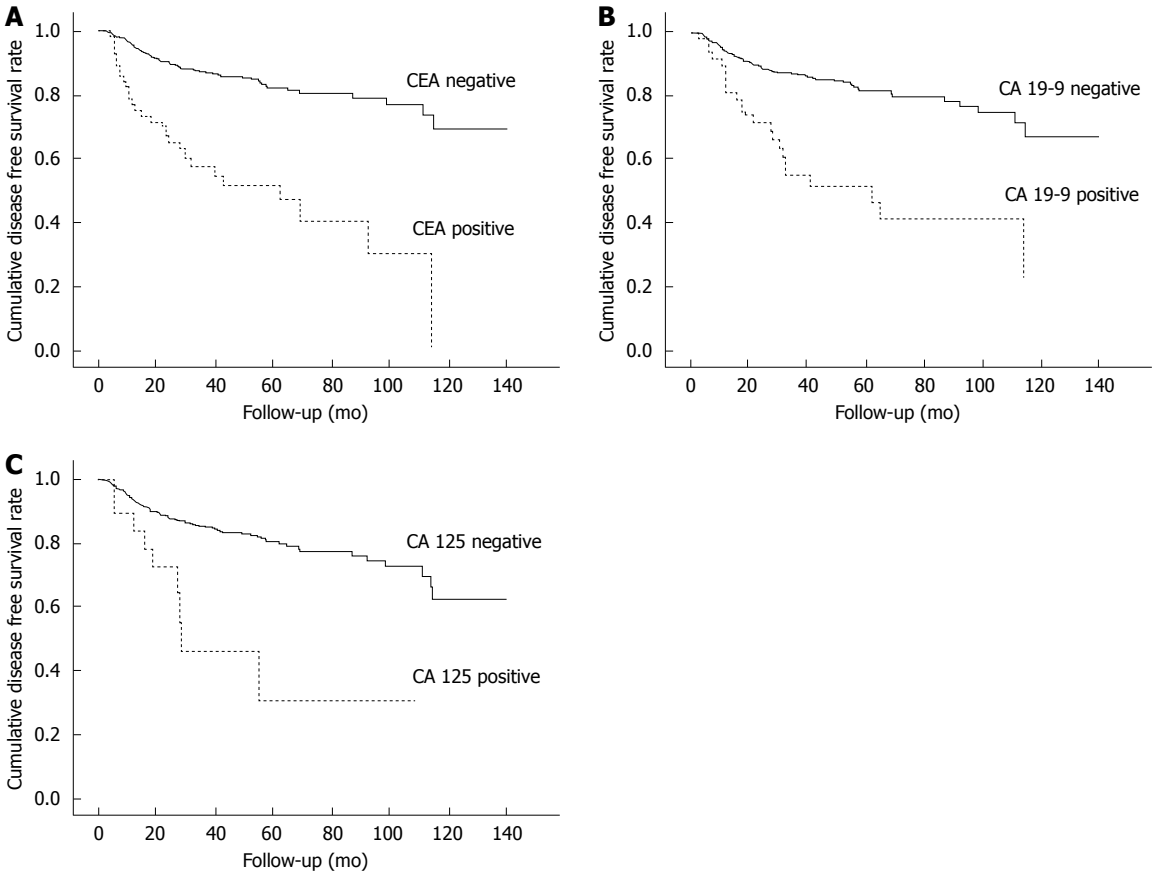

Figure 1 Disease free survival curve according to positivity of Carcinoembryonic antigen (A), Carbohydrate antigen 19-9 (B) and Carbohydrate antigen 125 (C).

Table 3 Univariateand multivariate analysis of prognostic risk factors for disease-free survival after curative operations

Variables	Univariate analysis		Multivariate analysis	
	5-year DFS	*P* value	HR (95%CI)	*P* value
Age, yr		0.865		0.282
< 60 (*n* =272)	78.3%			
⩾ 60 (*n* = 364)	80.1%		1.250 (0.833-1.877)	
Sex		0.194		0.037
Male (*n* = 417)	78.3%			
Female (*n* = 219)	81.7%		0.616 (0.391-0.971)	
Differentiation		0.020		0.292
Differentiated (*n* = 382)	81.6%			
Undifferentiated (*n* = 254)	75.6%		0.797 (0.524-1.215)	
Location		0.004		0.454
Lower 1/3 (*n* = 359)	76.0%			
Middle 1/3 (*n* = 212)	86.8%		0.797 (0.489-1.297)	
Upper 1/3 (*n* = 60)	70.8%		1.172 (0.643-2.135)	
Whole (*n* = 5)	40.0%		0.487 (0.138-1.720)	
Lymphovascular invasion		< 0.001		0.709
Negative (*n* = 513)	81.8%			
Positive (*n* = 123)	69.4%		1.085 (0.708-1.662)	
Perineural invasion		< 0.001		0.072
Negative (*n* = 582)	81.4%			
Positive (*n* = 54)	54.4%		1.629 (0.958-2.770)	
Stage		< 0.001		< 0.001
Ⅰ A (*n* = 292)	98.7%			
Ⅰ B (*n* = 102)	89.4%		6.613 (1.703-25.674)	0.006
Ⅱ (*n* = 75)	79.8%		15.415 (4.297-55.300)	< 0.001
ⅢA (*n* = 69)	49.5%		43.857 (12.999-147.966)	< 0.001
ⅢB (*n* = 39)	46.2%		66.090 (18.643-234.289)	< 0.001
Ⅳ (*n* = 59)	32.7%		93.720 (27.252-319.103)	< 0.001
CEA		< 0.001		0.073
Negative (*n* = 579)	82.1%		1.559 (0.959-2.535)	
Positive (*n* = 57)	51.3%			
CA 19-9		< 0.001		0.694
Negative (*n* = 586)	81.7%			
Positive (*n* = 50)	51.7%		0.901 (0.535-1.516)	
CA 125		< 0.001		0.020
Negative (*n* = 616)	80.6%			
Positive (*n* = 20)	30.8%		2.431 (1.153-5.123)	

15.549; $P < 0.001$) were independent risk factors for a non-curative operation. (Table 2) Recurrences after curative operation were 124 cases among 636 patients, and 64 patients had two or more recurrence site; hematogenous ($n = 72$), peritoneal ($n = 50$), loco-regional ($n = 46$) and distant lymph node metastases ($n = 41$). The 5-year disease-free survival rate after curative operation was 77.9%, and the 5-year disease-free survival rate of CEA, CA 19-9 and CA 125 are 51.3%, 51.7% and 30.8%, respectively (Figure 1). Risk factors of recurrence were tumor location, differentiation, lymphovascular invasion, perineural invasion, stage, and CEA, CA 19-9, and CA 125. In a multivariate analysis, gender, stage, and positivity of CA 125 (HR = 2.431; $P = 0.020$) were independent risk factors for recurrence (Table 3).

DISCUSSION

Depth of invasion and lymph node metastasis were most important independent prognostic risk factors in gastric cancer, and currently used TNM stage is useful to predicting survival rate in each stage. However, it was difficult to estimate N stage, to predict survival rate in each stage and to decide treatment of modality in preoperative clinical TNM stage. Many prognostic risk factors of gastric cancer were investigated, and tumor marker is one of the prognostic factors of gastric cancer. CEA and CA 19-9 are the most common tumor markers for predicting prognosis in gastric cancer[14]. The sensitivities of CEA and CA 19-9 in gastric cancer are 16%-58.4% and 34.1%-64.9%[15-17]. Preoperative positivity for CEA and CA 19-9 is associated with a poor prognosis[4,5,18], but there is a few report for preoperative CA 125 in gastric cancer. The preoperative CEA and CA 19-9 were not prognostic factor for palliative gastric surgery, but it was independent prognostic factors in curative surgery[4]. The postoperative CEA positivity in early gastric cancer and postoperative CEA and CA 72-4 positivity in advanced gastric cancer were independent prognostic factors[18]. In our study, the preoperative CEA and CA 19-9 were not independent prognostic risk factors for recurrence

as other study[19], interestingly preoperative CA 125 was an independent risk factor for recurrence with a HR of 2.431. In early stage (data was not shown), no recurrence observed in the patients with positivity of CA 125 in stage IA (n = 2), but there were two cases of recurrences among 4 patients with positivity of CA 125 in stage IB, one is peritoneal carcinomatosis, and the other is loco-regional recurrence, respectively. We think that this finding may be related with recurrence patterns of gastric cancer. The sensitivity of CA 125 is 6%-31.6%[20,21], and it is related with peritoneal metastasis[22]. In the Japan Clinical Oncology Group (JCOG9501) trial, recurrence patterns were peritoneal recurrence, regional lymph node recurrence, hepatic, and others were: 38.1%, 21.9%, 20.9%, and 19.7%, respectively[23]. In Korean study, recurrent patterns were observed according to time period. Hematogenous recurrence was the most common recurrent pattern in the 1990s, whereas peritoneal recurrence was the most common recurrent pattern in the 2000s. Furthermore, peritoneal dissemination was the most frequent recurrence pattern (32.1%) during the entire period[10]. In our study, peritoneal dissemination and hematogenous metastasis were the main recurrence patterns. The positivity of CA 125 may be reflected recurrent patterns of peritoneal dissemination.

In our study, stage Ⅰ and Ⅱ are 61.8%, this is why non-curative operation smaller (6.4%) than other studies (17.9%-35.8%)[24,25]. In general, diagnostic accuracy of preoperative T stage in gastric cancer is 65%-92.1%[26]. and sensitivity and specificity of hematogenous metastasis especially liver are 87.5% and 99.0%[27]. Diagnostic accuracy of preoperative for peritoneal metastasis is 30%-100%[27], but sensitivity is only 28.8%[11]. Diagnostic laparoscopy maybe useful for border line peritoneal dissemination in image study. Kapiev *et al*[28] reported that 29.5% of patients with borderline unresectable gastric cancer were diagnosed as peritoneal metastasis by diagnostic laparoscopy. In our study, the proportion of non-curative resection with peritoneal dissemination is low (3.1%), according to our data, diagnostic laparoscopy is not necessary even in far advanced gastric cancer. Peritoneal metastasis is the main cause of non-curative operation in our study and to avoid unnecessary operation precise diagnosis of peritoneal dissemination is important. In general, tumor markers (CEA, CA 19-9 and CA 125) are related with peritoneal dissemination[8,9,22]. There is a few report of relationship between curative resection and preoperative tumor marker. Pectasides *et al*[16] reported that the sensitivity of CEA and CA 19-9 in inoperable or metastatic disease gastric cancer were 48.6% and 64.9%, retrospectively. As compared to our study, their study did not checked tumor marker preoperative, and there was no data of CA 125. In our study, 24.2% and 42.9% of patients with increased levels of CA 19-9 and CA 125 received a non-curative operation. The HRs of CA 19-9 and CA 125 for non-curative operations were 4.153 and 10.796, each representing statistically significant levels. We think that the positivity of CA 19-9 and CA 125 may reflect peritoneal dissemination. Thus, it would be useful biomarker to avoid unnecessary laparotomy for patients with borderline resectability on preoperative imaging test if they show increased CA 19-9 and CA 125 levels.

In far advanced gastric cancer, the positivity of CA 19-9 and CA 125 showed a higher frequency of receiving non-curative operations, a more careful approach is necessary. Additionally, a more aggressive treatment is required even if a curative operation is performed, as preoperative increased CA 125 is related to possible recurrence.

COMMENTS

Background

Preoperative image study is a useful diagnostic tool for staging in gastric cancer. But its role in resectability and prognosis in a part of gastric cancer patients is questionable. The aim of this study was to investigate the correlation among tumor markers especially carbohydrate antigen (CA) 125, curative resection, and recurrence in gastric cancer.

Research frontiers

CA 125 is related to peritoneal dissemination, and it has been reported that CA 125 is related with peritoneal dissemination in gastric cancer. However, the clinical significant of CA 125 in gastric cancer is not clarified. The current hot spot is to clarify the role of CA 125 in gastric cancer.

Innovations and breakthroughs

Previous studies showed that CA 125 is related with peritoneal carcinomatosis in gastric cancer. But, there were few or no studies concerning the relation between preoperative CA 125 and curative operation or prognosis. In this study, the preoperative CA 125 is related with non-curative operation and poor prognosis.

Applications

In far advanced gastric cancer, the positivity of CA 125 showed a higher frequency of receiving non-curative operations, a more careful approach is necessary. Additionally, a more aggressive treatment is required even if a curative operation is performed, as preoperative increases in CA 125 are related to possible recurrence.

Peer review

This is a retrospective study that analyzes clinical significance of tumor marker in gastric cancer. The results are interesting and show that the positivity of preoperative CA 125 associated with non-curative operations and poor prognosis. This finding suggest that CA 125 is a useful tumor marker predicting non-curative operation and poor prognosis.

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ORIGINAL ARTICLE

Retrospective Study

Survival in gastric cancer in relation to postoperative adjuvant therapy and determinants

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Abstract

AIM: To evaluate survival data in patients with gastric cancer in relation to postoperative adjuvant therapy and survival determinants

METHODS: A total of 201 patients (mean ± SD age: 56.0 ± 11.9 years, 69.7% were males) with gastric carcinoma who were operated and followed up at Lutfi Kirdar Kartal Training and Research Hospital between 1998 and 2010 were included in this retrospective study. Follow up was evaluated divided into two consecutive periods (before 2008 and 2008-2010, respectively) based on introduction of 3-D conformal technique in radiotherapy at our clinic in 2008. Data on patient demographics, clinical and histopathological characteristics of gastric carcinoma and the type of treatment applied after surgery [postoperative adjuvant treatment protocols including chemoradiotherapy (CRT) and chemotherapy (CT), supportive therapy or follow up without any treatment] were recorded. The median duration and determinants of local recurrence free (LRF) survival, distant metastasis free (DMF) survival and overall survival were evaluated in the overall population as well as with respect to follow up years [1998-2008 (n = 127) *vs* 2008-2010 (n = 74)].

RESULTS: Median duration for LRF survival, DMF survival and overall survival were 31.9, 24.1 and 31.9 mo, respectively in patients with postoperative adjuvant CRT. No significant difference was noted in median duration for LRF survival, DMF survival and overall survival with respect to treatment protocols in the overall population and also with respect to followed up periods. In the overall population, CT protocols FUFA [5-fluorouracil (400 mg/m^2) and leucovorin-folinic acid (FA, 20 mg/m^2)] (29.9 mo) and UFT® + Antrex® [a fixed combination of the oral FU prodrug tegafur (flouroprymidine, FT, 300 mg/m^2 per day) with FA (Antrex®), 15 mg tablet, two times a day] (42.5 mo) was significantly associated with longer LRF survival times than other CT protocols (P = 0.036), while no difference was noted between CT protocols in terms of DMF survival and overall survival. Among patients received CRT, overall survival was significantly longer in patients with negative than positive surgical margin (27.7 mo *vs* 22.4 mo, P = 0.016) in the overall

study population, while time of radiotherapy initiation had no significant impact on survival times. Nodal stage was determined to be independent predictor of LRF survival in the overall study population with 4.959 fold (P = 0.042) increase in mortality in patients with nodal stage N2 compared to patients with nodal stage N0, and independent predictor of overall survival with 5.132 fold (P = 0.006), 5.263 fold (P = 0.027) and 4.056 fold (P = 0.009) increase in the mortality in patients with nodal stage N3a (before 2008), N3b (before 2008) and N2 (overall study population) when compared to patients with N0 stage, respectively.

CONCLUSION: Our findings emphasize the likelihood of postoperative adjuvant CRT to have a survival benefit in patients with resectable gastric carcinoma.

Key words: Gastric carcinoma; Local recurrence free survival; Distant metastasis free survival; Postoperative adjuvant therapy; Overall survival

Core tip: This retrospective single centre analysis of survival data in patients with resected gastric carcinoma revealed median 31.9 mo of local recurrence free (LRF) survival, 24.1 mo of distant metastasis free survival and 31.9 mo of overall survival *via* postoperative adjuvant chemoradiotherapy during follow up from 1998 to 2010. Use of 5-fluorouracil and leucovorin-folinic acid and uracil/tegafur based chemotherapy protocols and the absence of positive surgical margin but not the interval between surgery and radiotherapy had a significant impact on survival times, while the nodal stage was the independent prognostic factor for LRF and overall survival.

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INTRODUCTION

Despite advances in surgical techniques, patients with gastric cancer show poor prognosis and cure rate remains dismal with 5-year survival rates of 8%-34% and locoregional recurrence of 40%-90% even after curative resection[1].

Accordingly implication of neoadjuvant or adjuvant therapy in patients with resectable gastric cancer mainly in the form of postoperative chemoradiotherapy (CRT) and perioperative chemotherapy (CT) has been considered by several studies in terms achievement of better therapeutic outcomes and shown to be associated with high-level evidence for improved survival in Western populations[2-5].

Besides, based on data from the phase Ⅲ, INT 0116-SWOG0008 study in which better survival rates were achieved by adding CT (5-fluorouracil and leucovorin-folinic acid) and concurrent 45 Gy radiotherapy to surgery[2], postoperative CRT has become a standard in gastric carcinoma, especially in United States[6].

Additionally, a past meta-analysis and the Surveillance, Epidemiology, and End Results database have demonstrated a favorable survival impact of radiotherapy in patients with resectable gastric cancer[7,8]. However, despite increasing evidence available for a survival advantage from adjuvant therapies, adjuvant treatment strategies in patients with resectable gastric cancer still remains debated[9,10] particularly in terms of favour of radiotherapy associated with CT, the adequacy of nodal dissection, the likelihood of CT related toxic effects and inconsistency of different therapeutic trials in terms of survival and relapse rates[11-13].

Given that radiotherapy has been included as a component of adjuvant therapy at our institution, the present single-centre retrospective study (1998-2010) was designed to analyze survival data in patients with gastric cancer after surgical resection in relation to efficacy of postoperative adjuvant therapy protocols and survival determinants.

MATERIALS AND METHODS

Study population

A total of 201 patients (mean ± SD age: 56.0 ± 11.9) years, 69.7% were male with gastric cancer who were operated and followed up at Lutfi Kirdar Kartal Training and Research Hospital between 1998 and 2010 were included in this retrospective study. In order to prevent the likelihood of misinterpretation of survival outcome, follow up was evaluated divided into two consecutive periods (before 2008 and 2008-2010, respectively) based on introduction of 3-D conformal technique in radiotherapy in 2008. All patients who were operated due to gastric cancer with stage T3 or T4 and/or any T level with positive lymph node (stage ⅠB-ⅢC) were included in the study except for 2 patients who had radiotherapy per se as the post adjuvant treatment.

While the present study was exempt from the requirement of ethical approval in relation to its retrospective design, the permission was obtained from our institutional ethics committee for the use of patient data for publication purposes.

Study parameters

Data on patient demographics, clinical and histopathological characteristics of gastric carcinoma and the type of treatment applied after surgery

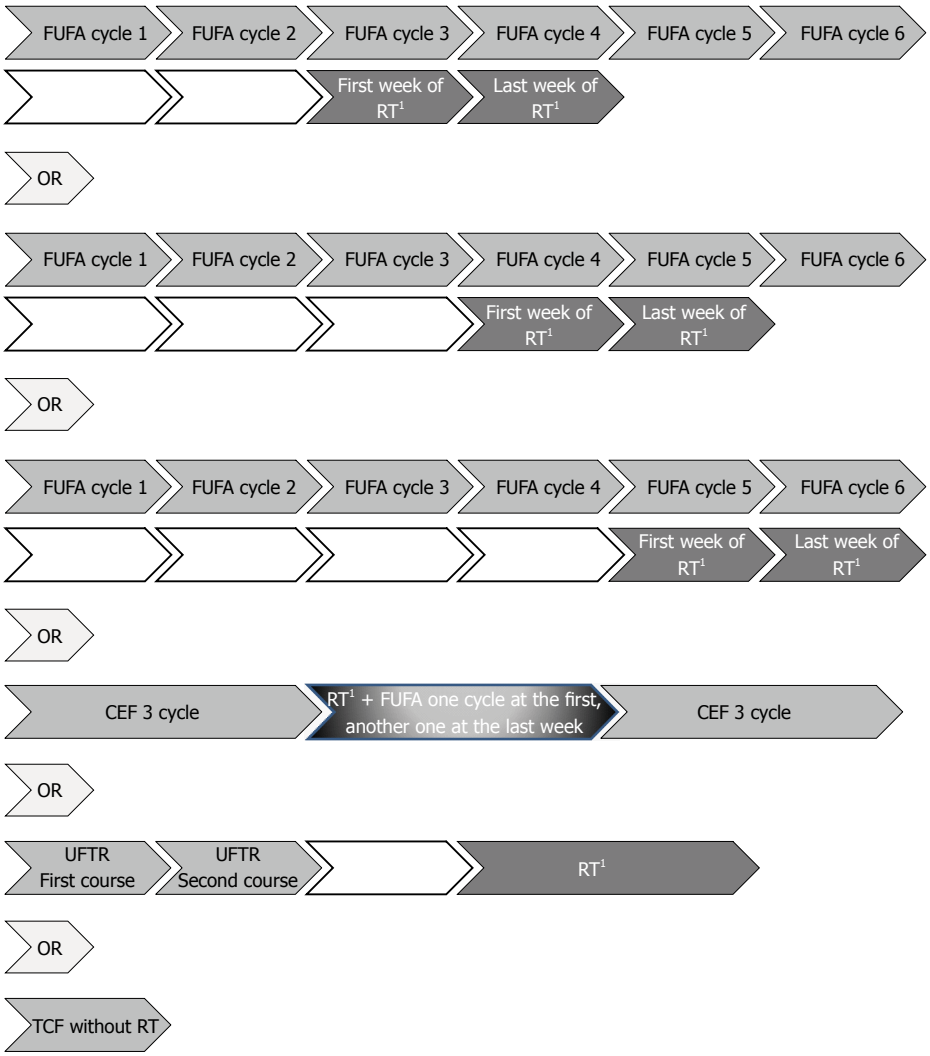

Figure 1 Schema of postoperative chemoradiotherapy. [1]Total duration of RT (45 Gy/25 fractions) = 5 wk. FUFA: 5-fluorouracil and leucovorin-folinic acid; CEF: Cyclophosphamide, epirubicin, and fluorouracil; UFTR: Uracil/tegafur; TCF: Docetaxel, cisplatin, and fluorouracil; RT: Radiation therapy.

(postoperative adjuvant treatment protocols including CRT, CT, supportive therapy or follow up without any treatment) were recorded and the rate and determinants of local recurrence free (LRF) survival, distant metastasis free (DMF) survival and overall survival were evaluated in the overall population as well as with respect to follow up years [1998-2008 (n = 127) *vs* 2008-2010 (n = 74)].

Staging

Clinical staging was performed according to American Joint Committee on Cancer (AJCC) Staging Manual, Sixth Edition (2002 and 2010), published by Springer Science + Business Media. Details of tumour site, histology and stage were recorded, as was the type of surgical resection on the basis of histopathological reports. Thorax and total abdominal computed tomography, complete blood counts including liver and renal function tests and bone scan if elevated alkaline phosphotase or bone pain present were performed for distant metastasis evaluation.

Chemoradiotherapy

Figure 1 illustrates the schema of postoperative chemoradiotherapy. The CT regimen involved 1-2 cycles of bolus FUFA [5-fluorouracil (5-FU, 400 mg/m^2 per day) and leucovorin-folinic acid (FA, 20 mg/m^2) D1-5 every 28 d]. Usually third and fourth or fourth and fifth cycles of FUFA or fifth and sixth cycles of FUFA according to performance status of patient and patient waiting list for machine, were applied concomitantly during first and last weeks of RT course, remaining cycles of CT were given in 4 wk after the completion of radiotherapy or CEF [(cisplatin 50 mg/m^2), eprubicin (50 mg/m^2) and 5-FU (500 mg/m^2), D1 every 21 d], followed by 45 Gy simulator planned concurrent radiotherapy in 25 daily fractions of for 5 wk. For CEF regime, usually after 3 cycles, concomitantly FUFA was applied during first and last week of radiotherapy the same as FUFA regime, after completion of CRT, remaining 3 cycles of CEF

was applied. At 3 weekly intervals 14 d of UFTR [a fixed combination of the oral FU prodrug tegafur (flouroprymidine, FT, 300 mg/m^2 per day in two divided doses) with FA (Antrex®), 15 mg tablet, two times a day] was given for two to three course and then the same doses with radiotherapy throughout the whole radiotherapy course excluding weekends for 5 wk. Few patients were applied TCF regime without radiotherapy including TCF (docetaxel 75 mg/m^2 in combination with cisplatin 75 mg/m^2 on Day 1 and fluorouracil 750 mg/m^2 per day by continuous infusion for five days).

Radiotherapy

Two dimensional (2D) treatment was applied with Saturn 41, 1996, France, GE using plan Target 2; 3D treatments were applied with Siemens Onco impression 2007, with XiO planning system, 2007 or DHX, varian, United States, eclips planning, 2007. Fields included tumor site, residual stomach and peripheral lymph nodes. All the rules of RTOG for organ at risks were strictly obeyed, no overdose was used.

Follow up

After completion of treatments every three months for two years, 6 mo up to 5 years, annually afterwards, controls of patients included physical examination, whole blood counts liver and renal function tests, tumor markers carcinoembryonic antigen and carbohydrate antigen 19-9, total abdominal ultrasonography or magnetic resonance imaging, chest X-ray when patient has any complaints.

Statistical analysis

Statistical analysis was made using MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013), χ^2 and Fisher-Exact tests were used for the comparison of categorical data, while numerica data were analyzed using Student-*t* test and Mann-Whitney *U* test for variables with normal distribution and for non-normally distributed variables, respectively. Survival analysis was made *via* Kaplan Meier analysis and comparisons were made *via* Log-Rank test. Correlates of survival were determined *via* Cox-Regression analysis with inclusion of independent variables with $P < 0.2$ significance in the univariate analysis into the model *via* Hosmer-Lemeshow method. Data were expressed as "mean ± SD", minimum-maximum and percent (%) where appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic and clinicopathological characteristics of patients

Adenocarcinoma (65.2%) was the most common histological type; the tumor was poorly differentiated in 64.2% of patients and located in the antrum in 47.2% with T3T4 stage in 68.7% and AJCC 2002 nodal stage of N1 in 48.3% and AJCC 2010 nodal stage of N1 in 21.9% (Table 1).

In patients followed up before 2008, adenocarcinoma type (72.4% *vs* 52.7%, $P = 0.001$) was more common and vascular involvement (64.8% *vs* 80.6%, $P = 0.033$) was less common than in patients followed up after 2008, while demographic and other clinicopathological characteristics were similar between the two groups (Table 1).

Postoperative adjuvant treatment protocols

CRT was the leading postoperative treatment applied in 73.1% of overall patients and more commonly in the follow up period of 2008-2010 compared to follow up before 2008 (85.1% *vs* 66.1%, $P = 0.023$). CT *per se*, supportive treatment and follow up without treatment were more prevalent in the follow up before 2008 compared with later years (Table 2).

FUFA (55.7% in the overall population, 55.1% before 2008, and 56.8% in 2008-2010) was the most commonly applied CT protocol regardless of the follow up period, as followed by CEF (14.9%) and UFT (11.9%). CEF in patients followed up in 2008-2010 (28.3% *vs* 7.1%, $P < 0.001$) and UFT in patients followed up before 2008 (18.1% *vs* 1.4%, $P < 0.001$) were more common CTs (Table 2).

Considering radiotherapy use of Co^{60} or Lineer accelerator 6-15 MV photon with 2-D simulator planning (62.7%) before 2008, while use of 6-23 MV photon lineer accelaretors with 3-D simulator planning (75.7%) after 2008 were more common ($P < 0.001$, Table 2).

Median survival with respect to study variables

Median duration for LRF survival, DMF survival and overall survival were 31.9, 24.1 and 31.9 mo, respectively in patients who received postoperative adjuvant CRT. Local recurrence occurred in 27 (13.7%) patients during the entire follow up and in 18 (14.4%) and 9 (12.5%) patients in the follow up periods of 1998-2008 and 2008-2010, respectively.

No significant difference was noted in median duration for LRF survival, DMF survival and overall survival with respect to treatment protocols during the entire follow up period as well as in patients followed up before or after 2008 (Table 3).

In the overall population, CT protocols FUFA (29.9 mo) and UFT (42.5 mo) were significantly associated with longer median duration for LRF survival than CEF (13.3 mo) and TCF (15.0 mo) ($P = 0.036$ for each), while no difference was noted between CT protocols in terms of DMF survival and overall survival (Table 3). Among patients received CRT, overall survival was significantly longer in patients with negative than positive surgical margin (27.7 mo

Table 1 Demographic and clinicopathologic characteristics of patients *n* (%)

	Follow up (yr)		Total (*n* = 201)	*P* value
	Before 2008 (*n* = 127)	2008-2010 (*n* = 74)		
Demographics				
Age (yr), mean ± SD	56.7 ± 12.7	54.7 ± 10.3	56.0 ± 11.9	0.270[1]
Gender				
Female	36 (28.3)	25 (33.8)	61 (30.3)	0.419[2]
Male	91 (71.7)	49 (66.8)	140 (69.7)	
Clinicopathologic characteristics				
Pathological type				
AdenoCa	92 (72.4)	39 (52.7)	131 (65.2)	0.001[2]
Signet ring	33 (26.0)	31 (41.9)	64 (31.8)	
Squamous	2 (1.6)	0	0	
Mucinous	0	4 (5.4)	4 (2.0)	
Tumor size				
< 5 cm	49 (43.0)	28 (47.5)	77 (44.5)	0.055[2]
5-10 cm	60 (52.6)	23 (39.0)	83 (48.0)	
> 10 cm	5 (4.4)	8 (13.6)	13 (7.5)	
Tumor location				
Antrum	56 (44.8)	37 (51.4)	93 (47.2)	0.249[2]
Cardia	29 (23.2)	15 (20.8)	44 (22.3)	
Corpus	37 (29.6)	15 (20.8)	52 (26.4)	
> 1 region	3 (2.4)	5 (6.9)	8 (4.1)	
Differentiation				
Well	2 (1.7)	1 (1.8)	3 (1.7)	0.885[2]
Moderate	41 (35.3)	18 (31.6)	59 (34.1)	
Poor	73 (62.9)	38 (66.7)	111 (64.2)	
Surgery type				
Total gastrectomy	64 (50.4)	37 (50.0)	101 (50.2)	0.957[2]
Subtotal gastrectomy	63 (49.6)	37 (50.0)	100 (49.8)	
Surgical margin				
Negative	114 (89.8)	61 (82.4)	175 (87.1)	0.135[2]
Positive	13 (10.2)	13 (17.6)	26 (12.9)	
Vascular involvement	81 (64.8)	58 (80.6)	139 (70.6)	0.033[3]
Perineural involvement	80 (64.0)	52 (72.2)	132 (68.4)	0.426[3]
TM stage				
T1T2	40 (31.5)	23 (31.1)	63 (31.3)	0.951[2]
T3T4	87 (68.5)	51 (68.9)	138 (68.7)	
Nodal stage				
N0	30 (24.2)	12 (16.7)	42 (21.4)	0.254[2]
N1	55 (44.4)	26 (36.1)	81 (41.3)	
N2	26 (21.0)	24 (33.3)	50 (25.5)	
N3	13 (10.5)	10 (13.9)	23 (11.7)	
AJCC 2002 nodal stage				
N0	32 (25.2)	14 (18.9)	46 (22.9)	0.364[2]
N1	62 (48.8)	35 (47.3)	97 (48.3)	
N2	26 (20.5)	15 (20.3)	41 (20.4)	
N3	6 (4.7)	8 (10.8)	14 (7.0)	
NX	1 (0.8)	2 (2.7)	3 (1.5)	
AJCC 2010 nodal stage				
N0	32 (25.2)	14 (18.9)	46 (22.9)	0.231[2]
N1	32 (25.2)	12 (16.2)	44 (21.9)	
N2	31 (24.4)	23 (31.1)	54 (26.9)	
N3a	25 (19.7)	15 (20.3)	40 (19.9)	
N3b	6 (4.7)	8 (10.8)	14 (7.0)	
Nx	1 (0.8)	2 (2.7)	3 (1.5)	
Dissected lymph nodes, median (IQR)	15 (13.0)	17.5 (13.0)	16 (14.0)	0.216[4]
LN1				
< 15	66 (52.4)	30 (40.5)	96 (48.0)	0.106[2]
⩾ 16	60 (47.6)	44 (59.5)	104 (52.0)	
LN2				
< 10	38 (30.2)	18 (24.3)	56 (28.0)	0.375[2]
⩾ 11	88 (69.8)	56 (75.7)	144 (72.0)	
Involved lymph nodes, median (IQR)	2 (6.0)	4 (7.0)	3 (6.0)	0.073[4]

[1]Student-t test; [2]χ^2 test; [3]Fisher-Exact test; [4]Mann-Whitney *U* test. AJCC: American Joint Committee on Cancer.

Table 2 Postoperative adjuvant treatment protocols *n* (%)

Treatment	Follow up (yr)		Total (*n* = 201)	*P* value[1]
	Before 2008 (*n* = 127)	2008-2010 (*n* = 74)		
Chemoradiotherapy	84 (66.1)	63 (85.1)	147 (73.1)	0.023
Chemotherapy	19 (15.0)	6 (8.1)	25 (12.4)	
Supportive treatment	10 (7.9)	2 (2.7)	12 (6.0)	
None (follow up)	14 (11.0)	3 (4.1)	17 (8.5)	
Radiotherapy				0.003
No	43 (33.9)	11 (14.9)	54 (26.9)	
Yes	84 (66.1)	63 (85.1)	147 (73.1)	
Chemotherapy protocol				< 0.001
FUFA	70 (55.1)	42 (56.8)	112 (55.7)	
CEF	9 (7.1)	21 (28.3)	30 (14.9)	
None	14 (11.0)	3 (4.1)	17 (8.5)	
UFT	23 (18.1)	1 (1.4)	24 (11.9)	
TCF	1 (0.8)	5 (6.8)	6 (3.0)	
Supportive	10 (7.9)	2 (2.7)	12 (6.0)	
Type of radiotherapy device				< 0.001
Co60	21 (25.3)	0 (0.0)	21 (14.4)	
Linak	58 (69.9)	7 (11.1)	65 (44.5)	
Varian-Siemens	4 (4.8)	56 (88.9)	60 (41.1)	
Simulator planning				< 0.001
2 dimensional	79 (62.7)	7 (9.5)	86 (43.0)	
3 dimensional	4 (3.2)	56 (75.7)	60 (30.0)	
No radiotherapy	43 (34.1)	11 (14.9)	54 (27.0)	

[1]χ^2 test. FUFA: 5-fluorouracil and leucovorin-folinic acid; CEF: Cyclophosphamide, epirubicin, and fluorouracil; UFT: Uracil/tegafur; TCF: Docetaxel, cisplatin, and fluorouracil.

Table 3 Median survival (mo) in study groups according to variables

	Total (*n* = 201)			Before 2008 (*n* = 127)			2008-2010 (*n* = 74)		
	LFS	DFS	OS	LFS	DFS	OS	LFS	DFS	OS
Treatment protocols									
Chemoradiotherapy	31.9	24.1	31.9	37.8	24.1	37.8	11.7	19.2	11.7
Chemotherapy	25.9	20.6	27.1	43.6	22.3	51.7	15.0	18.2	15.3
P value[1]	0.793	0.834	0.597	0.792	0.656	0.630	0.959	0.848	0.715
Chemotherapy protocols[a]									
FUFA	29.9	23.8	31.9						
UFT	42.5	20.6	53.0						
CEF	13.3	16.0	13.3						
TCF	15.0	1.6	15.0						
P value (FUFA-UFT)[1]	0.036	0.6	0.477						
Surgical margin[2]									
Positive	20.6	21.4	22.4	43.8	20.5	52.2	15.0	19.1	15.0
Negative	26.0	19.2	27.7	41.5	48.2	50.9	15.0	18.3	15.3
P value[1]	0.509	0.511	0.016	0.239	0.126	0.053	0.519	0.699	0.185
RT simulator planning[b]									
2 dimensional	42.4	23.1	50.4						
3 dimensional	14.1	16.0	14.1						
P value[3]	NA	NA	NA						
Time of RT initiation									
< 4 mo	36.3	20.6	40.9						
⩾ 4 mo	39.8	16.8	39.8						
P value	0.058[1]	NA	0.370[4]						

[1]Kaplan Meier-Log rank test; [2]Based on patients on chemoradiotherapy; [3]Not calculated to exclude potential bias since mean follow up duration was significantly longer in the 2 dimensional simulator planning group. Analysis was performed in the overall population, since [a]Only 1 patient recevied FUFA in the "after 2008" group; [b]2-dimensional planning was the leading option before 2008 and 3 dimensional planning after 2008; [4]Breslow test. FUFA: 5-fluorouracil and leucovorin-folinic acid; CEF: Cyclophosphamide, epirubicin, and fluorouracil; UFT: Uracil/tegafur; TCF: Docetaxel, cisplatin, and fluorouracil; RT: Radiation therapy; NA: Not available; OS: Overall survival; PFS: Progression-free survival; DFS: Disease-free survival.

vs 22.4 mo, *P* = 0.016), while the interval between surgery and radiotherapy had no significant impact on survival times (Table 3).

Univariate analysis for the correlates of survival

In the univariate analysis, vascular involvement (*P* = 0.005 in follow up before 2008), AJCC 2002 nodal

Table 4 Univariate analysis for the correlates of median local recurrence free, distant metastasis free and overall survival

	Local recurrence free survival (mo)			Distant metastasis free survival (mo)			Overall survival (mo)		
	Before 2008 (*n* = 127)	2008-2010 (*n* = 74)	Total (*n* = 201)	Before 2008 (*n* = 127)	2008-2010 (*n* = 74)	Total (*n* = 201)	Before 2008 (*n* = 127)	2008-2010 (*n* = 74)	Total (*n* = 201)
Age									
≤ 50 yr	44.1	20.6	49.8	14.0	23.1	14.0	28.7	20.6	33.7
> 50 yr	39.1	26.0	43.6	15.9	18.3	15.9	26.0	22.2	26.7
P value	0.066	0.471	0.312	0.747	0.925	0.48	0.044	0.65	0.181
Type of treatment									
Chemoradiotherapy	37.8	24.1	37.8	11.7	19.2	11.7	31.9	24.1	31.9
Chemotherapy	43.6	22.3	51.7	15.0	18.2	15.3	25.9	20.6	27.1
P value	0.792	0.656	0.63	0.959	0.848	0.715	0.793	0.834	0.597
Type of gastrectomy surgery									
Total	39.8	15.9	26.7	29.8	18.3	24.0	43.9	15.9	29.1
Subtotal	43.3	13.8	25.9	14.9	19.1	16.0	46.9	13.8	28.5
P value	0.885	0.122	0.888	0.395	0.636	0.393	0.318	0.1	0.092
Vascular involvement									
No	49.7	11.5	39.7	26.0	-	26.0	49.8	11.5	43.9
Yes	35.0	16.0	23.5	24.0	18.3	21.4	37.8	16.2	24.0
P value	0.697	0.005	0.594	0.858	-	0.81	< 0.001	0.795	< 0.001
Perineural involvement									
No	45.9	14.9	31.3	24.0	-	24.0	48.6	14.9	32.5
Yes	41.2	15.0	25.0	22.4	16.0	20.5	43.6	15.2	25.9
P value	0.706	0.353	0.822	0.609	-	0.999	0.122	0.275	0.058
T stage									
T1	55.4	20.2	48.6	27.6	-	27.6	55.4	20.2	48.6
T2	54.4	16.4	28.3	29.4	30.2	29.8	54.4	16.9	32.4
T3	37.0	14.1	25.1	24.1	18.3	22.1	41.1	14.1	26.0
T4	30.9	24.4	24.4	18.6	12.6	16.6	31.8	30.6	30.6
P value	0.054	0.959	0.128	0.668	0.223	0.353	0.04	0.28	0.002
Nodal stage									
N0	54.9	13.7	45.7	41.1	22.2	41.1	54.9	14.8	47.8
N1	46.9	14.6	29.1	30.6	21.0	27.7	53.7	14.8	37.0
N2	23.2	16.0	19.2	16.6	18.3	16.6	24.1	16.0	19.2
N3	27.1	13.4	16.6	16.1	12.6	15.0	35.0	13.4	20.7
P value	0.515	0.504	0.647	0.02	0.887	0.014	< 0.001	0.439	< 0.001
Nodal stage (AJCC 2002)									
N0	54.9	11.3	42.1	41.1	22.2	41.1	54.9	11.3	45.7
N1	42.4	16.9	28.3	30.6	20.5	26.0	49.2	16.9	29.1
N2	19.3	15.5	16.1	14.2	13.8	14.2	20.1	15.5	19.1
N3	31.0	10.7	15.0	19.5	19.8	19.8	31.0	10.7	15.0
P value	0.057	0.283	0.024	0.011	0.817	0.001	< 0.001	0.224	< 0.001
LN1									
< 15	40.5	14.9	30.5	24.0	19.1	23.1	45.7	14.9	33.2
> 16	42.7	15.0	25.4	24.0	18.3	20.5	44.9	15.2	25.7
P value	0.181	0.768	0.423	0.309	0.742	0.664	0.724	0.461	0.523
LN2									
< 10	34.0	15.9	25.4	20.9	22.2	22.2	40.5	16.2	31.0
> 11	46.4	14.6	26.8	24.1	16.0	22.2	47.8	14.6	28.0
P value	0.169	0.768	0.373	0.204	0.742	0.349	0.985	0.464	0.822
Nodal stage (AJCC 2010)									
N0	54.9	11.3	42.1	41.1	22.2	41.1	54.9	11.3	45.7
N1	51.0	16.9	39.1	29.4	36.8	33.1	53.8	16.9	44.9
N2	32.5	16.0	25.8	28.0	18.3	22.3	37.8	17.0	25.9
N3a	19.6	15.0	16.1	15.4	13.8	14.6	20.7	15.0	17.7
N3b	31.0	15.0	16.3	19.5	19.8	19.8	31.0	15.0	16.3
P value	0.131	0.402	0.057	0.06	0.849	0.039	< 0.001	0.098	< 0.001
Involved lymph nodes (*n*)									
≤ 5	48.6	35.5	52.2	15.9	24.1	16.2	35.5	31.9	38.3
> 5	20.7	16.9	26.0	15.0	16.0	15.2	16.1	16.3	16.6
P value	0.002	0.564	< 0.001	0.036	0.191	0.218	0.23	0.001	< 0.001

AJCC: American Joint Commission on Cancer; LN: Lymph nodes.

stage (P = 0.024 in overall study population) and the number of involved lymph nodes (P = 0.002 in follow up before 2008 and P < 0.001 in the overall study population) were significantly associated with LRF survival (Table 4).

Nodal stage (P = 0.020 in follow up before 2008,

Table 5 Multivariate Cox regression analysis for the correlates of local recurrence free, distant metastasis free and overall survival

	Local recurrence free survival						Distant metastasis free survival						Overall survival					
	Before 2008		2008-2010		Overall		Before 2008		2008-2010		Overall		Before 2008		2008-2010		Overall	
	Sig.	Exp(B)	Sig.	Exp(B)	Sig.	Exp(B)	Sig.	Exp(B)	Sig.	Exp(B)	Sig.	Exp(B)	Sig.	Exp(B)	Sig.	Exp(B)	Sig.	Exp(B)
Age (yr)																		
< 50 *vs* > 50	0.748	1.191			0.387	1.468							0.122	1.742				
Operaton type																		
Total *vs* subtotal			0.118	0.284											0.147	0.426		
Vascular involvement																		
Yes *vs* No			0.748	0.707									0.099	2.375			0.085	2.106
Perinueral involvement																		
Yes *vs* No													0.656	0.820			0.326	0.707
T stage	0.772				0.318								0.781				0.762	
T1	0.938	10856.260			0.936	18774.638							0.995	0.995			0.967	0.967
T2	0.934	21845.678			0.935	23091.062							0.674	1.425			0.651	1.447
T3	0.933	24325.273			0.928	62667.340							0.969	0.962			0.734	1.369
LN1																		
< 15 *vs* > 16	0.458	2.278					0.722	0.863										
LN2																		
< 10 *vs* > 11	0.073	0.146																
Nodal stage (AJCC 2010)	0.782				0.167		0.994				0.052		0.033				0.104	
N1	0.255	3.526			0.217	2.748	0.754	1.196			0.831	1.118	0.404	1.624			0.162	2.184
N2	0.232	3.853			0.042[1]	4.959	0.969	1.025			0.313	1.614	0.122	2.439			0.009[1]	4.056
N3a	0.452	3.407			0.786	1.380	0.814	1.266			0.014	3.218	0.006[1]	5.132			0.059	4.733
N3b	0.344	5.380			0.502	2.330	0.761	1.471			0.060	3.446	0.027[1]	5.263			0.098	4.292
İnvolved lymph nodes (n)																		
⩽ 5 *vs* > 5	0.464	2.322			0.242	2.573	0.283	2.677	0.207	2.245					0.303	1.742	0.726	1.245
Significance of the model	*P* = 0.053		*P* = 0.218		*P* = 0.017		*P* = 0.153		*P* = 0.196		*P* = 0.054		*P* = 0.001		*P* = 0.151		*P* < 0.001[1]	

[1]Indicate statistical significance at $\alpha = 0.05$ level. Only the variables with $P < 0.2$ significance in the univariate analysis were included into the Cox-regression model on the basis of Hosmer-Lemeshow method. Since both American Joint Commission on Cancer (AJCC) 2002 and 2010 nodal staging variables met the criteria to be included in the multivariate analysis, only AJCC 2010 nodal staging was included.

P = 0.014 in the overall study population), AJCC 2002 nodal stage (*P* = 0.011 in follow up before 2008, *P* = 0.001 in the overall study population), AJCC 2010 nodal stage (*P* = 0.039 in the overall study population) and the number of involved lymph nodes (*P* = 0.036 in follow up before 2008) were significantly associated with DMF survival (Table 4).

Age (*P* = 0.014 in the overall study population), vascular involvement (*P* < 0.001 in follow up before 2008 and in the overall study population), T stage (*P* = 0.04 in follow up before 2008 and *P* = 0.002 in the overall study population), nodal stage, AJCC 2002 nodal stage and AJCC 2010 nodal stage in follow up before 2008 and in the overall study population, *P* < 0.001 for each) and the number of involved lymph nodes (*P* = 0.001 in follow up before 2008 and *P* < 0.001 in the overall study population) were the significant correlates of overall survival (Table 4).

Multivariate Cox regression analysis for the determinants of survival

None of the variables determined to be significantly associated with LRF survival in the univariate analysis (vascular involvement and number of involved lymph

nodes) was significant correlates of LRF survival in the multivariate analysis in patients followed before 2008. Multivariate analysis confirmed the association between nodal stage and LRF survival in the overall study population with 4.959 fold (P = 0.042) increase in mortality in patients with nodal stage N2 compared to patients with nodal stage N0, while no significant association was noted in terms of number of involved lymph nodes (Table 5).

None of the variables determined to be significantly associated with DMF survival in the univariate analysis (nodal stage and number of involved lymph nodes) was significant correlates of DMF in the multivariate analysis in patients followed before 2008 or in the overall study population (Table 5).

Except for 5.132 fold (P = 0.006), 5.263 fold (P = 0.027) and 4.056 fold (P = 0.009) increase in the mortality in patients with nodal stage N3a (before 2008), N3b (before 2008) and N2 (overall study population) when compared to patients with N0 stage, respectively, none of the variables significantly associated with overall survival in the univariate analysis was significant correlates of overall survival in the in the multivariate analysis in patients followed before 2008 and in the overall study population (Table 5).

DISCUSSION

The present retrospective single centre analysis of survival data (1998-2010) in patients with gastric carcinoma revealed median 31.9 mo of LRF survival, 24.1 mo of DMF survival and 31.9 mo of overall survival *via* postoperative adjuvant CRT during follow up from 1998 to 2010 with local recurrence rate of 13.7% in the overall study population. No significant difference was observed in median duration of LRF survival, DMF survival and overall survival with respect to treatment protocols (CRT *vs* CT), interval between surgery and radiotherapy and the type of radiotherapy (2-D *vs* 3-D), while FUFA (29.9 mo) and UFT (42.5 mo) based CT protocols and absence of positive surgical margin (27.7 mo) were associated with significantly longer median durations of LRF survival and overall survival, respectively. Multivariate analysis revealed higher nodal stage to be a significant determinant of LRF in the overall study population, while to predict overall survival both in patients followed up in 1998-2008 and in overall study population.

Regardless of the follow up period, adenocarcinoma type, location in antrum, poor differentiation, T3T4 and N1-N2 stage were the leading histopathological characteristics of the tumor as identified in most of patients. Patients followed up in 2008-2010 period were associated with significantly higher rate of signet ring cell type of carcinoma, vascular involvement, use of CRT and CEF based CT protocols as well as 3-D conformal technique in RT when compared to patients followed up before 2008, while the two groups of follow up were homogenous in terms of demographic and other clinicopathological characteristics.

Although the demonstration of the efficacy of postoperative CRT for locally advanced gastric cancer in randomized clinical trials provide a basis for the consideration of this therapy as the standard of care for resectable high-risk disease, local recurrence rates have been indicated to remain at 19% even after adjuvant CRT[2,4], which seems in line with the local recurrence rate (13.7%) demonstrated in our study population.

Although the efficacy of postoperative CT following complete resection has been associated with a significant survival benefit in some studies especially with fluoropyrimidine based regimens[2,3,14].

Nonetheless, preceding the landmark Intergroup Trial INT-0116 on the effect of surgery plus postoperative CRT on the survival of patients resected for adenocarcinoma of the stomach after a 10-year median follow-up[2], postoperative adjuvant CRT has consistently been associated with improved overall and relapse free survivals rates in comparison to patients without CRT in several clinical trials[2,15-17].

Past studies concerning direct comparison of CT plus radiotherapy with CT-only in patients with gastric cancer revealed significant improvement in disease free survival[12] and in median duration of relapse-free survival with 30 mo *vs* 19 mo[2] and 50 mo *vs* 36 mo)[18], while a significant increase (36 mo *vs* 27 mo)[2] as well as no significant improvement (58 mo *vs* 48 mo)[18] were noted for overall survival in CRT *vs* CT-only arm.

Accordingly, albeit not statistically significant, a tendency for higher rates for LRF survival (31.9 mo *vs* 25.9 mo), DMF survival (24.1 mo *vs* 20.6 mo) and overall survival (31.9 mo *vs* 27.1 mo) with postoperative CRT *vs* CT in our study population are in agreement with the survival benefit of CRT indicated in the past studies.

Indeed, due to variability of accepted standards for incorporation of postoperative CRT into the routine clinical practice in different countries, the ideal oral chemotherapeutic agent to be used in CRT protocol has not yet been defined[6].

Tried primarily in advanced stage gastric cancer as an alternative to FU, UFT was shown to be as effective as FU in postoperative therapy in the past studies[19,20]. Notably, type of CT protocol had significant influence on LRF survival but not on DMF survival and overall survival in our study population with significantly improved LRF survival rates obtained similarly in patients received UFT (42.5 mo) and FUFA (29.9 mo) based regimens. This finding seems in line with the previously emphasized survival benefit of fluoropyrimidine (FT) as well as

FU[13,15] based regimens, while also supports that concurrent UFT with radiotherapy is an equally effective regimen in the postoperative treatment of gastric adenocarcinoma when compared to FUFA[6].

It should also be noted that restriction of the radiation dose to the intra-abdominal target volume which to 45-50 Gy due to adjacent dose-limiting organs in conventional RT has been suggested not be sufficient for disease control in patients with locally advanced gastric adenocarcinoma[4,21]. Nonetheless, use of escalated radiation doses with concurrent CT in an adjuvant setting has currently been considered as a strategy that deserves to be optimized and further evaluated in randomized clinical trials[4].

Extended interval between surgery and radiation has been considered to allow accelerate proliferation of cancer cells under stress and thus delivery of a larger dose early in the course of treatment has been suggested to further improve disease control of gastric cancer after surgical resection[4]. However, in our study population, the interval between surgery and radiotherapy initiation had no significant impact on survival times and similar values for overall survival was noted in patients who underwent radiotherapy within 4 mo (40.9 mo) *vs* after 4 mo (39.8 mo) of surgery.

Divided based on introduction of 3-D conformal technique in radiotherapy in 2008 at our clinic, the two consecutive periods of follow up (from 1998 to 2008 and from 2008 to 2010) in our study showed distinct median durations for LRF survival (37.8 mo *vs* 24.1 mo), DMF survival (11.7 mo *vs* 19.2 mo) and overall survival (31.9 mo *vs* 24.1 mo) with postoperative adjuvant CRT. However one must remain prudent when comparing these results, given that no significant difference was noted in median duration of survival with respect to treatment protocols (CRT *vs* CT) as well as type of radiotherapy (2-D *vs* 3D) along with higher proportion of patients under UFT therapy in the 1998-2008 group, and more importantly the remarkable difference between these groups in terms of duration of follow up (10 years *vs* 3 years). In this regard, longer term follow-up is needed to determine the actual treatment outcome and thereby the optimal therapy in our patients with gastric cancer.

Additionally, it should be emphasized that increasing evidence for a survival advantage from adjuvant therapies seems to enable postoperative CRT to become standard practice in patients with resectable gastric cancer only if treatment-related complications are minimized to ensure the maintenance of the survival advantage[2,15,17].

Lymph node metastasis was reported amongst the prognostic factors for gastric cancer in several studies[22-24]. In patients with gastric cancer, the 5-year survival rate N0, N1, N2, N3a and N3b after D2 lymph node dissection were reported to be 89.7%, 73.6%, 54.9%, 23.1% and 5.4%, respectively in a recent study[25], while positive lymph node and TNM stage were documented as independent prognostic factors for gastric cancer in a recent multivariate analysis[18]. Likewise, our findings indicated higher nodal stage as the common predictor of LRF survival in the overall population while of overall survival both in 1998-2008 group and in the overall study population

While higher nodal stage, T stage, and the number of involved lymph nodes were amongst the factors significantly associated with overall survival according to univariate analysis in our study population, these findings were not confirmed in the multivariate analysis. Larger scale studies with longer term follow up are needed to clarify prognostic determinants in patients with gastric carcinoma who received postoperative adjuvant CRT.

Certain limitations to this study should be considered. The major limitation seems to be the difference among study groups with respect to duration of follow up. Due to switching from 2-D to 3-D conformal technique in radiotherapy at our clinic in 2008, overall population was evaluated as divided into two consecutive periods of follow up including periods from 1998 to 2008 and between 2008 and 2010. However since data from patients in the first group are based on remarkably longer follow up of 10 years when compared to data from patients in the second group with 3 years of follow up, difference in survival times between two groups should be cautiously interpreted, given that no difference was noted in median duration of survival with respect to type of either post-adjuvant treatment protocol or the radiotherapy. Secondly, retrospective design seems to be another pitfall of our study which disabled to apply standard inclusion criteria and to enable patients to be prospectively randomized into treatment groups. Nevertheless, while based on a retrospective analysis of a single institution, our findings represent a solid ground for future larger scale prospective studies on comparison of different postoperative adjuvant treatment protocols in patients with gastric cancer in the longer term follow up.

In conclusion, based on identification of median 31.9 mo of LRF survival, 24.1 mo of DMF survival and 31.9 mo of overall survival *via* postoperative adjuvant CRT during follow up from 1998 to 2010 in our study population, our findings emphasize the likelihood of postoperative adjuvant CRT to have a survival benefit in patients with resectable gastric carcinoma. Use of FUFA and UFT based CT protocols and the absence of positive surgical margin in patients received CRT seems to be in favor of LRF survival and overall survival, respectively, while no significant difference was observed in duration of

survival with respect to treatment protocols (CRT *vs* CT), interval between surgery and radiotherapy and the type of radiotherapy (2-D *vs* 3-D). Nodal stage was the independent prognostic factor for LRF and overall survival, while concluding the efficacy of post adjuvant CRT and exact determinants of survival in gastric cancer patients seem to depend on conduction of future prospective randomized trials on comparison of surgery only and postoperative CRT within a longer period of follow-up.

COMMENTS

Background

Implication of neoadjuvant or adjuvant therapy in patients with resectable gastric cancer mainly in the form of postoperative chemoradiotherapy (CRT) and perioperative chemotherapy (CT) has been considered by several studies in terms achievement of better therapeutic outcomes and shown to be associated with high-level evidence for improved survival in Western populations.

Research frontiers

Despite increasing evidence available for a survival advantage from adjuvant therapies, adjuvant treatment strategies in patients with resectable gastric cancer still remains debated particularly in terms of favour of radiotherapy associated with CT, the adequacy of nodal dissection, the likelihood of CT related toxic effects and inconsistency of different therapeutic trials in terms of survival and relapse rates.

Innovations and breakthroughs

The present retrospective single centre analysis of survival data (1998-2010) in patients with gastric carcinoma revealed median 31.9 mo of local recurrence free (LRF) survival, 24.1 mo of distant metastasis free (DMF) survival and 31.9 mo of overall survival *via* postoperative adjuvant CRT during follow up from 1998 to 2010 with local recurrence rate of 13.7% in the overall study population. Albeit not statistically significant, a tendency for higher rates for LRF survival, DMF survival and overall survival with postoperative CRT *vs* CT in the study population are in agreement with the survival benefit of CRT indicated in the past studies. Extended interval between surgery and radiation has been considered to allow accelerate proliferation of cancer cells under stress and thus delivery of a larger dose early in the course of treatment has been suggested to further improve disease control of gastric cancer after surgical resection.

Applications

The findings emphasize the likelihood of postoperative adjuvant CRT to have a survival benefit in patients with resectable gastric carcinoma. Use of 5-fluorouracil and leucovorin-folinic acid and uracil/tegafur based CT protocols and the absence of positive surgical margin in patients received CRT seems to be in favor of LRF survival and overall survival, respectively, while no significant difference was observed in duration of survival with respect to treatment protocols (CRT *vs* CT), interval between surgery and radiotherapy and the type of radiotherapy (2-D *vs* 3-D) and nodal stage was the independent prognostic factor for LRF and overall survival.

Terminology

Patients with gastric cancer show poor prognosis and cure rate remains dismal with 5-year survival rates of 8%-34% and locoregional recurrence of 40%-90% even after curative resection. The implication of neoadjuvant or adjuvant therapy in patients with resectable gastric cancer mainly in the form of postoperative CRT and perioperative CT has been considered by several studies in terms achievement of better therapeutic outcomes.

Peer review

The authors performed retrospective single centre analysis of survival data (1998-2010) in patients with gastric carcinoma after curative resection. Theirs findings emphasize the likelihood of postoperative adjuvant CRT to have a survival benefit in patients with resectable gastric carcinoma. The authors concluded that prognosis of gastric cancer cases after curative resection with postoperative adjuvant chemoradiotherapy was better that that of cases without postoperative adjuvant.

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ORIGINAL ARTICLE

Retrospective Study

Hospital type- and volume-outcome relationships in esophageal cancer patients receiving non-surgical treatments

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Abstract

AIM: To study the "hospital type-outcome" and "volume-outcome" relationships in patients with esophageal cancer who receive non-surgical treatments.

METHODS: A total of 6106 patients with esophageal cancer diagnosed between 2008 and 2011 were identified from a national population-based cancer registry in Taiwan. The hospital types were defined as medical center and non-medical center. The threshold for high-volume hospitals was based on a median volume of 225 cases between 2008 and 2011 (annual volume, > 56 cases) or an upper quartile (> 75%) volume of 377 cases (annual volume > 94 cases). Cox regression analyses were used to determine the effects of hospital type and volume outcome on patient survival.

RESULTS: A total of 3955 non-surgically treated patients were included in the survival analysis. In the unadjusted analysis, the significant prognostic factors included cT, cN, cM stage, hospital type and hospital volume (annual volume, > 94 *vs* ⩽ 94). The 1- and 3-year overall survival rates in the non-medical centers (36.2% and 13.2%, respectively) were significantly higher than those in the medical centers (33.5% and 11.3%, respectively; $P = 0.027$). The 1- and 3-year overall survival rates in hospitals with an annual volume of ⩽ 94 (35.3% and 12.6%, respectively) were significantly higher than those with an annual volume of > 94 (31.1% and 9.4%, respectively; $P = 0.001$). However, in the multivariate analysis, the hospital type was not statistically significant. Only cT, cN, and cM stages and hospital volume (annual volume > 94 *vs* ⩽ 94) were independent prognostic factors.

CONCLUSION: Whether the treatment occurs in medical

centers is not a significant prognostic factor. High-volume hospitals were not associated with better survival rates compared with low-volume hospitals.

Key words: Cancer registry; Esophageal cancer; Hospital volume; Hospital type; Survival

Core tip: The hospital type-outcome and volume-outcome relationships in patients with esophageal cancer who receive surgical resection are well established. However, little is known concerning the hospital type- and volume-outcome relationships in patients without surgical resection. Our population-based study, including 3955 non-surgically treated patients, showed that the medical center is not a significant prognostic factor. Moreover, the high-volume hospitals were not associated with better survival rates compared with the low-volume hospitals.

Hsu PK, Chen HS, Wang BY, Wu SC, Liu CY, Shih CH, Liu CC. Hospital type- and volume-outcome relationships in esophageal cancer patients receiving non-surgical treatments. *World J Gastroenterol* 2015; 21(4): 1234-1242 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1234.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1234

INTRODUCTION

Even though multidisciplinary approaches and several combinations of therapies, such as surgery, chemotherapy, and radiotherapy, have been applied to treat esophageal cancer, the prognosis of patients with esophageal cancer is poor. Moreover, a large number of patients develop either locoregional recurrence or distant metastasis shortly after curative treatments; the prognosis for these patients is dismal[1]. To improve outcome, centralized care for esophageal cancer patients has been proposed. Several authors have suggested that referring patients to specialized units that have healthcare professionals with adequate experience may improve the quality of care as well as patient survival[2-4]. Indeed, hospital type-outcome analyses have demonstrated better outcome in university hospitals. For example, Dikken *et al*[5] reported that the 3-mo mortality rate after esophagectomy was 2.5% in university hospitals and 4.4% in non-university teaching hospitals, which was a significant difference ($P < 0.05$). Moreover, Verhoef *et al*[6] reported that the 5-year survival rate for surgical patients was 49.2% for the university hospitals versus 32.6% for the teaching non-university hospitals and 27.3% for the non-teaching hospitals ($P < 0.05$). The results of the hospital volume-outcome analysis supported the impact of volume on patient survival. For example, Birkmeyer *et al*[7] analyzed the Surveillance, Epidemiology, and End Results database and reported an absolute difference in 5-year likelihood of survival rates after esophagectomy for cancer between the low-volume hospitals (17%) and the high-volume hospitals (34%). A recent meta-analysis also demonstrated a long-term survival benefit after esophageal cancer resection for the high-volume hospitals (HR = 0.82; 95%CI: 0.75-0.90) compared with their low-volume counterparts[8].

However, the majority of the reports focused on the effect of hospital type and volume among patients who had undergone esophagectomy. There are few studies concerning how hospital type and volume influence the survival rate in patients without surgical resection. Therefore, we aimed to study the differences in patient and tumor characteristics according to hospital type and volume categories in this population-based study. We emphasized whether hospital type or volume affected the prognosis in patients with esophageal cancer who received non-surgical treatments.

MATERIALS AND METHODS

The patient data were obtained from the Taiwan Cancer Registry, which is a national population-based cancer registration database organized and funded by the Health Promotion Administration, Ministry of Health and Welfare, the executive branch of the central government. The hospitals with greater than 50-bed capacity, which provide outpatient care and hospitalized cancer care, are recruited to participate in reporting all newly diagnosed malignant neoplasms to the registry. The data were collected and verified by cancer registrars at each hospital. The clinical details including sex, date of birth, date of hospitalization, care facilities, date of diagnosis, clinical stage, surgical method, surgical margin, pathological stage, treatment modality, radiation dose, and survival status were recorded. Using the International Classification of Diseases for Oncology (ICD-O-3) site codes (C15.0, C15.1, C15.2, C15.3, C15.4, C15.5, C15.8, and C15.9) and morphology codes (8052, 8070, 8071, 8072, 8073, 8074, 8076, 8077, 8083 and 8084), 6106 patients who were diagnosed with esophageal squamous cell carcinoma (ESCC) between January 1, 2008 and December 31, 2011 were identified. The treatment modalities included the following: (1) neoadjuvant chemoradiation followed by surgery (n = 850); (2) surgery alone (n = 679); (3) surgery followed by chemotherapy or/and radiotherapy (n = 622); (4) definitive chemoradiation (n = 3020); (5) radiotherapy alone (n = 442); (6) chemotherapy alone (n = 333); and (7) unknown (n = 160).

To study the hospital type-outcome relationship,

Table 1 Patient characteristics according to hospital type *n* (%)

Variables	Total	Medical center or not		
		Yes	No	*P* value
Patient number	6106	4180	1926	
Hospital number	62	19	43	
Sex				0.963
Male	5768 (94.5)	3949 (94.5)	1819 (94.4)	
Female	338 (5.5)	231 (5.5)	107 (5.6)	
Age (yr)				< 0.001
< 40	189 (3.1)	133 (3.2)	56 (2.9)	
40-49	1413 (23.1)	908 (21.7)	505 (26.2)	
50-59	2272 (37.1)	1577 (37.7)	695 (36.1)	
60-69	1278 (20.9)	915 (21.9)	363 (18.9)	
70-79	658 (10.8)	433 (10.4)	225 (11.7)	
⩾ 80	296 (4.9)	214 (5.1)	82 (4.3)	
Tumor length (cm)				0.006
mean ± SD	5.3 ± 3.1	5.4 ± 3.0	5.1 ± 3.2	
cT stage				0.009
1	531 (8.7)	340 (8.1)	191 (9.9)	
2	838 (13.7)	544 (13.0)	294 (15.3)	
3	2994 (49.0)	2086 (49.9)	908 (47.1)	
4	1339 (21.9)	901 (21.6)	438 (22.7)	
Unknown	404 (6.6)	309 (7.4)	95 (4.9)	
cN stage				< 0.001
0	1385 (22.7)	862 (20.6)	523 (27.2)	
1/2/3	4488 (73.5)	3141 (75.1)	1347 (69.9)	
Unknown	233 (3.8)	177 (4.2)	56 (2.9)	
cM stage				0.424
0	4261 (69.8)	2931 (70.1)	1330 (69.1)	
1	1731 (28.4)	1167 (27.9)	564 (29.3)	
Unknown	114 (1.9)	82 (2.0)	32 (1.7)	
Tumor location				< 0.001
Upper third	1445 (23.7)	936 (22.4)	509 (26.4)	
Middle third	2152 (35.2)	1390 (33.3)	762 (39.6)	
Lower third	1134 (186)	758 (18.1)	376 (19.5)	
Unknown	1375 (22.5)	1096 (26.2)	279 (14.5)	
Tumor differentiation				< 0.001
Well	150 (2.5)	108 (2.6)	42 (2.2)	
Moderate	2759 (45.2)	1781 (42.6)	978 (50.8)	
Poorly	1309 (21.4)	916 (21.9)	393 (20.4)	
Unknown	1888 (30.9)	1375 (32.9)	513 (26.6)	
Treatment modality				0.009
Surgery with neoadjuvant chemoradiation	850 (13.9)	610 (14.6)	240 (12.5)	
Surgery without neoadjuvant chemoradiation	1301 (21.3)	914 (21.9)	387 (20.1)	
No surgery	3955 (64.8)	2656 (63.5)	1299 (67.5)	
Hospital volume				< 0.001
Q1-Q2	3137	1504 (36.0)	1633 (84.8)	
Q3-Q4	2969	2676 (64.0)	293 (15.2)	
Hospital volume				< 0.001
Q1-Q3	4717	2791 (66.8)	1926 (100.0)	
Q4	1389	1389 (33.2)	-	

the hospital types were defined as medical center and non-medical center according to Taiwan Joint Commission on Hospital Accreditation (http://www.tjcha.org.tw) based on the quality of process and outcome in healthcare performance. There are a total of 19 medical centers in Taiwan. To study the volume-outcome relationship, the hospitals were divided into quartiles (Q1-Q4) of total hospital volume between 2008 and 2011. The threshold for high-volume hospitals was based on the median (Q3-4, > 50%) volume of 225 cases between 2008 and 2011 (annual volume, > 56 cases) or upper quartile (Q4, > 75%) volume of 377 cases (annual volume, > 94 cases). A subset of 3955 patients who were treated without surgical resection was included in the outcome analysis. The outcome measures were 1- and 3-year overall survival. The survival time was defined as the number of days between the date of diagnosis and the date of death or the end of the study on December 31, 2012, whichever occurred first.

Statistical analysis

The categorical and continuous variables were compared using the χ^2 test and Student's *t*-test, respectively. The survival curves were plotted using the Kaplan-Meier method and were compared using the log-rank test. The differences in survival estimates were calculated using the Cox proportional hazards regression model, stratified for hospital type or volume, and adjusted for known prognostic factors. All of the statistical calculations were performed using SAS version 9.3 (SAS Institute, Inc, Cary, NC) and SPSS version 17.0 (SPSS Inc, Chicago, IL). *P* less than 0.05 was considered to be statistically significant.

RESULTS

The characteristics of patients according to hospital type are presented in Table 1. A total of 6106 patients received treatments for ESCC in 62 hospitals, whereas 4180 (68.5%) of 6106 patients were treated in 19 medical centers and 1926 patients (31.5%) were treated in non-medical centers. The patients who were treated in medical centers were more likely to be older than those treated in non-medical centers (57.45 ± 11.4 years *vs* 56.89 ± 11.6 years). As for tumor characteristics, a higher percentage of patients with advanced stage tumors was found in medical centers. The patients who were treated in medical centers had tumors of larger size (5.4 ± 3.0 cm *vs* 5.1 ± 3.2 cm, $P = 0.006$), and a higher frequency was noted to be cT3/4 (71.5% *vs* 69.8%, $P = 0.009$) and clinical node-positive tumors (75.1% *vs* 69.9%, $P < 0.001$). Furthermore, a higher proportion of the patients in medical centers than in non-medical centers received surgical resection (36.5% *vs* 32.6%, $P = 0.009$).

Table 2 presents the characteristics of patients according to hospital volume. There were 8 hospitals, including 7 medical centers, in quartiled 3-4, whereas only 3 medical centers were in quartile 4. There was no difference in the distribution of age and sex between high- and low-volume hospitals. However, the patients who were treated in high-volume hospitals had tumors of larger size (Q4 *vs*

Table 2 Patient characteristics according to hospital volume *n* (%)

Variables	Hospital volume			Hospital volume		
	Q1-Q2	Q3-Q4	*P* value	Q1-Q3	Q4	*P* value
No. of annual cases	⩽ 225	> 225		⩽ 377	> 377	
No. of patients	3137	2969		4717	1389	
No. of hospitals	54	8		59	3	
Sex			0.295			0.295
Male	2954 (94.2)	2814 (94.8)		4465 (94.7)	1303 (93.8)	
Female	183 (5.8)	155 (5.2)		252 (5.3)	86 (6.2)	
Age (yr)			0.182			0.56
< 40	88 (2.8)	101 (3.4)		142 (3.0)	47 (3.3)	
40-49	715 (22.8)	698 (23.5)		1108 (23.3)	305 (22.0)	
50-59	1194 (36.9)	1078 (36.3)		1762 (37.1)	510 (36.7)	
60-69	629 (20.1)	649 (21.9)		976 (20.6)	302 (21.8)	
70-79	352 (11.3)	306 (10.3)		496 (10.5)	162 (11.7)	
⩾ 80	159 (5.1)	137 (4.6)		233 (4.9)	63 (4.5)	
Tumor length (cm)			0.845			< 0.001
mean ± SD	5.3 ± 3.2	5.3 ± 2.9		5.2 ± 3.0	5.7 ± 3.0	
cT stage			0.005			< 0.001
1	245 (7.8)	286 (9.6)		412 (8.7)	119 (8.6)	
2	466 (14.9)	372 (12.5)		711 (15.1)	127 (9.1)	
3	1531 (48.8)	1463 (49.3)		2167 (45.9)	827 (59.5)	
4	710 (22.6)	629 (21.2)		1073 (22.8)	266 (19.2)	
Unknown	185 (5.9)	219 (7.4)		354 (7.5)	50 (3.6)	
cN stage			< 0.001			< 0.001
0	820 (26.1)	565 (19.0)		1183 (25.1)	202 (14.5)	
1/2/3	2210 (70.5)	2278 (76.7)		3340 (70.8)	1148 (82.7)	
Unknown	107 (3.4)	126 (4.2)		194 (4.1)	39 (4.1)	
cM stage			0.002			0.014
0	2177 (69.4)	2084 (70.2)		3257 (69.1)	1004 (72.3)	
1	919 (29.3)	812 (27.4)		1362 (28.9)	369 (26.6)	
Unknown	41 (1.3)	73 (2.5)		98 (2.1)	16 (1.2)	
Tumor location			< 0.001			< 0.001
Upper third	801 (25.5)	644 (21.7)		1192 (25.3)	253 (18.2)	
Middle third	1223 (39.0)	929 (31.3)		1785 (37.8)	367 (26.4)	
Lower third	600 (19.1)	534 (18.0)		894 (19.0)	240 (17.3)	
Unknown	513 (16.4)	862 (29.0)		846 (17.9)	529 (38.1)	
Tumor differentiation			< 0.001			< 0.001
Well	76 (2.4)	74 (2.5)		128 (2.7)	22 (1.6)	
Moderate	1611 (51.4)	1148 (38.7)		2256 (47.8)	503 (36.2)	
Poorly	677 (21.6)	632 (21.3)		892 (18.9)	417 (30.0)	
Unknown	773 (24.6)	1115 (37.6)		1441 (30.6)	447 (32.2)	
Treatment modality			< 0.001			< 0.001
Surgery with neoadjuvant chemoradiation	381 (12.1)	469 (15.8)		524 (11.1)	326 (23.5)	
Surgery without neoadjuvant chemoradiation	643 (20.4)	658 (22.2)		1056 (22.4)	245 (17.6)	
No surgery	2113 (67.4)	1842 (62.0)		3137 (66.5)	818 (58.9)	
Hospital type			< 0.001			< 0.001
Medical center	1504 (47.9)	2676 (90.1)		2791 (59.2)	1389 (100.0)	
Not center	1633 (52.1)	293 (9.9)		1926 (40.8)	0	

Q1-3: 5.7 ± 3.0 cm *vs* 5.2 ± 3.0 cm, $P < 0.001$), a higher frequency was noted to be cT3/4 (Q4 *vs* Q1-3: 78.7% *vs* 68.7%, $P < 0.001$) and clinical node-positive tumors (Q4 *vs* Q1-3: 82.7% *vs* 70.8%, $P < 0.001$). There was also a higher proportion of patients in high-volume hospitals than in low-volume hospitals who received surgical resections (Q4 *vs* Q1-3: 41.1% *vs* 33.5%, $P < 0.001$).

Table 3 summarized the characteristics of patients according to hospital volume. There was lower frequency of clinical stage Ⅱ (10.2% *vs* 15.4%) and higher frequency of stage Ⅲ (44.6% *vs* 39.2%) patients in medical center compared to non-medical center hospital. There was also more clinical stage Ⅱ (Q1-2 *vs* Q3-4: 14.1% *vs* 9.5%; Q1-3 *vs* Q4: 12.9% *vs* 9.3%) and less stage Ⅲ (Q1-2 *vs* Q3-4: 40.5% *vs* 45.0%; Q1-3 *vs* Q4: 42.4% *vs* 43.9%) patients in low volume hospitals compared to high volume hospitals. Besides, the percentage of radiotherapy alone treatment was higher in non-medical center (14.1% *vs* 9.8% in medical center) hospitals and low volume (13.4% in Q-2 *vs* 8.7% in Q3-4; 12.5% in Q1-3 *vs* 7.6% in Q4) hospitals.

In the survival analysis of 3955 patients with non-surgical treatments, the significant prognostic factors included cT stage, cN stage, cM stage, hospital type and hospital volume (Q4 *vs* Q1-3) (Table 4). The prognosis of patients without resection seemed to be

Table 3 Patient characteristics according to hospital type and hospital volume in non-surgical treatment patients (*n* = 3955) *n* (%)

Variables	Medical center			Hospital volume			Hospital volume		
	Yes	No	*P* value	Q1-2	Q3-4	*P* value	Q1-3	Q4	*P* value
Sex			NS			NS			NS
Male	2523 (95.0)	1220 (94.0)		1917 (94.2)	1826 (95.1)		2845 (94.8)	898 (94.2)	
Female	133 (5.0)	79 (6.0)		118 (5.8)	94 (4.9)		157 (5.2)	55 (5.8)	
Age (yr)			< 0.05[a]			NS			NS
< 40	84 (3.2)	29 (2.2)		50 (2.5)	63 (3.3)		83 (2.8)	30 (3.1)	
40-49	536 (20.2)	315 (24.2)		425 (20.1)	426 (22.2)		636 (21.2)	215 (22.6)	
50-59	957 (36.0)	433 (33.3)		723 (35.5)	667 (34.7)		1052 (35.0)	338 (35.5)	
60-69	587 (22.1)	255 (19.6)		417 (20.5)	425 (22.1)		645 (21.5)	197 (20.7)	
70-79	304 (11.4)	187 (14.4)		270 (13.3)	221 (11.5)		371 (12.4)	120 (12.6)	
⩾ 80	188 (7.1)	80 (6.2)		150 (7.4)	118 (6.1)		215 (7.2)	53 (5.6)	
Tumor location			NS			< 0.01[d]			NS
Upper third	759 (28.6)	430 (33.1)		680 (33.4)	509 (26.5)		642 (21.4)	174 (18.3)	
Middle third	841 (31.7)	501 (38.6)		773 (38.0)	569 (29.6)		1079 (35.9)	263 (27.6)	
Lower third	336 (12.7)	169 (13.0)		222 (10.9)	283 (14.7)		694 (23.1)	184 (19.3)	
Unknown	720 (27.1)	199 (15.3)		360 (17.7)	559 (29.1)		587 (19.6)	332 (34.8)	
Tumor differentiation			NS			< 0.05[c]			< 0.01[d]
Well	50 (1.9)	27 (2.1)		43 (2.1)	34 (1.8)		66 (2.2)	11 (1.1)	
Moderate	985 (37.1)	577 (44.4)		910 (44.7)	652 (34.0)		1176 (39.1)	386 (40.5)	
Poorly	547 (20.1)	264 (20.3)		428 (21.0)	383 (19.9)		534 (17.8)	277 (29.1)	
Unknown	1074 (40.4)	431 (33.2)		654 (32.1)	851 (44.3)		1226 (40.8)	279 (29.3)	
Clinical stage			< 0.01[b]			< 0.01[d]			< 0.05[c]
0/ Ⅰ	70 (2.7)	67 (5.2)		77 (3.6)	60 (3.3)		111 (3.9)	26 (2.5)	
Ⅱ	272 (10.2)	200 (15.4)		298 (14.1)	174 (9.5)		375 (12.9)	97 (9.3)	
Ⅲ	1184 (44.6)	509 (39.2)		865 (40.9)	828 (45.0)		1233 (42.4)	460 (43.9)	
Ⅳ	1071 (40.3)	507 (39.0)		841 (39.8)	737 (40.0)		1138 (39.1)	440 (42.0)	
Unknown	59 (2.2)	16 (1.2)		32 (1.5)	43 (2.3)		51 (1.8)	24 (2.3)	
Treatment modality			< 0.05[a]			< 0.01[d]			< 0.01[d]
Chemotherapy	234 (8.8)	99 (7.6)		175 (8.3)	158 (8.6)		228 (7.8)	105 (10.0)	
Radiotherapy	259 (9.8)	183 (14.1)		282 (13.4)	160 (8.7)		362 (12.5)	80 (7.6)	
Chemoradiation	2040 (76.8)	980 (75.4)		1572 (74.4)	1448 (78.6)		2206 (75.9)	814 (77.8)	
Unknown	123 (4.6)	37 (2.9)		84 (4.0)	76 (4.1)		112 (3.9)	48 (4.6)	
Stage-specific 3-yr survival									
Stage Ⅰ	33.44%	48.35%		41.81%	43.03%		42.23%	45.86%	
Stage Ⅱ	21.1%	21.12%		22.53%	18.74%		22.60%	15.76%	
Stage Ⅲ	11.84%	14.11%		12.61%	12.32%		12.61%	12.03%	
Stage Ⅳ	6.6%	5.05%		6.2%	6.03%		6.04%	6.36%	

[a]*P* < 0.05, patients in medical center *vs* non-medical center hospital; [b]*P* < 0.01, patients in medical center *vs* non-medical center hospital; [c]*P* < 0.05, patients in low volume hospitals *vs* high volume hospitals; [d]*P* < 0.01, patients in low volume hospitals *vs* high volume hospitals. NS: Not significant.

better in the non-center hospitals (HR = 0.92, 95% CI: 0.86-0.99; *P* = 0.028). The Kaplan-Meier plot demonstrated that the 1- and 3-year overall survival rates in the non-medical centers (36.2% and 13.2%, respectively) were significantly higher than those in the medical centers (33.5% and 11.3%, respectively; *P* = 0.027) (Figure 1A). However, after adjustment for clinicopathological factors, the hospital type was not a significant prognostic factor of survival (*P* = 0.447) (Table 4, model 1). As for the volume-outcome analysis, there was no survival difference when comparing quartiles 3-4 to quartiles 1-2 (*P* = 0.315) (Table 4). The 1-/3-year overall survival rates were 33.8%/11.3%, respectively, in hospitals in quartiles 3-4 and 35.0%/12.6%, respectively, in hospitals in quartiles 1-2 (*P* = 0.315) (Figure 1B). However, when comparing hospitals in quartile 4 to quartiles 1-3, a significant survival benefit was noted in quartiles 1-3 (Table 4) (HR = 0.87; 95%CI: 0.80-0.94; *P* = 0.001). The Kaplan-Meier plot demonstrated that the 1- and 3-year overall survival rates in quartiles 1-3 (35.3% and 12.6%, respectively) were significantly higher than those in quartile 4 (31.1% and 9.4%, respectively; *P* = 0.001) (Figure 1C). After adjustment for age, sex and clinical tumor-node-metastasis (TNM) stage, hospital volume (Q1-3 *vs* Q4) remained statistically significant independent prognostic factors (HR = 0.91; 95%CI: 0.83-0.99; *P* = 0.028) (Table 3, model 4).

DISCUSSION

Since Luft *et al*[9] published the first study on volume-outcome relationship in surgery in 1987, subsequent studies have investigated the volume-outcome relationship in esophageal cancer surgery[10-13]. The results of meta-analyses demonstrated an inverse correlation between hospital volume and short-term postoperative outcomes, including mortality and

Table 4 Cox regression analysis of the relationship between prognostic factors and outcome in patients without esophagectomy

Variables	Univariate analysis			Multivariate analysis								
				Model 1			Model 2			Model 3		
	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value
Age (yr)												
< 55	1	-		1	-							
⩾ 55	0.94	0.88-1.01	0.091	0.96	0.90-1.03	0.288	0.96	0.90-1.03	0.297	0.96	0.90-1.04	0.322
Sex												
Male	1	-		1	-							
Female	0.86	0.73-1.00	0.053	0.9	0.76-1.06	0.19	0.9	0.76-1.05	0.185	0.89	0.76-1.05	0.176
cT stage												
T1/2	1	-		1	-							
T3/4	1.77	1.61-1.96	< 0.001	1.55	1.40-1.73	< 0.001	1.56	1.40-1.73	< 0.001	1.55	1.39-1.73	< 0.001
cN stage												
N negative	1	-		1	-							
N positive	1.64	1.48-1.81	< 0.001	1.22	1.09-1.37	0.001	1.22	1.09-1.37	0.001	1.21	1.08-1.36	0.001
cM stage												
0	1	-		1	-							
1	1.67	1.56-1.80	< 0.001	1.52	1.41-1.64	< 0.001	1.52	1.41-1.64	< 0.001	1.52	1.41-1.64	< 0.001
Hospital type												
Medical center	1	-		1	-							
Not center	0.92	0.86-0.99	0.028	0.97	0.90-1.05	0.447						
Hospital volume												
Q3-4	1	-		1	-							
Q1-2	0.97	0.90-1.03	0.315				0.99	0.92-1.06	0.694			
Hospital volume												
Q4	1	-										
Q1-3	0.87	0.80-0.94	0.001							0.91	0.83-0.99	0.028

Model 1: Impact of hospital type adjusted for age, sex and clinical tumor-node-metastasis (TNM) stages; Model 2: Impact of hospital volume (Q3-4 *vs* Q1-2) adjusted for age, sex and clinical TNM stages; Model 3: Impact of hospital volume (Q4 *vs* Q1-3) adjusted for age, sex and clinical TNM stages.

complication rates in esophageal cancer surgery[10-13]. Although the impact of hospital volume on long-term survival after esophagectomy is controversial, the recent meta-analysis by Brusselaer *et al*[8] showed that high-volume surgery results in better long-term survival than low-volume surgery. To promote high-value health care, the Leapfrog Group has advocated that esophagectomy be performed only in institutions with an annual caseload of at least 13[14]. The research in the Netherlands also provided evidence that the centralization of esophageal cancer patients to specialized care would lead to better outcome. In a retrospective study, van de Poll-Franse *et al*[2] showed that 63.2% of patients had surgery in high-volume hospitals after the centralization of esophageal cancer patients, whereas only 17.2% of patients still underwent resections in low-volume hospitals. The 3-year survival rates increased from 32.0% to 45.1% for patients who had surgery (P = 0.004), and from 13.1% to 17.9% for all of the patients included in the study (P = 0.026). They concluded that the centralization of patients with esophageal and gastric cardia cancer surgery was associated with improvements in the overall survival rate for surgically as well as non-surgically treated patients. The majority of the volume-outcome relationship studies supported the idea of "practice makes perfect," which means more experience gained in hospitals that treat a greater number of patients could lead to improvements in the management of patients as well as improvements in the advantages in patient survival; moreover, the volume-outcome relationship studies supported the idea of "selective referral pattern," which means hospitals with better outcomes receive more referrals, leading to higher volumes. These two theories did not explain our results that patients without resection had worse prognosis when treated in very high-volume hospitals (> 75%, Q4). One likely reason may be due to a higher percentage of patients with advanced stage tumors in high-volume hospitals, which suggests that patients with poor performance or higher-risk for treatments were more likely to be referred to high-volume hospitals. Furthermore, our observation that worse prognosis in high-volume hospitals was compatible with the findings in the report by Rouvelas *et al*[15], which showed that patients operated by low-volume surgeons had the highest 30-d mortality risk compared with those operated by medium- and high-volume surgeons; however, this risk did not decrease with an additional increase in workload, which indicates that the volume factor is not the only determinant for patient outcome.

As for the hospital type-outcome relationship, the results in the literature were conflicting. Theoretically, the patients who are managed at a higher-level hospital are more likely to receive a wider range

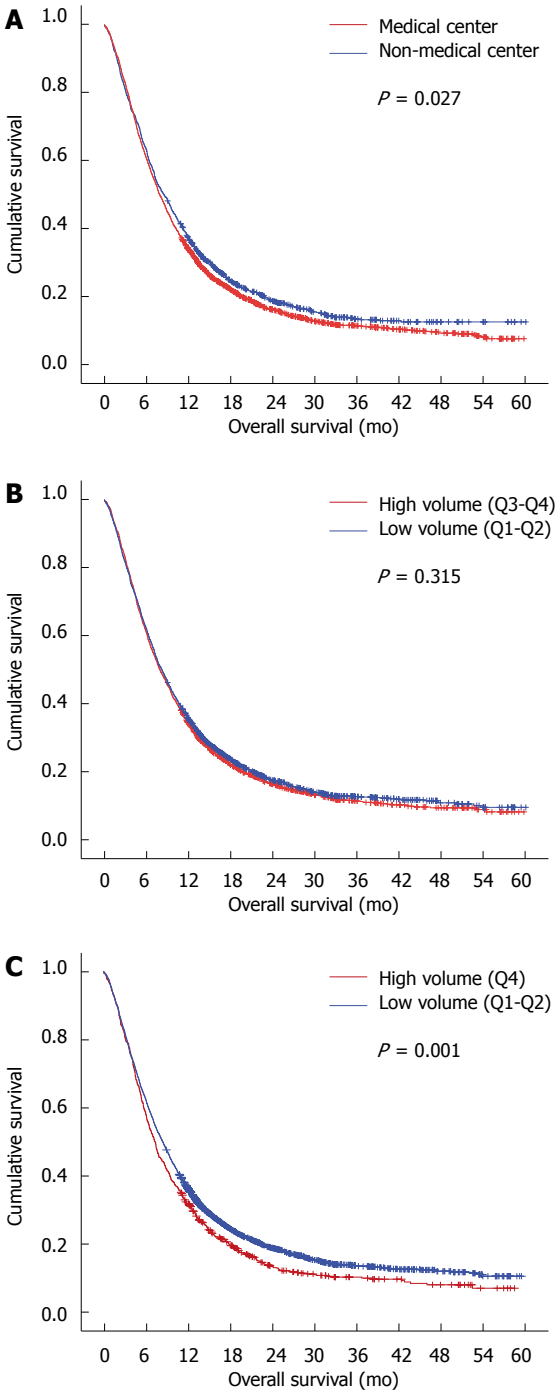

Figure 1 Kaplan-Meier plot. A: Showing that the overall survival was better in the non-medical centers (blue line) compared with the medical centers (red line) (P = 0.027, non-medical centers *vs* medical centers); B: Showing that no overall survival difference was noted between the hospitals in Q3-4 (red line) and Q1-2 (blue line) (P = 0.315, Q3-4 *vs* Q1-2); C: Showing that the overall survival was better in the low-volume hospitals (Q1-3, blue line) compared with the very high-volume hospitals [Q4, red line (P = 0.001), low volume hospitals *vs* high volume hospitals].

of diagnostic investigations, such as PET/CT scans and endoscopic ultrasound scans, which would result in accurate staging of a greater proportion of tumors and the appropriate use of combined oncological treatment modalities. Therefore, the hospital type may be a likely surrogate for quality of care. For example, Dikken *et al*[5] and Verhoef *et al*[6] have demonstrated that patients undergoing esophagectomy in university hospitals exhibited better outcome in terms of 3-mo mortality rates and 5-year survival rates. However, several studies have reported opposing results. Viklund *et al*[16] demonstrated no decreased risk of overall complications at university hospitals compared with nonuniversity hospitals. Similarly, Rodgers *et al*[17] showed that urban hospitals did not demonstrate better inpatient mortality than did rural hospitals. Although the teaching status appeared to confer benefit in the univariate analysis, this significance was lost once hospital volume was included. Consistent with Rodgers' results, Bachmann *et al*[4] found that teaching hospital status was not independently associated with postoperative mortality rate in the case-mix adjusted survival analysis. Whereas the hospital-type relationship remains uncertain in patients undergoing surgical resection, our present study demonstrated that the hospital type, medical center *vs* non-medical center, did not influence outcome in patients with non-surgical treatments. Instead, the clinical TNM stages were significant prognostic factors. Our findings suggested that among those non-surgically treated patients, due to either unresectable tumors or their unsuitable status for surgery, the nature of the tumor apparently had a greater influence on the likelihood of long-term survival.

In our cohort, there was a higher proportion of patients in medical centers or high-volume hospitals who received surgical resections despite a higher frequency of advanced stage tumors. The population-based study by Bachmann *et al*[4] showed that patients treated in high-volume hospitals or by high-volume doctors were more likely to undergo surgical resection. In Coupland's report, increasing resection rates were associated with lower mortality for all of the patients, including with and without surgical resection, with an HR of 0.86 (95%CI: 0.84-0.89) in the highest resection quintile compared with the lowest resection quintile[18]. However, our study has contradictory results: the higher resection rates in medical centers and high-volume hospitals did not translate into better outcomes in those patients without resection in our study.

Our study has both strengths and weaknesses. Its strengths include the population-based design and the large number of patients. We focused on cancers with squamous cell carcinoma histology, which is in contrast to the adenocarcinoma-predominant databases from Western countries. We emphasized the hospital type- and volume-outcome relationships in patients with non-surgical treatments, which has never been exclusively discussed. However, the results of our study may be limited by the nature of the population-based study.

No detailed information was collected on the use of diagnostic tools and the protocol of chemotherapy, radiotherapy or any other non-surgical treatments when surgery was not an option.

In conclusion, the present population-based study, including 3955 non-surgically treated patients with esophageal cancer, demonstrated that the hospital type is not a significant prognostic factor. Moreover, the high-volume hospitals are not associated with better survival compared with the low-volume hospitals. For patients with esophageal cancer who receive non-surgical treatments, the nature of the tumor, *i.e.*, the clinical TNM stage, constitutes a significant prognostic factor.

COMMENTS

Background

The prognosis of esophageal cancer is extremely poor. To improve outcome, centralization of care for patients with esophageal cancer has been proposed. Many reports have focused on the hospital type-outcome and volume-outcome relationships in esophageal cancer after esophagectomy. Little is known about the hospital type- and volume-outcome relationships in patients without surgical resection. Therefore, the authors studied the differences in patient and tumor characteristics according to the hospital type and the volume categories in this population-based study. The authors aimed to investigate whether the hospital type or volume would affect the prognosis in patients with esophageal cancer who receive non-surgical treatments.

Research frontiers

Hospital type-outcome analyses have shown better outcome in university hospitals. Previous studies demonstrated a higher 5-year survival rate for surgical patients in university hospitals compared with patients in teaching non-university and non-teaching hospitals. The hospital volume-outcome analyses also support the impact of volume on patient survival. The meta-analysis results have demonstrated a long-term survival benefit after esophageal cancer resection for high-volume hospitals compared with the low-volume counterparts.

Innovations and breakthroughs

Instead of investigating the outcome after esophagectomy, we focused on patients with esophageal cancer who receive non-surgical treatments. The data showed that hospital volume (annual volume, > 94 *vs* ≤ 94) remained statistically significant (HR = 0.91, 95%CI: 0.83-0.99; *P* = 0.028) in the multivariate analysis. The authors concluded that receiving non-surgical treatments for esophageal cancer in medical centers or high-volume hospitals is not associated with better survival. The nature of the tumor, *i.e.*, the clinical TNM stages, was a significant prognostic factor.

Applications

For patients with non-surgically treated esophageal cancer, medical centers or high-volume hospitals were not associated with better survival. The disease aggressiveness, *i.e.*, TNM stages, had a greater impact on patient survival.

Terminology

Hospital types were defined as medical centers and non-medical centers according to the Taiwan Joint Commission on Hospital Accreditation (http://www.tjcha.org.tw) based on the quality of process and outcome in healthcare performance. The threshold for high-volume hospitals was based on the median (Q3-4, > 50%; annual volume, > 56 cases) or upper quartile (Q4, > 75%; annual volume, > 94 cases).

Peer review

The present article delivers an important cautionary recommendation that the data regarding surgical patients with esophageal cancer should not be casually extrapolated to non-surgical patients with esophageal cancer.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic implications of estrogen receptor 1 and vascular endothelial growth factor A expression in primary gallbladder carcinoma

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Abstract

AIM: To investigate the prognostic significance of estrogen receptor 1 (ER1) and vascular endothelial growth factor A (VEGF-A) expression in primary gallbladder carcinoma (GBC) to identify new prognostic markers for this malignancy.

METHODS: Using immunohistochemistry, we investigated ER1 and VEGF-A expression in 78 GBC and 78 cholelithiasis (CS) tissues. The results were correlated with clinicopathological features. Univariate and multivariate analyses were performed to evaluate the relationship between ER1 and VEGF-A expression and patients' prognosis. Further Kaplan-Meier survival analysis was also performed.

RESULTS: ER1 and VEGF-A expression was significantly higher in GBC compared with CS (47/78 *vs* 28/78, $P < 0.05$; 51/78 *vs* 33/78, $P < 0.05$). ER1 expression was correlated with gender ($P < 0.05$) and VEGF-A expression was correlated with tumor differentiation in GBC patients ($P < 0.05$). In univariate analysis, age and tumor node metastasis (TNM) stage were factors associated with GBC prognosis ($P < 0.05$). Although there was no statistical difference between the expression of ER1 or VEGF-A and overall survival, the high expression of ER1 combined with VEGF-A predicted a poor prognosis for GBC patients (16.30 ± 1.87 *vs* 24.97 ± 2.09, log-rank $P < 0.05$). In multivariate analysis, combined expression of ER1 and VEGF-A and TNM stage were independent prognostic factors for GBC patients ($P < 0.05$).

CONCLUSION: Combined expression of ER1 and VEGF-A is a potential prognostic marker for GBC patients. Clinical detection of ER1 and VEGF-A in surgically resected GBC tissues would provide an

important reference for decision-making of post-operative treatment programs.

Key words: Gallbladder carcinoma; Estrogen receptor 1; Vascular endothelial growth factor A

Core tip: Gallbladder carcinoma (GBC) is a serious threat to public health for its poor prognosis. The authors found that estrogen receptor 1 (ER1) and vascular endothelial growth factor A (VEGF-A) expression was significantly higher in GBC than in cholelithiasis tissues, and high expression of ER1 combined with VEGF-A conferred a poor prognosis in GBC patients after surgery. Combined expression of ER1 and VEGF-A was an independent factor associated with GBC prognosis. Clinical detection of ER1 and VEGF-A may guide postoperative clinical treatment of GBC patients.

Zhang LQ, Xu XS, Wan Y, Song SD, Wang RT, Chen W, Wang ZX, Chang HL, Wei JC, Dong YF, Liu C. Prognostic implications of estrogen receptor 1 and vascular endothelial growth factor A expression in primary gallbladder carcinoma. *World J Gastroenterol* 2015; 21(4): 1243-1250 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1243.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1243

INTRODUCTION

Primary gallbladder carcinoma (GBC), originating from the bile duct epithelium, is characterized by poor prognosis[1,2]. Most of GBC patients were asymptomatic until the disease has progressed to an advanced and non-curative stage. According to epidemiological investigations, the 5-year survival rate for GBC patients was less than 10%, with the overall mean survival time of 6 mo. In clinical practice, the tumor node metastasis (TNM) staging system sometimes could not predict GBC patients' prognosis accurately. In spite of this, except for the TNM staging system, there were no other molecular markers available to facilitate the evaluation of GBC prognosis. Therefore, it is imperative to explore new predictive factors to guide the postoperative treatments for GBC patients.

Due to the female predominance in GBC incidence, it is speculated that estrogen may play important roles in the genesis and progression of GBC[3-5]. Estrogen executes its biological functions by binding to estrogen receptor (ER), and a number of studies have reported that ER was associated with carcinogenesis[6-10]. ER includes two subtypes, ER1 (or ER-α) and ER2 (or ER-β). In spite of similar molecular structure, ER1 and ER2 exhibited an antagonistic effect in some biological processes. As far as our knowledge, ER1 is able to promote tumor development and indicates poor prognosis, while ER2 usually suppresses tumor progression and prefigures good survival[11-13]. Therefore, some researchers assumed that ER1 possibly keep a subtle balance with ER2 in normal conditions[14]. Sumi *et al*[13] have reported the relationship between ER2 and GBC prognosis. However, although ER1 has been detected in GBC samples, its clinical significance is still equivocal.

Angiogenesis is essential for cancer growth, invasion and metastasis. It is well known that vascular endothelial growth factor (VEGF) is a potent vascular active molecule which directly stimulates the proliferation of vascular endothelial cells[15]. Accumulating evidence suggested that VEGF plays important roles in many kinds of tumors by inducing neoangiogenesis. In human cholangiocarcinoma, VEGF-A was positively expressed and was considered to mediate the proliferative effects of estrogen[16]. Similar to other tumors, adequate blood supply and sufficient angiogenesis are fundamental requirements for the growth of GBC. In GBC, the VEGF-A single nucleotide polymorphisms were implicated in GBC risk[17]. There was also investigations indicating that VEGF-A was highly expressed in GBC and was correlated with a poor prognosis[18]. Nevertheless, Giatromanolaki *et al*[19] reported that VEGF was not associated with GBC patient survival, but combined VEGF and thymidine phosphorylase expression was considered an unfavorable prognostic factor. Therefore, it is still controversial with regards to the prognostic significance of VEGF in GBC.

ER1 and VEGF-A play important roles in GBC. Estrogen can modulate VEGF expression[20-23]. However, there have been no relevant reports about the prognostic significance of ER1 and VEGF-A in GBC. Hence, we investigated the expression status of ER1 and VEGF-A in resected human GBC tissues, and to evaluate their prognostic value in GBC.

MATERIALS AND METHODS

Tissue specimens

In the present study, tissue specimens were collected from 156 patients who had undergone surgical resection at the First Affiliated Hospital of Medical College, Xi'an Jiaotong University (Xi'an, China) between October 2009 and October 2010, including 78 patients with GBC confirmed by postoperative pathological diagnosis, and 78 patients with cholelithiasis (CS) who underwent cholecystectomy. None of them received any preoperative radiochemotherapy. The two groups were matched in age and gender. The clinicopathological information was obtained from the hospital's medical records. The following data of each patient was included: age, gender, gallstone status, tumor differentiation, and TNM stage. All GBC patients were closely followed after surgery for

4 to 53 mo, and we defined that GBC patient's death was the only positive outcome in our study.

Immunohistochemical staining

The streptavidin-peroxidase (SP) method was performed using rabbit polyclonal antibody to ER1 and VEGF-A obtained from Santa Cruz Biotechnology to detect the expression of ER1 and VEGF-A in GBC and CS tissues. Formalin-fixed and paraffin-embedded specimens were cut into 4-μm sections, mounted onto slides treated with poly-L-lysine, deparaffinized, and rehydrated. The slides were heated at 96-98 ℃ in a microwave for 15 min in a citrate buffer solution at pH 6.0 and cooled for 30 min at room temperature to retrieve antigen. To quench the endogenous peroxidase activity, sections were treated with 0.3% H_2O_2 for 30 min. Subsequently, the sections were treated with 5% normal goat serum in phosphate-buffered saline for 1 h to block nonspecific sites. All sections in a humidified box were incubated overnight at 4 ℃ with specific antibodies detecting ER1 and VEGF-A, and then incubated with biotinylated anti-rabbit IgG and avidin-biotin-peroxidase complex, respectively. Antibody binding was visualized by exposure to diaminobenzidine. Hematoxylin was used to weakly counterstain sections. The sections were dehydrated in graded alcohol and cleared in xylene. Finally, all sections were mounted with neutral gum.

Immunohistochemical assessment of ER1 and VEGF-A

According to the previous literature[18,24,25], a semi-quantitative manner was used to evaluate the staining of ER1 and VEGF-A. All of the sections were assessed independently by two investigators in a blind manner under a transmission light microscope. The intensity of staining (IS) and the percentage of positively stained (PS) cells were evaluated. The IS was scored as 0 (absent), l (weak), 2 (moderate), and 3 (strong). The percentage of PS cells was scored as 0 (none), 1 (1%-25%), 2 (26%-50%), 3 (51%-75%), and 4 (76%-100%). Five fields per case and 100 tumor cells per × 40 field were examined. The mean value obtained was the final score for each case. A final score (FS) was calculated using the formula: FS = IS + PS. Finally, all the sections were defined as "low" expression if FS was 0-4 or "high" expression if FS was 5-7 for assessment of ER1 and VFGF-A staining. The typical histology corresponding to each histological score used in this study is shown in Figure 1.

Statistical analysis

Fisher's exact test or χ^2 test as appropriate was performed to assess the associations between the ER1 and VEGF-A expression and clinicopathological variables. Kaplan-Meier method was used to plot survival curves, and the log-rank test was used to determine statistical differences. Multivariate analysis was performed using Cox proportional hazard model. A *P*-value less than 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 13.0 program.

RESULTS

ER1 and VEGF-A expression is significantly higher in GBC tissues compared with CS tissues

The expression status of ER1 and VEGF-A is shown in Figure 2. VEGF-A was expressed in the cytoplasmic compartment, and ER1 was expressed in the nucleus. The expression of both ER1 and VEGF-A was significantly higher in GBC compared with CS (Table 1). Higher ER1 expression was observed in more GBC (47/78, 60.3%) than in CS tissues (28/78, 35.9%) (P = 0.002). Similarly, higher expression of VEGF-A was observed in more GBC (51/78, 65.4%) than in CS tissues (33/78, 42.3%) (P = 0.004). In GBC patients, there was no statistical significance between the histological scores of ER1 and VEGF-A (r = 0.176, P = 0.124).

Relationship between the expression of ER1 and VEGF-A and clinicopathological features of GBC

ER1 expression was associated with gender. ER1 expression was more frequent in females than males (P = 0.022). In addition, VEGF-A expression was correlated with tumor differentiation (P = 0.01). No significant difference was found between the expression of ER1 and VEGF-A and other clinicopathological factors (Table 2).

Expression of ER1 and VEGF-A and GBC prognosis

Univariate analysis (Table 3) revealed that age and TNM stage were significantly associated with GBC prognosis (P < 0.05). Patients with stage 2 GBC had a better survival than those with stages 3 and 4 disease (Figure 3A). Although there was no statistical difference between ER1 or VEGF-A expression status and GBC prognosis (Figure 3B and 3C, P > 0.05), combined expression of ER1 and VEGF-A was correlated with postoperative survival of GBC patients (Figures 3D and 4, P < 0.05). GBC patients with simultaneous high expression of ER1 and VEGF-A had a poorer prognosis. By multivariate analysis, TNM stage and combined ER1 and VEGF-A expression were identified as independent prognostic factors (P < 0.05) (Table 4). There was no statistical significance between ER1 and VEGF-A expression and GBC recurrence (P > 0.05).

DISCUSSION

The present study examined the expression of ER1 and VEGF-A in resected human GBC and CS tissues. The main findings are: (1) ER1 and VEGF-A

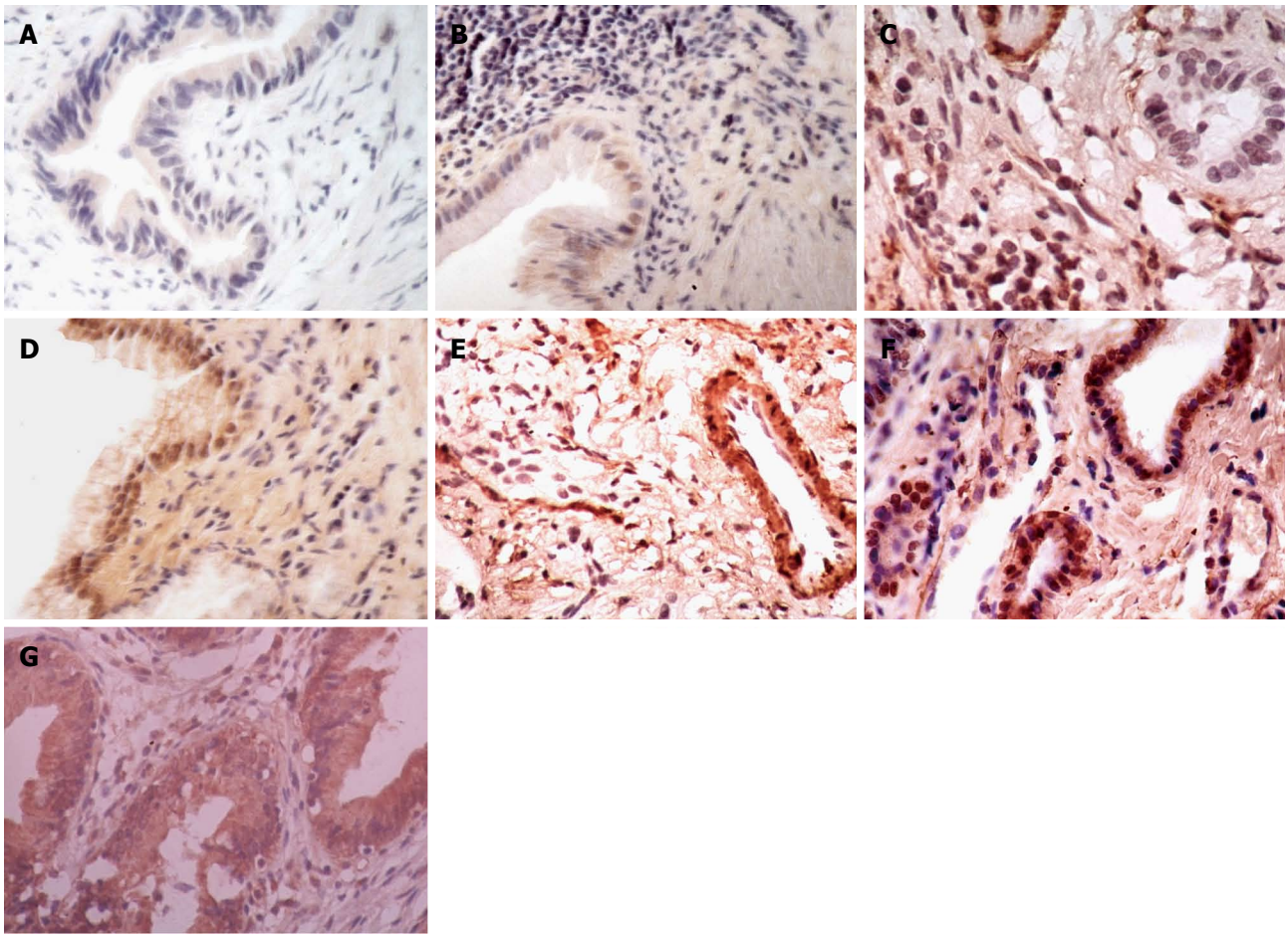

Figure 1 Typical histology corresponding to each histological score. A, B, C, D, E, F, and G were scored as 0, 2, 3, 4, 5, 6, and 7, respectively.

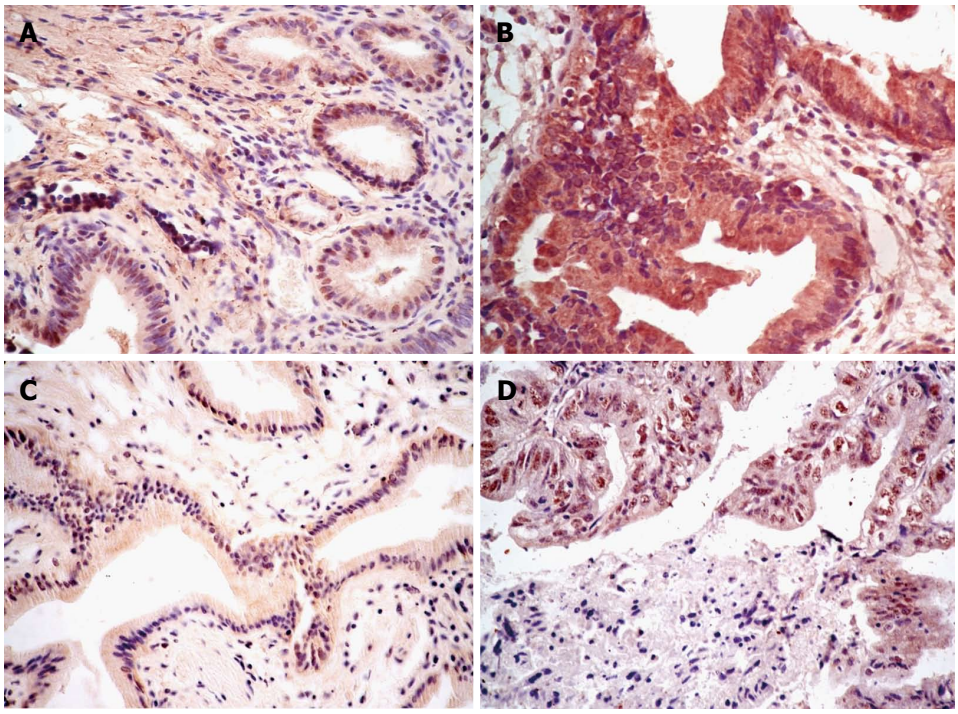

Figure 2 Immunohistochemical staining of estrogen receptor 1 and vascular endothelial growth factor A in gallbladder carcinoma and cholelithiasis specimens. A: Low vascular endothelial growth factor A (VEGF-A) expression in cholelithiasis (CS) tissue; B: High VEGF-A expression in gallbladder carcinoma (GBC) tissue; C: Low estrogen receptor 1 (ER1) expression in CS tissue; D: High ER1 expression in GBC tissue.

Table 1 Comparison of expression of estrogen receptor 1 and vascular endothelial growth factor A between gallbladder carcinoma and cholelithiasis

Group	ER1 expression		*P*-value	VEGF-A expression		*P*-value
	High	Low		High	Low	
GBC	47	31	0.002	51	27	0.004
CS	28	50		33	45	

GBC: Gallbladder carcinoma; CS: Cholelithiasis; ER1: Estrogen receptor 1; VEGF-A: Vascular endothelial growth factor A.

Table 2 Association between estrogen receptor 1 and vascular endothelial growth factor A expression and clinicopathological characteristics of gallbladder carcinoma

Characteristic	ER1 expression		*P*-value	VEGF-A expression		*P*-value
	High	Low		High	Low	
Gender			0.022			0.099
Male	15	18		25	8	
Female	32	13		26	19	
Age (yr)			0.151			0.095
≤ 55	18	17		23	12	
> 55	29	14		28	15	
Gallstones			0.370			0.056
Present	32	24		33	23	
Absent	15	7		18	4	
TNM stage			0.177			0.781
Ⅱ	17	16		21	12	
Ⅲ/Ⅳ	30	15		30	15	
Differentiation			0.205			0.010
Well	16	15		15	16	
Moderate/poor	31	16		36	11	

ER1: Estrogen receptor 1; VEGF-A: Vascular endothelial growth factor A; TNM: Tumor node metastasis.

Table 3 Univariate analysis of prognostic factors associated with overall survival in patients with gallbladder carcinoma

Risk factor	Survival time(month) (mean ± SE)	*P*-value (Log-rank test)
Gender		0.682
Male	23.24 ± 4.09	
Female	21.97 ± 1.72	
Age (yr)		0.015
≤ 55	26.34 ± 2.71	
> 55	18.77 ± 1.71	
Gallstones		0.068
Present	17.69 ± 2.06	
Absent	23.96 ± 2.02	
TNM stage		0.007
Ⅱ	27.16 ± 2.77	
Ⅲ/Ⅳ	18.59 ± 1.74	
Differentiation		0.685
Well	23.19 ± 2.94	
Moderate/poor	21.65 ± 1.86	
ER1 level		0.053
High	19.81 ± 1.79	
Low	25.85 ± 2.86	
VEGF-A level		0.155
High	20.35 ± 1.87	
Low	25.65 ± 2.86	
ER1 combined with VEGF-A		0.007
+/+	16.30 ± 1.87	
+/-, -/+, -/-	24.97 ± 2.09	

ER1: Estrogen receptor 1; VEGF-A: Vascular endothelial growth factor A; TNM: Tumor node metastasis; +/+: High ER1 expression and high VEGF-A expression; +/-: High ER1 expression and low VEGF-A expression; -/+: Low ER1 expression and high VEGF-A expression; -/-: Low ER1 expression and low VEGF-A expression.

expression were significantly higher in GBC than in CS tissues, ER1 expression was significantly associated with gender, and VEGF-A expression was associated with tumor differentiation; and (2) high expression of ER1 combined with VEGF-A in GBC predicted a poor prognosis. This is the first study to report prognostic significance of expression of ER1 combined with VEGF-A in GBC.

The poor prognosis of GBC has caused wide public attentions. Despite rapid improvement in medical technology over past decades, the survival time of GBC patients are far from satisfactory. Based on many clinical and molecular investigations about GBC, we speculated that the dismal prognosis of GBC patients may be attributed to the following aspects: (1) early diagnosis is difficult and most GBC cases are diagnosed at an advanced stage and have lost the best surgical chance; (2) GBC is relatively resistant to chemotherapy and radiation; apart from surgical resection, other effective measures are lacking; and (3) postoperative therapy for GBC patients should be selected according to patients' prognosis. Despite a number of studies have been conducted about the molecular mechanisms of GBC, there have been no effective prognostic biomarkers for GBC to guide postoperative treatment. The present study exhibited that combined ER1 VEGF-A expression was associated with GBC prognosis, which would favor postoperative treatment.

ER1 as a promising prognostic factor has been investigated in several tumors. In ER-negative breast cancer, ER1 expression was necessary and sufficient in the bone marrow-derived cells themselves to promote tumor formation in response to estrogen[12]. In biliary tract cancers (including tumors of the gallbladder, bile duct and ampulla of Vater), the single nucleotide polymorphisms of the gene coding ER1 were correlated with risks of these tumors[26]. In our study, the results showed that ER1 expression was significantly higher in GBC compared with CS. This indicated that ER1 probably plays an important role in GBC, despite that the exact mechanisms are unclear at present. In addition, ER1 expression in GBC tissue exhibited a female predominance. It is well known that the overall level of estrogen in females is obviously higher than in males. It is likely that estrogen induces ER overexpression in females. Thus, our findings may partially explain why GBC is more frequent in females. Nevertheless, there was no significant correlation between ER1 expression

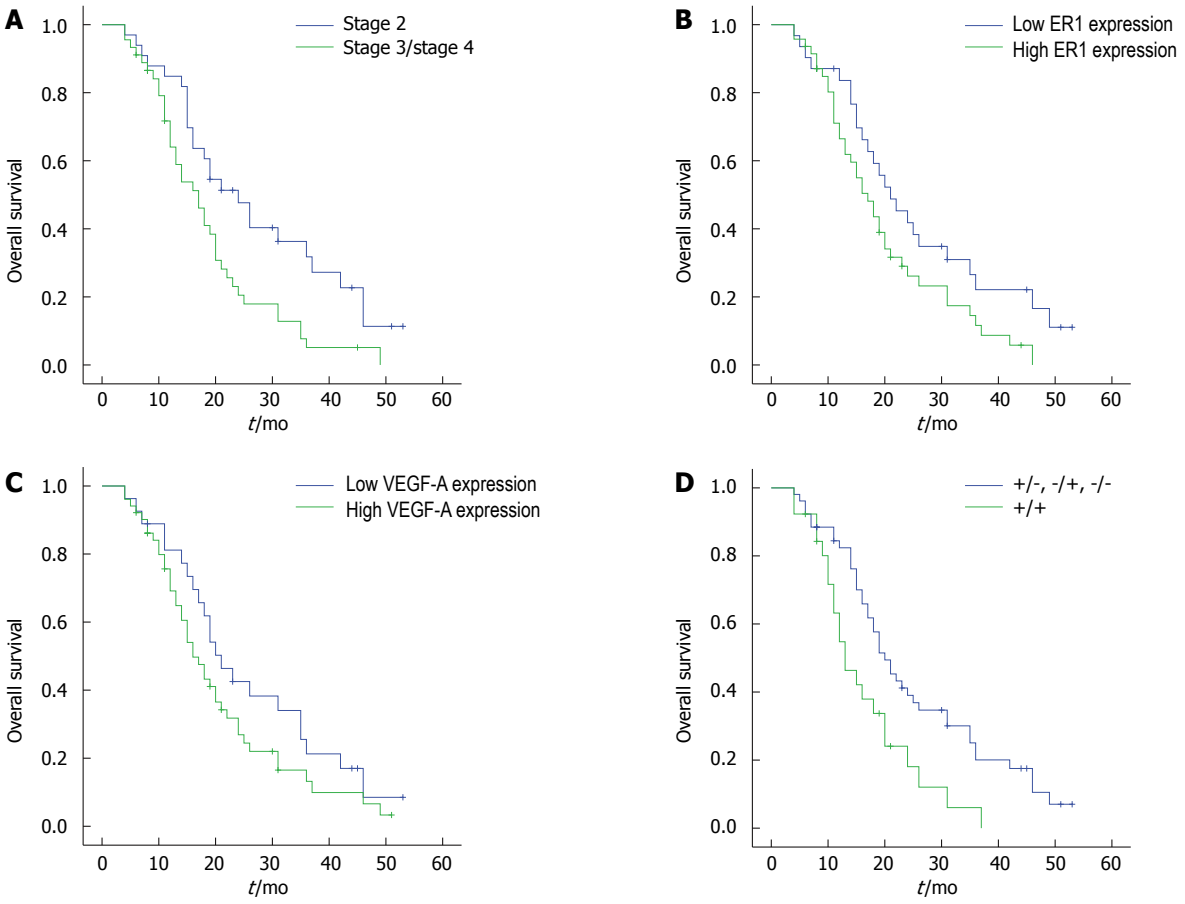

Figure 3 Kaplan-Meier survival curves. A: Stratified for tumor node metastasis stage. Patients with stage 2 disease had a better prognosis than patients with stages 3 and 4 disease (P = 0.07); B: Stratified for estrogen receptor 1 expression status. Low estrogen receptor 1 (ER1) expression was associated with a better survival time than high ER1 expression, but there was no statistical significance between the two groups (P = 0.053); C: Stratified for vascular endothelial growth factor A expression status. Low vascular endothelial growth factor A (VEGF-A) expression was associated with a better survival time than high VEGF-A expression, but there was no statistical significance between two groups (P = 0.155); D: Stratified for estrogen receptor and vascular endothelial growth factor A expression. All patients were clarified into two groups: high expression of estrogen receptor1 (ER1) and vascular endothelial growth factor A (VEGF-A) group (+/+), and low expression of ER1 and VEGF-A group (+/-, -/+, -/-). Patients in high expression of ER1 and VEGF-A group had a worse prognosis than low expression of ER1 and VEGF-A group (P = 0.009). +/+: high ER1 expression and high VEGF-A expression; +/-: high ER1 expression and low VEGF-A expression; -/+: low ER1 expression and high VEGF-A expression; -/-: low ER1 expression and low VEGF-A expression.

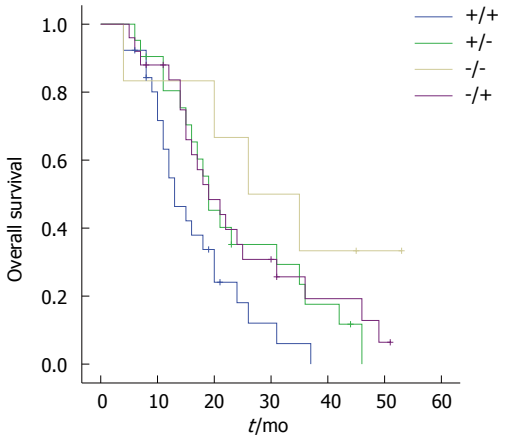

Figure 4 Kaplan-Meier survival curves stratified for estrogen receptor 1 and vascular endothelial growth factor A expression. Patients with high expression of estrogen receptor1 (ER1) combined with vascular endothelial growth factor A (VEGF-A) (+/+) had worst prognosis than other groups (+/-,-/+,-/-) (P = 0.007). +/+: high ER1 expression and high VEGF-A expression; +/-: high ER1 expression and low VEGF-A expression; -/+: low ER1 expression and high VEGF-A expression; -/-: low ER1 expression and low VEGF-A expression.

Table 4 Multivariate analysis of factors associated with survival in patients with gallbladder carcinoma

Item	Hazard ratio	95% CI	*P*-value
Age, yr			0.076
≤ 55 *vs* > 55	0.615	0.359-1.053	
TNM stage			0.031
Ⅲ/Ⅳ *vs* Ⅱ	1.781	1.054-3.011	
ER1 combined with VEGF-A			0.042
+/+ *vs* +/-, -/+, -/-	1.773	1.021-3.080	

ER1: Estrogen receptor 1; VEGF-A: Vascular endothelial growth factor; TNM: Tumor node metastasis; +/+: High ER1 expression and high VEGF-A expression; +/-: High ER1 expression and low VEGF-A expression; -/+: Low ER1 expression and high VEGF-A expression; -/-: Low ER1 expression and low VEGF-A expression.

and postoperative survival.

VEGF-A, a classic biological molecule in angiogenesis, has been investigated in various kinds of cancer. In human intra-hepatic cholangiocarcinoma, VEGF-A mediated the proliferative effect of estrogen to

promote cholangiocarcinoma growth[16]. As to VEGF-A and GBC, there have been many reports[17,18,24,25]. Recently, a study revealed that VEGF-A was highly expressed in GBC and correlated with poor prognosis[18]. Additionally, another study showed that VEGF-A expression in GBC tissues is correlated with histologic differentiation and is an independent prognostic factor[24]. Our results were inconsistent with these previous investigations. Nevertheless, of note in our results, the high expression of ER1 combined with VEGF-A in GBC tissues predicted a poor prognosis. Based on this finding, we speculate that there were potentially synergistic effects between VEGF-A and ER1 in GBC progression. From the perspective of biological significance, this assumption is possible. Estrogen binding to ER can promote production of VEGF as mentioned before. Increasing VEGF can induce angiogenesis to provide plenty of oxygen and nutrients, and thus promote GBC growth, invasion and metastasis, finally leading to a poor survival. Of course, this assumption needs to be confirmed by further investigations.

Some limitations of this study should be taken into account. The sample size of this study was small. In addition, our study was not mechanistic, and there was very little information about molecular mechanisms.

In conclusion, our study suggested that expression of ER1 combined with VEGF-A confers a particularly poor postoperative survival outcome, and represents a potential prognostic biomarker for GBC. Clinical detection of ER1 and VEGF-A in surgically resected GBC tissues may provide a reference for decision-making of postoperative treatment programs. GBC patients having high expression of ER1 and VEGF-A deserve a close surveillance to reduce postoperative mortality.

Prospect

Although ER1 and VEGF-A have been considered to be involved in progression of many kinds of tumors, their roles in GBC development have not been reported. Further investigations are required to explore the potential roles of ER1 and VEGF-A in GBC progression to clarify the molecular mechanism of GBC. In addition, ER1 and VEGF-A may represent potential therapeutic targets and adjuvant endocrine therapy may be new approaches for GBC.

COMMENTS

Background

Primary gallbladder carcinoma (GBC) is characterized by poor prognosis. In clinical practice, there has been no effective biomarker to predict the prognosis of GBC patients. Estrogen receptor 1 (ER1) and vascular endothelial growth factor A (VEGF-A) are involved in the progression of several kinds of malignancies. However, the prognostic significance of ER1 and VEGF-A in GBC is controversial, and needs further investigation.

Research frontiers

According to epidemiology, the 5-year postoperative survival of GBC patients is less than 10%. Therefore, it is a current hotspot that exploring effective prognostic markers to guide postoperative treatment for GBC patients so as to improve survival after surgery.

Applications

Clinical detection of ER1 and VEGF-A expression can predict prognosis of GBC patients, and provides a reference for making-decision of postoperative treatment programs. In addition, the identification of ER1 and VEGF-A expression in human GBC tissues would help to investigate the molecular mechanisms of GBC.

Terminology

ER1 also named estrogen receptor alpha, is a ligand-regulated transcription factor and mediates biological actions of estrogen. ER1 is implicated in several kinds of tumors. VEGF-A (vascular endothelial growth factor A) can promote physiological and pathological angiogenesis, and is believed to play an important role in various tumors.

Peer review

The authors reported that expression of ER1 combined with VEGF-A assessed by immunohistochemistry was a potential prognostic marker for GBC patients after surgery. Their findings were useful for the postoperative clinical treatment of GBC patients.

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ORIGINAL ARTICLE

Observational Study

Colectomy is a risk factor for venous thromboembolism in ulcerative colitis

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Author contributions: Kaplan GG conceived of the study; Kaplan GG, Nguyen GC, Leung Y, Seow CH, Debruyn J, Panaccione R, and Ghosh S participated in the design of the study; Lim A and Moran GW collected the data and developed the database; Kaplan GG and Hubbard J performed the statistical analysis; all authors interpreted the findings, helped to draft the manuscript and approved the final manuscript. Kaplan GG had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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Abstract

AIM: To compare venous thromboembolism (VTE) in hospitalized ulcerative colitis (UC) patients who respond to medical management to patients requiring colectomy.

METHODS: Population-based surveillance from 1997 to 2009 was used to identify all adults admitted to hospital for a flare of UC and those patients who underwent colectomy. All medical charts were reviewed to confirm the diagnosis and extract clinically relevant information. UC patients were stratified by: (1) responsive to inpatient medical therapy (n = 382); (2) medically refractory requiring emergent colectomy (n = 309); and (3) elective colectomy (n = 329). The primary outcome was the development of VTE during hospitalization or within 6 mo of discharge. Heparin prophylaxis to prevent VTE was assessed. Logistic regression analysis determined the effect of disease course (*i.e.*, responsive to medical therapy, medically refractory, and elective colectomy) on VTE after adjusting for confounders including age, sex, smoking, disease activity, comorbidities, extent of disease, and IBD medications (*i.e.*, corticosteroids, mesalamine, azathioprine, and infliximab). Point estimates were presented as odds ratios (OR) with 95%CI.

RESULTS: The prevalence of VTE among patients with UC who responded to medical therapy was 1.3% and only 16% of these patients received heparin

prophylaxis. In contrast, VTE was higher among patients who underwent an emergent (8.7%) and elective (4.9%) colectomy, despite greater than 90% of patients receiving postoperative heparin prophylaxis. The most common site of VTE was intra-abdominal (45.8%) followed by lower extremity (19.6%). VTE was diagnosed after discharge from hospital in 16.7% of cases. Elective (adjusted OR = 3.69; 95%CI: 1.30-10.44) and emergent colectomy (adjusted OR = 5.28; 95%CI: 1.93-14.45) were significant risk factors for VTE as compared to medically responsive UC patients. Furthermore, the odds of a VTE significantly increased across time (adjusted OR = 1.10; 95%CI: 1.01-1.20). Age, sex, comorbidities, disease extent, disease activity, smoking, corticosteroids, mesalamine, azathioprine, and infliximab were not independently associated with the development of VTE.

CONCLUSION: VTE was associated with colectomy, particularly, among UC patients who failed medical management. VTE prophylaxis may not be sufficient to prevent VTE in patients undergoing colectomy.

Key words: Inflammatory bowel diseases; Ulcerative colitis; Deep vein thrombosis; Pulmonary embolism; Surgery

Core tip: The occurrence of venous thromboembolism (VTE) in our population-based cohort was about 5%, which highlights the importance of this complication among hospitalized ulcerative colitis (UC) patients. However, the risk of VTE was low (about 1%) among flaring ulcerative colitis patients who responded to medical management. In contrast, UC patients who underwent an elective (5%) or emergent colectomy (8.7%) had higher occurrence of VTE. After adjusting for covariates the leading risk factor for VTE was the need for colectomy. VTE occurred in colectomy patients despite > 90% postoperative VTE prophylaxis. Thus, heparin prophylaxis may not be sufficient to prevent VTE in patients undergoing colectomy.

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INTRODUCTION

In the western countries the prevalence of ulcerative colitis (UC) has been reported as high as 500 cases per 100000 persons[1]. Approximately, 16% of UC patients will require colectomy within the first 10 years of diagnosis; though, colectomy rates have been decreasing overtime[2,3]. Colectomy for UC has been associated with postoperative morbidity and mortality[4-6]. An important contributor to postoperative complications is the occurrence of venous thromboembolism (VTE)[7,8], VTE carries risk of significant morbidity and mortality, particularly due to pulmonary embolism[9-11].

The inflammatory bowel diseases (IBD) have been established as an independent risk factor for developing VTE as well as recurrent VTE[12,13]. In recent years, several population-based studies showed that IBD patients are at increased risk of developing VTE[7,14]. Bernstein *et al*[7] demonstrated that IBD patients have a 3-4 fold increased risk of developing a VTE requiring hospitalization. Similar rates have been described in both ambulatory and hospitalized IBD patients[12,15], while specific studies focusing on hospitalized IBD patients showed an increased risk of 1.5-2 fold compared to hospitalized non-IBD patients[16]. In a meta-analysis the risk of VTE among IBD patients was 2-fold higher than individuals without IBD[17].

Studies have also highlighted some of the potential risk factors behind the development of VTE in IBD. One study demonstrated that IBD patients experiencing an acute flare that required steroid use were associated with an over 8-fold increased risk of VTE when compared to non-IBD patients[15]. The risk of VTE in IBD was driven by disease severity with the risk steadily increasing across IBD patients in remission, in a flare managed as an outpatient, and a flare managed in-hospital[18]. Among hospitalized IBD patients risk factors for VTE included UC, advanced age, IBD-related surgery, malnutrition, and medical co-morbidities[16,19]. Further, the prevalence of VTE among asymptomatic flaring IBD patients was low, which suggests that in-hospital factors drive the development of VTE[20]. Surgery has been recognized as an important risk factor for VTE among IBD patients[21,22]. However, studies are needed to explore the impact of VTE in UC patients who respond to in-hospital medical management as compared to those who are refractory to medical management and require colectomy.

Guidelines recommend prophylaxis for VTE using anti-coagulants (*e.g.*, subcutaneous heparin) in IBD patients who have been hospitalized for a flare of disease and following surgery[23,24]. While the use of VTE prophylactic doses of heparin in UC is recognized as safe[25,26], utilization of VTE prophylaxis for hospitalized UC patients in clinical practice may be suboptimal[27,28]. In a tertiary care referral center, nearly half of UC patients admitted to medical service were not provided VTE prophylaxis as compared to over 90% who were prescribed in a surgical service[27]. However, population-based studies that include VTE prescribing practices in community and

academic hospitals have not been explored.

We designed a large population-based study to determine the rate of VTE among UC patients who underwent an emergent or elective colectomy as compared to those who responded to in-hospital medical management. Furthermore, we explored heparin utilization in clinical practice and the efficacy of VTE prophylaxis in this population.

MATERIALS AND METHODS

Data source

The Data Integration, Measurement and Reporting (DIMR) hospital discharge abstract administrative database captures all hospitalizations in the Calgary Health Zone of Alberta Health Services, Canada. The Calgary Health Zone is a population-based health authority under a public, single payer system and provides all levels of medical and surgical care to the residents of the city of Calgary and over 20 nearby smaller cities, towns, villages, and hamlets. The estimated population of the Calgary Health Zone in 2009 was 1326115. The DIMR database contains 42 diagnostic and 25 procedural coding fields. The International Classification of Disease, Ninth Revision, Clinical Modification (ICD-9-CM) was used up to 2001, while ICD-10-CA and the Canadian Classification of Health Intervention (CCI) coding have been used after 2001[29].

Study population

The DIMR administrative discharge database was used to identify all adults (≥ 18 years of age) who were admitted to hospital with a diagnosis of UC (ICD-9-CM 556.X or ICD-10-CA K51.X) and underwent colectomy (ICD-9-CM: 45.7 and 45.8 or CCI: 1.NM.87, 1.NM.89, 1.NM.91, 1.NQ.89, 1. NQ. 90) between January 1st, 1997 and December 31, 2009[4]. Medical chart review confirmed all cases of UC and colectomy[29]. UC patients who underwent a colectomy were further stratified by emergent versus elective operation. An operation was defined as "elective" if the decision to operate on the UC patient was made prior to admission to hospital. In contrast, the decision for an "emergent" colectomy was determined during the hospital admission (*e.g.*, acute complication or refractory to in-hospital medical management)[4]. For a control group, we searched the administrative databases for all patients admitted to hospital from 1997 to 2009 with UC (ICD-9-CM 556.X or ICD-10-CA K51.X) coded in the primary diagnostic position and a random subset with UC coded in the second or third diagnostic position. Chart review confirmed that these patients were admitted for a flare of UC, but were discharged after responding to medical management. These patients never underwent colectomy during the course of the study period (1997 to 2009)[29].

Outcomes

The primary outcome was the development of VTE. Occurrence of VTE was defined according to anatomic location: (1) deep vein thrombosis in a limb; (2) deep vein thrombosis outside a limb including splenic, hepatic, mesenteric, renal, cephalic, subclavian, or portal vein thrombosis; and (3) pulmonary embolism, diagnosed in isolation or with a co-existing site. The diagnosis of VTE was evaluated in two settings: (1) diagnosis occurring during hospitalization; and (2) readmission to hospital for VTE within 6 mo of discharge. Readmission to hospital for VTE was included in the study population to capture VTE that developed early after admission or during in-hospital admission, but only recognized after discharge. Among UC patients who underwent an emergent colectomy, VTE were classified as preoperative or postoperative.

Variables

The primary variable of interest was the disease course, which was evaluated in 3 settings: (1) UC patients admitted to hospital for a flare of disease and discharged from hospital without colectomy after responding to medical mangement; (2) UC patients admitted to hospital for flare of UC requiring emergent colectomy; and (3) UC patients admitted to hospital for elective colectomy. The primary colectomy was recorded as the index date and no other UC-related bowel surgery was performed prior to the index date. In addition, data extracted from hospital records included: age at admission to hospital, stratified by the tercile of the entire cohort; sex; smoking status at hosptial admission (current, ex-smoker, or never smoker); disease duration (time from diagnosis of UC to admission to hospital); disease activity (> 5 stool/d and the presence of blood in stool versus ≤ 5 stool/d or absence of blood); extent of disease (left-sided colitis, pancolitis, or unknown); and a non-VTE in-hospital complication.

All comorbidities observed in our patients were recorded in the following groups as previously described[29]: coronary artery disease; congestive heart failure; other cardiovascular conditions (*e.g.*, arrhythmia); cancer; diabetes; gastrointestinal (*e.g.*, pancreatitis); haematological (*e.g.*, anemia); hypertension; liver disease (*e.g.,* primary sclerosing cholangitis); neurological conditions (*e.g.*, stroke); pulmonary disease (*e.g.*, asthma); renal disease (*e.g.,* dialysis); rheumatological (*e.g.*, rheumatoid arthritis); and thyroid or adrenal disease (*e.g.*, hypothyroidism). Comorbidities were classified as having 0 or ≥ 1 and have been validated in this population[29].

The chart reviewer determined the use (at time of admission or past history) of the following medications: mesalamine or sulfasalazine; azathioprine or 6-mercaptopurine; prednisone; and infliximab. VTE prophylaxis was evaluated for each UC patient in

the following settings: (1) in-hospital VTE prophylaxis among medically responsive UC flare patients; (2) pre- and postoperative VTE prophylaxis among UC patients who underwent emergent colectomy; and (3) postoperative VTE prophylaxis among UC patients who underwent elective colectomy. The primary medical VTE prophylaxis used was subcutaneous heparin; however, those using low molecular weight heparin were included and classified as VTE prophylaxis.

Statistical analysis

We described the occurrence of VTE stratified by disease course (*i.e.*, medically responsive to in-hospital management, emergent colectomy, and elective colectomy). For each outcome and study variable, descriptive statistics were performed using the Fisher Exact test for proportions; continuous variables were expressed as medians with interquartile ranges and compared using the Kruskal-Wallis test. Multivariate logistic regression analysis was performed to examine the association between disease course (*i.e.*, medically responsive, emergent colectomy, and elective colectomy) and VTE after adjusting for other clinical variables. Disease course and age were *a priori* forced into the regression model. The following variables were subsequently evaluated for independent effects on VTE development using the stepwise selection approach with 0.1 as the entry and exit *P* value: sex; year of admission; smoking status; comorbidities; disease duration; extent of disease; disease activity; in-hospital complication at anytime during admission (*i.e.*, pre- or postoperatively for surgical groups), but excluding VTE; and IBD-related medications (*i.e.*, mesalamine/sulfasalazine, prednisone, azathioprine/6-mercaptopurine, and infliximab) prescribed prior to admission. These variables were considered significant if the two-sided *P* value was < 0.05. We used a stepwise selection approach to adjust our model for significant variables, while optimizing the efficiency of the model by avoiding overfitting of covariates.

In a subanalysis, we compared medically responsive UC patients to UC patients who underwent an emergent colectomy (*i.e.*, elective colectomy patients were excluded). We repeated the multivariate logistic regression analysis described above, but included a variable in the model for the use of VTE prophylaxis prior to discharge (*i.e.,*medically responsive) and prior to colectomy. Second, we compared UC patients undergoing an elective colectomy to UC patients who underwent an emergent colectomy. Similarly, we included a variable in the logistic regression model that represented VTE prophylaxis for UC patients undergoing colectomy.

All statistical analyses were conducted using SAS version 9.3 (SAS Institute Inc., Cary, United States).

Ethical considerations

The study was approved by the Conjoint Health Research Ethics Board of the University of Calgary.

RESULTS

Between January 1st 1997 and December 31st 2009, 638 individuals with UC who underwent a colectomy (309 emergent and 329 elective) were identified. A further 382 UC patients were admitted emergently with a flare, but were discharged after responding to in-hospital medical management. The characteristics of patients with UC are presented in Table 1. The overall VTE rate was 4.7%, and 18.8% of these individuals experienced a pulmonary embolism. The most common site of VTE was intra-abdominal (45.8%) followed by lower extremity (19.6%). Table 2 describes the location of VTE.

VTE occurred in 1.3% of medically responsive, 8.7% of emergent colectomy, and 4.9% of elective colectomy UC patients (Table 1). Among the emergent colectomy group 88.9% were diagnosed with a VTE postoperatively. Among the 48 UC patients who were diagnosed with VTE, 16.7% were diagnosed after discharge from hospital. No patients died as a complication of the VTE. Postoperative VTE prophylaxis with anticoagulants was prescribed in over 90% of patients. However, VTE prophylaxis was prescribed preoperatively or prior to discharge in less than 20% of patients with UC who underwent emergent colectomy or were emergently admitted to hospital (*i.e.*, medically responsive), respectively (Table 1).

Among all UC patients (n = 1020), UC patients requiring elective colectomy (adjusted OR = 3.69; 95%CI: 1.30-10.44) and emergent colectomy (adjusted OR = 5.28; 95%CI: 1.93-14.45) were significantly more likely to be diagnosed with a VTE as compared to medically responsive UC patients (Table 3). Furthermore, the odds of a VTE significantly increased across time (adjusted OR = 1.10; 95%CI: 1.01-1.20). Patients who underwent emergent colectomy (*i.e.*, elective colectomy excluded) were significantly more likely to develop a VTE (adjusted OR = 4.62; 95%CI: 1.66-12.88) as compared to UC patients who were medically responsive. Furthermore, an in-hospital complication was associated with an increased odds of VTE (adjusted OR = 2.68; 95%CI: 1.42-5.05) (Table 3).

DISCUSSION

This study identified a population-based cohort of over 1000 hospitalised UC patients. All charts were identified to confirm the diagnosis of VTE both in-hospital and following discharge. The study population was stratified according to the indication for admission (*i.e.*, medically responsive UC flare,

Table 1 Characteristics of patients hospitalized for a ulcerative colitis flare, or undergoing a colectomy *n* (%)

Characteristics	Total cohort (*n* = 1020)	Elective colectomy (*n* = 329)	Emergency colectomy (*n* = 309)	Medically responsive (*n* = 382)	*P* value
Thrombosis					< 0.001
No	95.3 (972)	95.1 (313)	91.3 (282)	98.7 (377)	
Yes	4.7 (48)	4.9 (16)	8.7 (27)	1.3 (5)	
Age at Admission (tercile)					0.002
18-32	33.8 (345)	28.6 (94)	31.7 (98)	40.1 (153)	
33-47	32.6 (333)	33.1 (109)	31.1 (96)	33.5 (128)	
48+	33.5 (342)	38.3 (126)	37.2 (115)	26.4 (101)	
Gender					0.022
Male	57.0 (581)	61.4 (202)	58.9 (182)	51.6 (197)	
Female	43.0 (439)	38.6 (127)	41.1 (127)	48.4 (185)	
Smoking					0.011
Current	9.8 (94)	7.9 (25)	7.7 (23)	13.3 (46)	
Ex-smokers	33.0 (317)	30.5 (96)	39.0 (117)	30.1 (104)	
Never	57.2 (549)	61.6 (194)	53.3 (160)	56.5 (195)	
Missing (*n*)	*n* = 60	*n* = 14	*n* = 9	*n* = 37	
Comorbidity					
0 comorbidities	46.3 (472)	48.3 (159)	40.5 (125)	49.2 (188)	0.047
⩾ 1 comorbidities	53.7 (548)	51.7 (170)	59.6 (184)	50.8 (194)	
Primary sclerosing cholangitis					0.572
No	97.6 (996)	97.0 (319)	97.7 (302)	98.2 (375)	
Yes	2.4 (24)	3.0 (10)	2.3 (7)	1.8 (7)	
Ankylosing spondylitis or sacroiliitis					0.222
No	99.0 (1010)	99.7 (328)	98.4 (304)	99.0 (378)	
Yes	1.0 (10)	0.3 (1)	1.6 (5)	1.0 (4)	
Episcleritis, uveitis and iritis					0.603
No	98.9 (1009)	98.5 (324)	99.4 (307)	99.0 (378)	
Yes	1.1 (11)	1.5 (5)	0.6 (2)	1.0 (4)	
Disease duration, years					< 0.001
Median (IQR)	3 (0-9)	6 (2-14)	2 (0-7)	1 (0-6)	
Missing (*n*)	*n* = 35	*n* = 18	*n* = 9	*n* = 8	
Cancer/dysplasia					-
No	-	83.0 (273)	-	-	
Yes		17.0 (56)			
Extent of disease					< 0.001
Left-sided	29.4 (265)	23.5 (73)	20.9 (63)	44.5 (129)	
Pancolitis	70.6 (637)	76.5 (237)	79.1 (239)	55.5 (161)	
Missing (*n*)	*n* = 118	*n* = 19	*n* = 7	*n* = 92	
Blood in Stool and Stool Frequency > 5/d[1]					< 0.001
No	27.0 (217)	46.4 (91)	18.9 (54)	22.3 (72)	
Yes	73.0 (588)	53.6 (105)	81.1 (232)	77.7 (251)	
Missing (*n*)	*n* = 215	*n* = 133	*n* = 23	*n* = 59	
5-ASA[2]					< 0.001
No	33.1 (336)	28.0 (91)	26.9 (83)	42.4 (162)	
Yes	66.9 (680)	72.0 (234)	73.1 (226)	57.6 (220)	
Missing (*n*)	*n* = 4	*n* = 4			
Prednisone[2]					< 0.001
No	31.3 (318)	16.6 (54)	22.0 (68)	51.3 (196)	
Yes	68.7 (698)	83.4 (271)	78.0 (241)	48.7 (186)	
Missing (*n*)	*n* = 4	*n* = 4			
Azathioprine/6-mercaptopurine[2]					< 0.001
No	80.7 (820)	69.2 (225)	80.3 (248)	90.8 (347)	
Yes	19.3 (196)	30.8 (100)	19.7 (61)	9.2 (35)	
Missing (*n*)	*n* = 4	*n* = 4			
Infliximab[2]					0.006
No	96.1 (976)	94.8 (308)	94.5 (292)	98.4 (376)	
Yes	3.9 (40)	5.2 (17)	5.5 (17)	1.6 (6)	
Missing (*n*)	*n* = 4	*n* = 4			
Complication in hospital[3]					< 0.001
0 or Clavien Ⅰ	80.2 (818)	79.9 (263)	64.1 (198)	93.5 (357)	
Clavien Ⅱ/Ⅲ/Ⅳ	19.8 (202)	20.1 (66)	35.9 (111)	6.5 (25)	
Postoperative heparin					0.641
No	6.9 (44)	6.4 (21)	7.4 (23)	-	
Yes	93.1 (593)	93.6 (307)	92.6 (286)		
Missing (*n*)	*n* = 1	*n* = 1			

Heparin prophylaxis prior to surgery/discharge[4]					0.310
No	82.9 (572)	-	81.2 (251)	84.3 (321)	
Yes	17.1 (118)		18.8 (58)	15.7 (60)	
Missing (*n*)	*n* = 1			*n* = 1	

[1]Missing data is defined as missing on either the stool frequency, the presence of blood in stool, or both. [2]Defined as medication used prior to or at the time of admission to hospital. No refers to no record of drug use in the medical chart. [3]Complication was classified as Clavien ≥ Ⅱ. [4]Emergent colectomy - heparin prophylaxis in hospital prior to colectomy. Medically Responsive - heparin prophylaxis in hospital prior to discharge.

Table 2 Location of venous thromboembolism *n* (%)

Location of VTE	Total (*n* = 48)	Elective colectomy (*n* = 16)	Emergency colectomy (*n* = 27)	Medically responsive (*n* = 5)
Upper extremity	8 (17.4)	1 (6.3)	6 (22.2)	1 (20.0)
Lower extremity	9 (19.6)	3 (18.8)	6 (22.2)	0
Intra-abdominal	22 (45.8)	11 (68.8)	10 (37.0)	1 (20.0)
Pulmonary embolism	9 (18.8)	1 (6.3)	5 (18.5)	3 (60.0)

VTE: Venous thromboembolism.

Table 3 Independent predictors of venous thromboembolism for all ulcerative colitis patients; those admitted emergently to hospital and were medically responsive or underwent emergent colectomy; and those who underwent an emergent or elective colectomy

	Full cohort VTE (*n* = 1020) Adjusted OR (95%CI)	Emergent and medically responsive cohort VTE (*n* = 690)[1] Adjusted OR (95%CI)	Emergent and elective cohort VTE (*n* = 637)[1] Adjusted OR (95%CI)
Age, yr			
18-32	1.00	1.00	1.00
33-47	1.56 (0.68-3.56)	2.44 (0.81-7.34)	1.35 (0.56-3.27)
≥ 48	1.63 (0.74-3.59)	2.29 (0.79-6.66)	1.65 (0.72-3.75)
Disease course			
Medical responsive	1.00	1.00	Not applicable
Elective colectomy	3.69 (1.30-10.44)	Not applicable	1.00
Emergent colectomy	5.28 (1.93-14.45)	4.62 (1.66-12.88)	1.59 (0.82-3.07)
VTE prophylaxis[2]			
No	Not applicable	1.00	1.00
Yes		2.24 (0.99-5.03)	1.38 (0.32-6.00)
In-hospital complication			
No	1.00	1.00	1.00
Yes	2.68 (1.42-5.05)	2.80 (1.26-6.24)	2.53 (1.32-4.85)
Year	1.10 (1.01-1.20)	Not significant[3]	Not significant[3]

[1]One patient was excluded in each of these cases due to missing data. [2]VTE prophylaxis with anticoagulants (*e.g.*, heparin) was assessed preoperatively/in-hospital for the emergent colectomy and medically responsive cohort, and postoperatively for the emergent and elective colectomy cohort. VTE prophylaxis was not modeled for the entire cohort. [3]*P* value was > 0.1 when fitted into the regression model using stepwise selection. The following variables were tested in the multivariate model, but were not independent predictors in any of the models: sex, disease duration, comorbidities, extra-intestinal manifestations, extent of disease, blood in stool and frequency of bowel movements. 5-ASA: Azathioprine, prednisone, and infliximab; VTE: Venous thromboembolism.

emergent colectomy, or elective colectomy) in order to explore the effect of surgery on the risk of VTE. We demonstrated that colectomy was significantly associated with the development of VTE. VTE occurred in about 1% of the medically responsive UC patients, whereas UC patients who failed to respond to medical management and underwent a colectomy had an over 5-fold increased odds of VTE. Similarly, UC patients undergoing elective colectomy had an over 3-fold increased odds of VTE when compared to medically responsive UC inpatients. In contrast, the risk of VTE was not associated with disease location, or drug utilization including the use of corticosteroids, immunomodulators, and biologics.

VTE occurred in 4.7% of UC patients. These occurred in a number of locations including extremity, intra-abdominal (*i.e.*, portal and hepatic vein thrombosis), and pulmonary embolism. VTE were confirmed using multiple different testing modalities including chest X-ray, D-Dimer, Doppler ultrasound of the extremities, V/Q scan, CT PE protocol, and angiography. While most VTE were identified following the manifestation of clinical symptoms, most of the intra-abdominal clots were detected incidentally in asymptomatic patients on CT scans performed to assess postoperative symptoms or complications. While incidental cases were primarily identified in postoperative patients following colectomy (11 cases among elective and 10 cases among emergent colectomy), one

incidental intra-abdominal VTE was identified in the medically responsive group. Thus, some cases of VTE that were asymptomatic may have been missed. However, the proportion of clinically relevant VTE that we missed was likely low. For example, a recent study of inpatient and outpatient UC patients who were actively flaring, but were asymptomatic for a DVT, were not found to have any incidentally diagnosed DVT on screening ultrasound[20]. Also, we evaluated every case for readmission to hospital for a VTE within 6 mo of the discharge from hospital admission. Thus, we were able to capture clinically meaningful cases of VTE that were missed in hospital or developed post-discharge. Sixteen percent of UC patients were readmitted to hospital for VTE following discharge from hospital including one patient who was readmitted 6 d after discharge for mesenteric vein thrombosis that led emergent resection of ischemic bowel. Consequently, physicians should be aware of this complication, recognize the signs and symptoms, and have a low threshold to screen for VTE in the extremities and in the abdomen in UC patients.

Current American College of Gastroenterology guidelines recommend VTE prophylaxis for all patients admitted to hospital with an IBD flare as well as all postoperative patients undergoing colectomy[23]. The American College of Chest Physicians (ACCP) guidelines recommend VTE prophylaxis in IBD patients confined to a bed[30]. Though, ambulation alone would not prevent intra-abdominal VTE. In our study, greater than 90% of emergent and elective colectomy patients received VTE prophylaxis postoperatively. Despite the high utilization of postoperative heparin, the occurrence of VTE remained high. Similarly, a smaller study found that despite adequate postoperative heparin use, rates of VTE in post-colectomy patients were over 7-fold higher in UC patients as compared to patients undergoing surgery for colorectal cancer[31]. A randomized controlled trial that included nearly 600 patients with inflammatory bowel disease demonstrated that low dose heparin had significantly lower rates of VTE (2.9%) as compared to patients randomized to enoxaparin (9.0%)[32].

The current ACCP guidelines recommend that patients undergoing abdominal or pelvic surgery for cancer, with a high risk of postoperative VTE (*i.e.*, > 6%) and who do not have increased risk of bleeding, should be considered for both mechanical and pharmacological VTE prophylaxis and extended duration of the latter[30]. Our study was unique from prior work because we explored VTE occurrence up to 6 mo following discharge from hospital. We demonstrated that 16% of our detected VTE were diagnosed after discharge from hospital. Our findings raise the issue of whether the same consideration for extended postoperative VTE prophylaxis should be given for IBD surgeries. Prior studies have reported lower DVT levels (< 3%) postoperatively for IBD surgeries[31,32], as compared to our study. However, nearly half of our VTE population experienced an intra-abdominal VTE. The clinical significance of intra-abdominal VTE is controversial and depends on the acuity, size, and location of the thrombosis[33,34]. Given the uncertainty, these findings warrant a randomized controlled trial to evaluate the efficacy of VTE prophylaxis for 4 wk postoperatively.

Only 19% of patients admitted with an UC flare who underwent emergent colectomy received preoperative VTE prophylaxis. Similarly, only 16% of patients who were admitted for an UC flare and discharged without colectomy received VTE prophylaxis in-hospital. A recent multicenter chart audit of 29 Canadian hospitals also demonstrated similarly low prophylaxis rates for hospitalized medical patients requiring VTE prophylaxis[35]. Moreover, patients who received VTE prophylaxis were more likely to be diagnosed with a VTE. However, this finding likely reflected disease severity (*i.e.,* confounding by indication) because a meta-analysis indicated that heparin is safe to use in an acute flare of UC[25,36] and randomized controlled trials support the use of VTE prophylaxis in IBD[32]. Thus, in the absence of hemodynamically significant haemorrhage VTE prophylaxis should be prescribed. However, the adequate agent, dose, and duration of VTE prophylaxis in medically managed UC patients need further evaluation.

Our study could not differentiate whether the increased risk of VTE among UC patients undergoing colectomy was due specifically to the colectomy or was caused by disease severity necessitating colectomy. Though, most likely it was due to the combination of disease severity and surgery. Thus, the optimal approach of preventing VTE is to induce and maintain remission in UC. The majority of UC patients were admitted to hospital prior to publications demonstrating efficacy of infliximab in the hospital[37] and outpatient setting[38]. Additionally, recent studies have demonstrated that infliximab significantly reduced the risk of colectomy for up to 3 years following initiation of treatment[39-41]. Because less than 4% of the patients in our study were prescribed infliximab, greater utilization of anti-TNF therapies may lead to reduced colectomy and hence, burden of VTE among UC patients.

Several limitations to this study should be considered. While VTE events that resulted in a readmission to hospital within 6 mo of hospital discharge were captured, UC patients diagnosed and managed for VTE as an outpatient following discharge from hospital would not have been accounted for in this study. Thus, the occurrence of VTE may have been underestimated. While chart review improves the accuracy of the data and minimizes misclassification bias that occur with studies using administrative databases[29,42], some

data were missed due to incomplete recording in the medical chart. For example, we were not able to account for the use of oral contraceptive pills prior to hospitalization due to unreliable recording of this data. This study was not able to confirm if other forms of VTE prophylaxis such as graduated compression stockings or intermittent pneumatic compression devices for hospitalized UC patients were utilised. However, these modalities are only recommended if heparin is contraindicated because they are less effective at preventing proximal extremity and intra-abdominal VTE. Also, due to the retrospective nature of chart reviews we were not able to assess the number of flares from diagnosis to admission to hospital or to define disease severity using a validated disease activity index (*e.g.*, Mayo score). Finally, despite the advantages of designing a population-based study, regional practice pattern differences may limit generalizability. Consequently, we recommend hospitals assess their own utilization of heparin prophylaxis and correlate these with VTE outcomes.

VTE occurred commonly in UC patients who underwent emergent (8.7%) and elective (4.9%) colectomy despite greater than 90% rate of postoperative heparin prophylaxis. In contrast, about 1% of medically responsive UC inpatients experienced a VTE, despite a 16% prophylaxis rate. Thus, VTE prophylaxis should be prescribed at time of admission in all IBD patients, and VTE prophylaxis for flaring UC patients should be considered a quality indicator of best clinical practice. This population-based study confirmed that the necessity of colectomy was significantly associated with VTE. The high rates of VTE in UC patients who underwent colectomy despite postoperative VTE prophylaxis highlights potentially serious outcomes associated with surgical management of UC. Consequently, these findings emphasize the importance of optimizing medical therapy for UC patients.

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COMMENTS

Background

Ulcerative colitis (UC) is a chronic inflammatory condition of the large colon that affects young individuals in the prime of their lives. Most patients with UC require daily medications to control inflammation. When these medications do not work UC patients require an operation (*i.e.*, colectomy) to remove their large bowel.

Research frontiers

Patients with UC are at increased risk of developing a venous thromboembolism (VTE), which is a potentially life-threatening complication. Potential risk factors for developing VTE among patients with UC include acute flares, hospitalization, advanced age, comorbidities, colectomy, and malnutrition. Understanding the leading risk factors of VTE for patients with UC will allow physicians to optimize prevention of VTE.

Innovations and breakthroughs

The overall occurrence of VTE among hospitalized patients with UC was nearly 5%. However, the risk of VTE was low (about 1%) among flaring UC patients who were responsive to medical in-hospital management. In contrast, patients with UC who underwent an elective colectomy (5%) or emergent colectomy (8.7%) had significantly higher occurrence of VTE. In contrast, about 1% of medically responsive UC inpatients experienced a VTE.

Applications

Prescription of VTE prophylaxis for UC patients hospitalized for flare was suboptimal (< 20%). In contrast, VTE prescriptions postoperatively were high (> 90%) following a colectomy for UC. Thus, this data supports both VTE prophylaxis and aggressive medical management of ulcerative colitis patients to prevent VTE formation.

Terminology

Venous thromboembolism is a blood clot that forms within the venous circulation. International Classification of Diseases is a set of codes used by hospital health records to document diseases. These coded data can be used for research and surveillance purposes.

Peer review

"This is an excellent original contribution analyzing cohort of 1020 hospitalised UC patients towards risk of VTE. The Authors determined that patients who underwent elective or emergent colectomy had 4-5-fold increased risk of VTE when compared to UC patients treated non-surgically."

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ORIGINAL ARTICLE

Observational Study

Post-partum reactivation of chronic hepatitis B virus infection among hepatitis B e-antigen-negative women

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Abstract

AIM: To investigate the frequency and timing of post-partum chronic hepatitis B virus (HBV) reactivation and identify its pre-partum predictors.

METHODS: Forty-one hepatitis B e antigen (HBeAg)-negative chronic HBV infected pregnant women were prospectively evaluated between the 28th and the 32nd week of gestation. Subjects were re-evaluated at 3-mo intervals during the first post-partum year and every 6 mo during the following years. HBV DNA was determined using real-time reverse transcription polymerase chain reaction (Cobas TaqMan HBV Test) with a lower detection limit of 8 IU/mL. Post-partum reactivation (PPR) was defined as abnormal alanine aminotransaminase (ALT) levels and HBV DNA above 2000 IU/mL.

RESULTS: Fourteen out of 41 women (34.1%) had pre-partum HBV DNA levels > 2000 IU/mL, 18 (43.9%) had levels < 2000 IU/mL and 9 (21.9%) had undetectable levels. Fourteen women were lost to follow-up (failure to return). PPR occurred in 8 of the 27 (29.6%) women evaluated, all within the first 6 mo after delivery (5 at month 3; 3 at month 6). Five of the 6 (83.3%) women with pre-partum HBV DNA > 10000 IU/mL exhibited PPR compared with 3 of the 21 (14.3%) women with HBV DNA < 10000 IU/mL (two with HBV DNA > 2000 and the third with HBV DNA of 1850 IU/mL), $P = 0.004$. An HBV DNA level $\geqslant$ 10000 IU/mL independently predicted post-partum HBV infection reactivation (OR = 57.02, $P = 0.033$). Mean pre-partum ALT levels presented a non-significant increase in PPR cases (47.3 IU/L *vs* 22.2 IU/L, respectively, $P = 0.094$).

CONCLUSION: In the present study, PPR occurred in approximately 30% of HBeAg-negative pregnant women; all events were observed during the first semester after delivery. Pre-partum HBV DNA level > 10000 IU/mL predicted PPR.

Key words: Hepatitis B; Pregnancy; Reactivation; Post-

Partum; Hepatitis B virus-DNA

Core tip: According to our prospective study, the post-partum reactivation of chronic hepatitis B occurs in approximately 30% of hepatitis B e antigen-negative women; all cases are observed during the first 6 mo after delivery. Among demographic, hematological, biochemical and viral characteristics, the only pre-partum parameter predictive for post-partum hepatitis B virus reactivation is whether the maternal viral load is greater than 10000 IU/mL between the 28th and the 32nd week of gestation.

Elefsiniotis I, Vezali E, Vrachatis D, Hatzianastasiou S, Pappas S, Farmakidis G, Vrioni G, Tsakris A. Post-partum reactivation of chronic hepatitis B virus infection among hepatitis B e-antigen-negative women. *World J Gastroenterol* 2015; 21(4): 1261-1267 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1261.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1261

INTRODUCTION

Chronic hepatitis B virus (HBV) infection in pregnancy is an important global health problem. Over 50% of the 350 million chronic HBV carriers acquire their infection perinatally, and the risk of progression to chronic infection is inversely proportional to the age at infection[1,2]. Women of childbearing age with chronic HBV infection remain a significant source of HBV transmission worldwide. Thus, the management of chronic HBV infection during pregnancy is essential to interrupt perinatal HBV transmission[3].

Several published reports and expert opinions conclude that the major risk factor for immunoprophylaxis failure is maternal HBV DNA levels during the third trimester of pregnancy[4-9]. Serum HBV DNA determination is suggested between weeks 28 and 32 of pregnancy to determine whether treatment with nucleoside or nucleotide analogues is needed in highly viremic women[6-9]. Moreover, the importance of maternal viremia has been clearly documented in the literature because it is positively associated with cord blood viremia, a parameter that seems to also affect pregnancy outcome[10,11].

Data concerning the effect of pregnancy on chronic HBV infection and HBV-related liver disease are limited. In general, there is usually no deterioration of HBV-related liver disease during pregnancy[3]. HBV is a non-cytopathic virus and the associated liver inflammation is mainly mediated by the host's immune response. Moreover, because of pregnancy-induced immune mediated changes as well as pregnancy-induced plasma volume expansion, serum aminotransferase levels seem to remain within normal values, even in pregnant women with pre-existing chronic liver disease[12,13]. However, there are reports of severe HBV flares resulting in liver failure during the peripartum period, mainly in hepatitis B e antigen (HBeAg)-positive Asian women[14]. Although data from Europe concerning the clinical course of chronic HBV-infected Caucasian pregnant women in late pregnancy and early postpartum period are limited[15], there are no data on post-partum HBV reactivation among HBeAg-negative chronic HBV-infected women during long-term follow-up.

The aim of the study was to prospectively evaluate the frequency and timing of post-partum HBV re-activation appearance and to identify any pre-partum virological or hematological-biochemical predictive factors.

MATERIALS AND METHODS

Between January 2007 and January 2008, a total of 60 chronic HBV-infected pregnant women were evaluated between the 28th and the 32nd week of gestation. Namely, clinical examination, hematological, biochemical and serological tests were performed at the Department of Obstetrics and Gynecology of "Elena Venizelou" Maternal and Perinatal Hospital of Athens, Greece. A total of 2.0 mL of serum was obtained from each woman with chronic hepatitis B and kept at -80 ℃ until further analyses. Viral load in a 0.5-mL sample was determined by real-time reverse transcription-polymerase chain reaction (COBAS® AmpliPrep/COBAS® TaqMan® HBV Test, v2.0, lower detection limit: 8 IU/mL, Roche, Basel, Switzerland).

Hepatitis B surface antigen, HBeAg, antibody to HBeAg, antibody to hepatitis B core antigen (IgM/total), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis C virus (HCV), antibody to hepatitis D virus (HDV) and antibodies to human immunodeficiency virus (HIV) were detected using commercially available enzyme immunoassays (Abbott Laboratories, Abbott Park, IL, United States). Routine hematological and biochemical tests were performed using automated techniques.

Pregnant women with acute hepatitis B, HBeAg-positive chronic infection, co-infections (HCV, HDV, and HIV), or any known pre-existing liver disease were excluded from the study. Additionally, women with known pregnancy-related complications (intrahepatic cholestasis of pregnancy, HELLP syndrome, preeclampsia, placental hemorrhage, *etc.*), women taking medications (except for iron, folic acid, calcium and other vitamins or diet supplements), as well as those with known bacterial, fungal, parasitic or viral infections during pregnancy, were also excluded from the final analysis. Treatment and prophylaxis with nucleos(t)ide analogues were also considered as exclusion criteria. Finally, failure

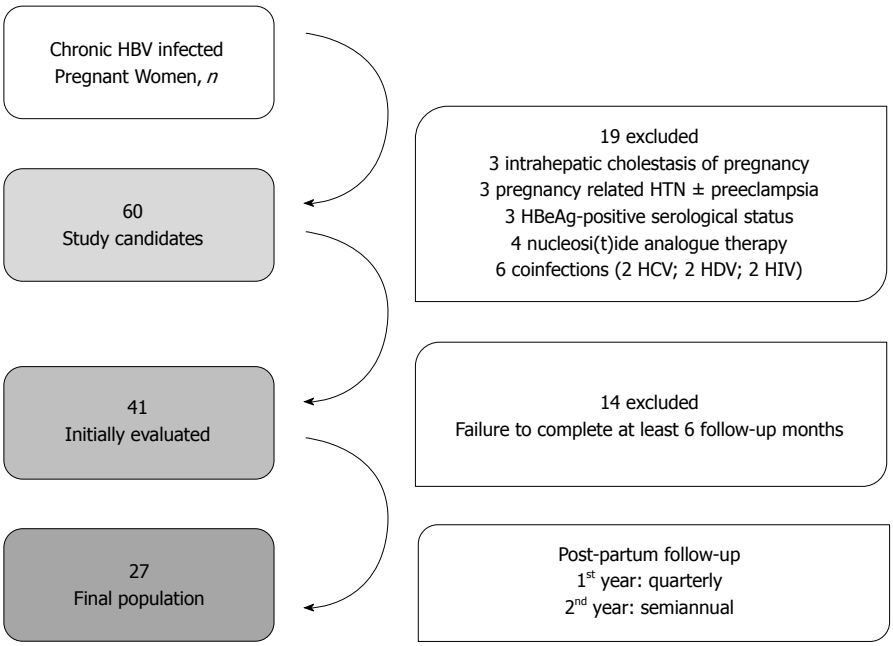


Figure 1 Flow chart diagram of the study population. HBV: Hepatitis B virus; HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; HDV: Hepatitis D virus.

to complete at least a 6-mo post-partum follow-up period also resulted in exclusion from the study.

All chronic hepatitis B-infected women were prospectively clinically, virologically and biochemically evaluated after delivery. In particular, all women were evaluated virologically [quantitative serum HBV DNA test, polymerase chain reaction (PCR)] and biochemically [serum alanine aminotransferase (ALT) levels] at the 3rd mo and the 6th mo of the post-partum period and then only biochemically (serum ALT levels) every 3 mo for the first post-partum year and every 6 mo for the following years. HBV DNA testing was repeated annually in those with ALT levels within the normal values proposed by our laboratory (< 35 IU/L). In those with abnormal serum ALT levels, HBV DNA was calculated immediately.

Post-partum HBV reactivation was defined as abnormal serum ALT levels and serum HBV DNA levels above 2000 IU/L, irrespective of the pre-partum levels.

Written informed consent was obtained from all patients. Study protocol was in accordance with the 1975 Declaration of Helsinki and was reviewed and approved by the "Elena Venizelou" Hospital Ethics Committee.

Statistical analysis

Continuous variables are presented as the mean ± SD unless stated otherwise. Because of the small number of patients, continuous variable differences between the groups presenting or not with HBV reactivation were evaluated as independent samples using the Mann-Whitney *U*-test. Categorical variable differences between the HBV reactivation and no-reactivation groups were evaluated using the χ^2 Fisher's exact test. The Kaplan-Meier plot was used to estimate cumulative hazard and event free time for post-partum HBV reactivation for patients according to their pre-partum serum HBV DNA levels (< or ⩾ than 10000 IU/mL); data regarding timing of events were interval censored.

Multivariate logistic regression analysis (enter method with forced entry of independent variables) was performed to further evaluate the association of HBV DNA levels ⩾ 10000 IU/mL with post-parturm HBV reactivation after adjustment for the percentage of polynuclear cells and lymphocytes within the total white blood cell count.

A *P*-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS 19 for MacOS (SPSS Inc, Chicago, Illinois, United States).

RESULTS

A total of 60 women were initially considered to be candidates for the study. Nineteen women were excluded from the final analysis per the study exclusion criteria. The flow chart diagram of the study population is presented in Figure 1.

Among the remainder of the 41 chronically infected pregnant women, 32/41 (78.1%) were HBV DNA-positive whereas 9/41 (21.9%) had undetectable HBV DNA levels in the pre-partum period. In particular, 18/41 (43.9%) women had detectable HBV DNA levels that were lower than 2000 IU/mL, and 14/41 (34.1%) had HBV DNA levels above 2000 IU/mL. Importantly, in a significant proportion of women with viremia above 2000 IU/mL, the HBV DNA levels

Table 1 Pre-partum (3rd trimester of pregnancy) hematological, biochemical and virological characteristics of chronic hepatitis B virus-infected patients

	Overall population	No-reactivation	HBV-reactivation	*P* value
Patients, *n*	27	19	8	
Hct	35.9% ± 4.0%	35.1% ± 3.0%	37.8% ± 5.6%	0.312
Hb, g/dL	12.0 ± 1.4	11.6 ± 1.1	12.7 ± 1.8	0.207
WBC, *n*	8.677 ± 2.835	8.835 ± 3.122	8.308 ± 2.231	0.444
PNL	67.3% ± 10.3%	68.8% ± 11.0%	62.3% ± 6.2%	0.008
LYMPHO	24.3% ± 9.1%	23.1% ± 9.8%	28.5% ± 5.2%	0.035
MONO	5.9% ± 1.8%	5.6% ± 1.9%	6.8% ± 1.0%	0.192
PLT, /10^3	209 ± 41	212 ± 45	202 ± 31	0.968
AST, IU/L	27.6 ± 14.7	23.8 ± 6.9	35.3 ± 22.8	0.585
ALT, IU/L	30.6 ± 28.2	22.2 ± 13.0	47.3 ± 42.4	0.094
GGT, IU/L	12.8 ± 6.8	14.2 ± 7.2	9.8 ± 5.4	0.210
LDH, IU/L	203.8 ± 85.6	180.6 ± 79.5	250.2 ± 88.8	0.214
TBIL, mg/dL	0.51 ± 0.29	0.53 ± 0.34	0.47 ± 0.19	1.000
DBIL, mg/dL	0.22 ± 0.20	0.26 ± 0.24	0.15 ± 0.06	0.462
TPROT, g/dL	6.67 ± 0.58	6.84 ± 0.48	6.23 ± 0.66	0.138
ALB, g/dL	3.59 ± 0.39	3.71 ± 0.32	3.25 ± 0.39	0.078
GLOB, g/dL	3.07 ± 0.39	3.10 ± 0.42	2.97 ± 0.34	0.661
HBV DNA ⩾ 10000 IU/mL, *n* (%)	6 (22.2)	1 (5.2)	5 (62.5)	0.004

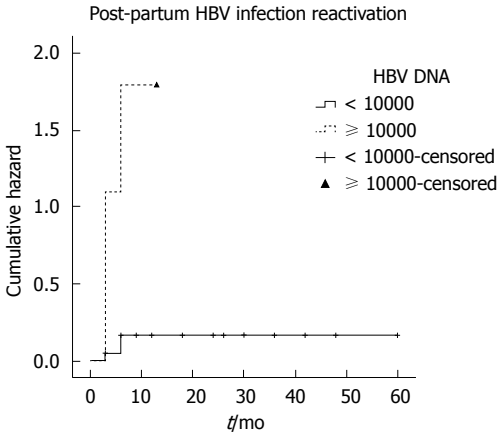
HBV: Hepatitis B virus; Hct: Hematocrit; Hb: Hemoglobin; WBC: White blood cells; PNL: Polynuclear cells; LYMPHO: Lymphocytes; MONO: Monocytes; PLT: Platelets; AST: Aspartate transaminase; ALT: Alanine transaminase; GGT: Gamma-glutamate transpeptidase; LDH: Lactate dehydrogenase; TBIL: Total bilirubin; DBIL: Direct bilirubin; TPROT: Total protein; ALB: Albumin; GLOB: Globulins.

were elevated more than >10000 IU/mL during the third trimester.

Fourteen women failed to follow-up after delivery and were excluded from the analysis. The remaining 27 women who were evaluated were followed for a period of 6 to 60 mo.

Eight out of the 27 (29.6%) HBeAg-negative chronic HBV-infected women showed a post-partum ALT flare with concomitant elevation in viremia above 2000 IU/mL. It is also important to note that all HBV reactivation cases were documented during the first 6 mo after delivery (5 cases were observed during the third month; the remaining 3 cases during the sixth month of follow-up). Women in whom HBV reactivation was not observed during this early post-partum period did not present an event during the follow-up period (17.6 ± 3.5 mo).

Age (27 ± 9.4 years *vs* 28.4 ± 5.8 years, *P* = 0.147), weight (70.8 ± 16.7 kg *vs* 69.2 ± 11.7 kg, *P* = 0.283), height (1.64 ± 0.05 m *vs* 1.64 ± 0.05 m, *P* = 1.00), body mass index (26.1 ± 5.3 kg/m^2 *vs* 25.6 ± 3.9 kg/m^2, *P* = 0.849), as well as weight gain during pregnancy (10.8 ± 0.83 kg *vs* 11.5 ± 3.59 kg, *P* = 0.594), were comparable among the women who exhibited HBV reactivation and those who did not. Detailed pre-partum hematological, biochemical and virological characteristics of the HBV-reactivation as well as no-reactivation cases are presented in Table 1.



HBV DNA, IU/mL	Patients, *n*	PPR, *n*	PPR free time in months (95%CI)
⩾ 10000	6	5	5.2 ± 1.5 (2.2, 8.1)
< 10000	21	3	51.6 ± 4.5 (42.8, 60.4)

Figure 2 Cumulative Hazard plot for post-partum hepatitis B virus reactivation. HBV DNA: Hepatitis B virus deoxyribonucleic acid; PPR: Post-partum reactivation.

The pre-partum peripheral white blood cell evaluation of the HBV-reactivation group revealed a lower percentage of neutrophils and higher percentage of lymphocytes (62.3% ± 6.2% *vs* 68.8% ± 11%, *P* = 0.008 and 28.5% ± 5.2% *vs* 23.1% ± 9.8%, *P* = 0.035, respectively) than in the no-reactivation group.

The women who exhibited post-partum HBV reactivation had comparable absolute lymphocyte counts (2099 ± 230 *vs* 1862 ± 107, *P* = 0.382) and exhibited a tendency toward lower absolute neutrophil counts (4582 ± 381 *vs* 6278 ± 710, *P* = 0.079) during the third trimester of pregnancy compared to women without HBV reactivation. The pre-partum serum aspartate transaminase, ALT and gamma-glutamate transpeptidase levels were comparable among the HBV-reactivation and no-reactivation cases, as shown in Table 1. It is important to note that the pre-partum serum ALT levels were within the normal range of our laboratory in the vast majority of patients, except for two women with HBV DNA levels of 40000 and 45000 IU/L, who had serum ALT levels of 120 and 95 IU/L, respectively. Both of them continued to have abnormal ALT and high HBV DNA levels during the early post-partum period (at month 3 of follow-up).

On one hand, none of the chronic HBV-infected pregnant women with undetectable HBV DNA levels during the pre-partum period presented HBV-reactivation. On the other hand, the majority of HBV-reactivation cases (5/8 women, 62.5%) had pre-partum HBV DNA levels above 10000 IU/mL, and the remaining three reactivation cases exhibited serum HBV DNA levels of 8620, 2550 and 1850 IU/mL. In the multivariate analysis, after adjustment for lymphocyte and neutrophil blood count, pre-partum serum HBV DNA levels above

10000 IU/mL continued to significantly predict HBV reactivation. Using the cut-off of 10000 IU/mL for pre-partum HBV DNA levels, it seems feasible to discriminate HBV-reactivation cases from non-reactivation cases (P = 0.004), as shown in Figure 2 and Table 1. Moreover, HBV DNA levels $\geqslant$ 10000 IU/mL independently predicted post-partum HBV infection reactivation after adjusting for the percentage (within total white blood cells) of neutrophils and lymphocytes (OR = 57.02, P = 0.033).

Five out of the 8 women with HBV reactivation initiated treatment with nucleos(t)ide analogues (three with tenofovir, one with entecavir and one with telbivudine), achieving long-term biochemical (normal ALT values) and virological (undetectable HBV DNA levels) responses. It is important to note that the remaining three women with HBV reactivation who declined treatment because of continuing lactation were followed up and had spontaneous disease remission. In 2 of these 3 women, HBV DNA decreased < 2000 IU/mL and ALT to normal levels in the following months of follow-up (36 and 24 mo, respectively), and only one had normal ALT levels and HBV DNA levels between 2000-10000 IU/mL during 24 mo of follow-up.

DISCUSSION

During pregnancy, several alterations in immune status allow women to tolerate the genetically different fetal tissues. Recently, there has been an increasing interest in the aspects and the possible mechanisms of a specific immunoregulation during pregnancy[15,16]. In general, a shift in the Th1-Th2 balance toward a Th2 response with increased amounts of regulatory T-cells is observed. This could also explain the tolerance against infectious agents, such as HBV. Pregnancy-induced endocrine and immune changes result in elevation of HBV DNA levels and normalization of liver tests between the first, second and third trimester of pregnancy in chronic HBV-infected women[17]. Moreover, the well-documented pregnancy-related plasma volume expansion and serum dilution[12,13], especially during the third trimester of pregnancy, might significantly affect serum HBV DNA levels as well as serum aminotransferase levels. Therefore, both parameters may be underestimated during late pregnancy. All these changes in immune status recover after delivery and the mother's immune system fully restores its function, a phenomenon that could be responsible for the observed post-partum exacerbation of chronic infections and autoimmune diseases[15,16].

Severe HBV reactivation cases causing fulminant liver failure during pregnancy have been reported in the literature. These cases mainly occurred in Asian HBeAg-positive or negative chronic HBV-infected pregnant women during the 2nd or the 3rd trimester of pregnancy[14], as well as in HBeAg-positive Caucasian women with high HBV DNA levels[18]. There is only one retrospective cohort study concerning the exacerbation of chronic HBV infection after delivery in a mixed population of 38 HBeAg-positive and negative chronic HBV-infected women[19]. In that study, a significant increase of liver disease activity was observed in 45% of cases after delivery, irrespective of pre-partum serum ALT or HBV DNA levels or the HBeAg status. It is important to note that 63% of the patients of the study of ter Borg *et al*[19] were HBeAg-positive and 45% were categorized in the immunotolerant phase of chronic HBV infection. Our study is the only study that specifically addresses the post-partum clinical outcome of HBeAg-negative chronic hepatitis B pregnant women. This population, characterized by lower serum HBV-DNA levels compared to their HBeAg-positive counterparts, represent the majority of chronic patients of reproductive age in Greece[20], as well as in other Mediterranean and Balkan countries. We found that approximately one-fifth of the study population (21.9%) presented undetectable serum HBV DNA levels during the third trimester of pregnancy using a sensitive PCR assay and that about one-third (34.1%) of the HBeAg-negative chronic HBV-infected pregnant women exhibited HBV DNA levels above 2000 IU/mL. These findings are consistent with previous studies in HBeAg-negative chronic HBV-infected pregnant women[11,20], among whom a considerable number of inactive carriers may exist. Distinguishing inactive carriers from chronic hepatitis B patients among the total HBeAg-negative chronic HBV-infected population is very difficult using only biochemical and virological parameters. In general, the differential diagnosis of HBeAg-negative chronic HBV-infected patients should be initially based on the combination of ALT activity and serum HBV-DNA levels. Patients who present with HBV-DNA levels above 2000 IU/mL almost always have elevated ALT values, as opposed to patients with lower HBV DNA levels who can be either inactive carriers or chronic hepatitis B cases[21]. Pregnancy-induced immune changes, pregnancy-related plasma volume expansion and serum dilution during the third trimester seem to further impair the differential diagnosis, based on virological and biochemical values. Only 2 out of 8 women with post-partum HBV reactivation had abnormal pre-partum ALT levels, whereas the majority of reactivation cases exhibited pre-partum HBV-DNA levels above 10000 IU/mL. Only one woman with HBV DNA < 2000 IU/mL and no women with undetectable pre-partum HBV DNA levels exhibited post-partum HBV reactivation.

Described early on by Rudolf Virchow, the physiologic leucocytosis of the third trimester of a normal, uncomplicated pregnancy that nor-

malized readily after delivery represents a well-known phenomenon[22-24]. Additionally, it has been reported that pregnant women present lower lymphocyte counts than non-pregnant women[25]. Lymphocyte proliferation and activation is a well-known phenomenon in patients with viral infections[26]. In our study, women who exhibited post-partum HBV reactivation had a significant difference in the percentage of neutrophils (62.3% ± 6.2% *vs* 68.8% ± 11%; $P = 0.008$) and lymphocytes (28.5% ± 5.2% *vs* 23.1% ± 9.8%; $P = 0.035$) among total white blood cells of the peripheral blood observed during the pre-partum period compared to non-reactivation cases. Moreover, the absolute lymphocyte count was comparable and the absolute neutrophil count was lower in the reactivation cases than in the non-reactivation cases. Although non-significant, this finding is most likely because of the major effect of pregnancy *per se* on the absolute neutrophil count. It may be that the level of maternal viremia affects the well-known pregnancy-induced leucocytosis, as well as the left shift in myeloid-neutrophilic lineage; a finding that needs further investigation in large scale studies.

Nevertheless, our present study has some limitations, such as the relatively small number of study subjects, of which a significant percentage were excluded from the final analysis because of either the exclusion criteria of the study or being lost to follow-up. It is well-known that being lost to follow-up frequently occurs even in large, randomized controlled trials of chronic HBV-infected pregnant women. Despite these limitations, we believe that the study population is able to represent the total HBeAg-negative chronic HBV-infected Caucasian population, prospectively examined in respect to post-partum viral reactivation, taking into account pre-partum virological, biochemical and hematological data.

In conclusion, post-partum HBV reactivation occurs in approximately 30% of HBeAg-negative chronic HBV-infected women and all events are recorded in the first semester after delivery. Pre-partum HBV DNA levels above 10000 IU/mL appear to be a significant predictor of post-partum HBV reactivation.

COMMENTS

Background

Perinatal transmission of chronic hepatitis B remains an important source of hepatitis B virus (HBV) worldwide, but the data concerning the effect of pregnancy on chronic HBV infection and HBV-related liver disease are limited. Additionally, the frequency and the timing of post-partum hepatitis B reactivation among hepatitis B e antigen (HBeAg)-negative women are not fully elucidated.

Research frontiers

During pregnancy, there is usually no deterioration of HBV-related liver disease. On the contrary, there are reports of severe HBV flares resulting in liver failure during the peripartum period, mainly in Asian women with the HBeAg-positive form of chronic HBV infection. In this study, the authors evaluate the frequency and timing of post-partum HBV reactivation, as well as its predictive factors.

Innovations and breakthroughs

Recent reports have highlighted the importance of maternal HBV DNA levels during the third trimester of pregnancy as the major risk factor for immunoprophylaxis failure. This is the first study demonstrating that post-partum HBV reactivation is observed in approximately 30% of HBeAg-negative women, all during 6 mo after delivery and mainly in women with serum HBV DNA > 10000 IU/mL during the third trimester of gestation.

Applications

The knowledge of timing and risk factors of chronic hepatitis B reactivation helps clinicians optimize the measurements of HBV DNA during pregnancy, modify the immunoprophylaxis of infants, and monitor women after delivery.

Terminology

Chronic hepatitis B may present either in HBeAg-positive or HBeAg-negative form. Without immunoprophylaxis, perinatal transmission occurs in 5% to 20% of infants born to HBeAg-negative mothers and in 70% to 90% of infants born to HBeAg-positive mothers. Maternal viral load in the third trimester is correlated with perinatal transmission.

Peer review

Data concerning post-partum reactivation of chronic HBV infection among HBeAg-negative women are rare. This study evaluated the frequency and timing of the appearance of post-partum HBV reactivation and identified its pre post-partum virological and biochemical predictors. The results will help clinicians optimize HBV management during pregnancy and identify women at risk for HBV reactivation after delivery.

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ORIGINAL ARTICLE

Observational Study

Diagnosis of early gastric cancer using narrow band imaging and acetic acid

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Abstract

AIM: To determine whether the endoscopic findings of depressed-type early gastric cancers (EGCs) could precisely predict the histological type.

METHODS: Ninety depressed-type EGCs in 72 patients were macroscopically and histologically identified. We evaluated the microvascular (MV) and mucosal surface (MS) patterns of depressed-type EGCs using magnifying endoscopy (ME) with narrow-band imaging (NBI) (NBI-ME) and ME enhanced by 1.5% acetic acid, respectively. First, depressed-type EGCs were classified according to MV pattern by NBI-ME. Subsequently, EGCs unclassified by MV pattern were classified according to MS pattern by enhanced ME (EME) images obtained from the same angle.

RESULTS: We classified the depressed-type EGCs into the following 2 MV patterns using NBI-ME: a fine-network pattern that indicated differentiated adenocarcinoma (25/25, 100%) and a corkscrew pattern that likely indicated undifferentiated adenocarcinoma (18/23, 78.3%). However, 42 of the 90 (46.7%) lesions could not be classified into MV patterns by NBI-ME. These unclassified lesions were then evaluated for MS patterns using EME, which classified 33 (81.0%) lesions as MS patterns, diagnosed as differentiated adenocarcinoma. As a result, 76 of the 90 (84.4%) lesions were matched with histological diagnoses using a combination of NBI-ME and EME.

CONCLUSION: A combination of NBI-ME and EME was useful in predicting the histological type of depressed-type EGC.

Key words: Narrow band imaging; Magnifying endoscopy; Acetic acid; Early gastric cancer; Diagnosis

Core tip: Prediction of the histological diagnosis of early gastric cancer (EGC) using endoscopy is important for determining the appropriate therapeutic approach. In

the present study, we combined magnifying endoscopy (ME) with narrow-band imaging (NBI) and enhanced ME (EME) to determine the associations between microvascular (MV) and mucosal surface (MS) patterns of depressed-type EGCs and the histological type. Indeed, 82 of the 90 lesions (91.1%) were classified according to MV or MS pattern, and 76 of the 90 lesions (84.4%) were diagnosed according to histological type. Therefore, our study suggested that the NBI-EME combination was useful for diagnosing the histological type in depressed-type EGC.

Matsuo K, Takedatsu H, Mukasa M, Sumie H, Yoshida H, Watanabe Y, Akiba J, Nakahara K, Tsuruta O, Torimura T. Diagnosis of early gastric cancer using narrow band imaging and acetic acid. *World J Gastroenterol* 2015; 21(4): 1268-1274 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1268.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1268

INTRODUCTION

Narrow-band imaging (NBI) is a video endoscopic imaging technique that enhances the display of microstructures and capillaries in the superficial mucosal layer, using narrow band filters that change the spectral feature of the observation light[1]. Microvascular (MV) patterns detected using standard magnifying endoscopy (ME) have been reported to be useful for the diagnosis of early gastric cancer (EGC)[2]. Furthermore, Nakayoshi *et al*[3] reported associations between images obtained by ME combined with NBI (NBI-ME) and histological findings. Their report concluded that the histological type of gastric cancer could be predicted using NBI-ME, which yielded clear images of the fine mucosal structure and the microvasculature of the gastric mucosa; however, endoscopic pathology by NBI-ME is not sufficient to replace conventional histology. Additional methods and techniques are necessary for predicting the histological type of EGC using endoscopy.

Enhanced-magnification endoscopy (EME) is a technique that combines ME with 1.5% acetic acid instillation. This technique was initially used to observe the specialized columnar epithelium of Barrett's esophagus[4], and it was later adopted for the assessment of gastric neoplasms[5,6]. When the epithelial surfaces are sprayed with acetic acid, they transiently whiten because of a reversible alteration in the tertiary structure of cellular proteins[5]. EME allows for the visualization of the actual villi and cryptal areas, which appear similar when observed with a stereoscopic microscope. Several studies have demonstrated that EME was useful in the diagnosis of EGC[7] and the detection of gastric cancer margins[8-10]. In contrast, only a few studies have reported on the associations between EME findings and histological diagnosis of EGC[11,12]. Lee *et al*[12] reported that the accuracy of EME in diagnosing undifferentiated adenocarcinoma seemed unsatisfactory when compared with its accuracy in diagnosing differentiated adenocarcinoma.

Therefore, in the present study, we combined NBI-ME and EME (NBI-EME combination) to determine whether the MV and mucosal surface (MS) patterns of depressed-type EGCs could precisely predict the histological type.

MATERIALS AND METHODS

Patients

The study group included 72 consecutive patients diagnosed with depressed-type EGC by four expert endoscopists at Kurume University Hospital between September 2007 and October 2011. Eighteen patients had two EGC lesions, and a total of 90 lesions were evaluated. Some of the clinical characteristics of the patients with EGC are summarized in Table 1. In all of the patients, the diagnosis of gastric cancer was based on the examination of biopsy specimens and was later confirmed by histopathology. The hospital ethics committee approved the study protocol, and all of the participating patients provided prior written informed consent.

NBI-EME combination

All of the procedures were performed using a GIF-H260Z magnifying endoscope and a CV260SL/CLV260SL endoscopic system (Olympus Medical Systems Co., Tokyo, Japan). The GIF-H260Z instrument not only maintains the capabilities of a standard videoendoscope, but it also affords a continuous range of image magnification adjustment. A black hood (MB-46, Olympus Medical Systems Co., Tokyo, Japan) was attached to the tip of the endoscope to maintain the focal distance during the procedure. The same endoscopy system settings (image enhancement mode-B8; and color enhancement mode-1) were maintained for all of the methods. For EME, 20-30 mL of 1.5% acetic acid were sprinkled onto the lesion using a syringe at low pressure, through the endoscope accessory channel. When the gastric mucosa whitened transiently, enhancing the contrast of the surface patterns, EME images were obtained from the same angle used to obtain the NBI-ME images. The shape and regularity on EME images were classified according to the form of the mucosal surface, and the width of crypt was classified by comparison with normal crypt size. All of the observations were made on optimal foci and at the highest achievable magnification ratios. Four endoscopists performed the endoscopic procedures, using a digital filing system to record the images. For each lesion, endoscopic NBI-ME and EME images were evaluated for MV and MS patterns, respectively,

Table 1 Characteristics of patients with depressed-type electrocardiograph

Characteristics	
No. of patients	72
Sex (M/F)	47/25
Tumors	90
Mean age	64 (28-89)
Size (major axis)	16.5 mm (5-52 mm)
Histological diagnosis	
Differentiated	67
Undifferentiated	23
Depth	
Mucosal	84
Submucosal	6

by four expert endoscopists.

Endoscopic treatment and histopathology

All of the EGC patients underwent endoscopic submucosal dissection (ESD) without any complications. The resected EGC specimens were then extended on boards with pins for fixation in 20% formalin. Each lesion, together with the surrounding mucosa, was cut into 2- to 5-mm-wide serial-step sections. The histologic criteria for diagnosing EGC were based on the Japanese classification of gastric carcinomas[13].

Statistical analysis

The NBI-ME/EME and histological findings were evaluated by Fisher's exact test and Pearson's χ^2 test. All of the statistical tests were two sided with a significance level of 0.05. Statistical analysis was performed using JMP software (JMP, version 10.0; SAS Institute Inc., Cary, NC, United States).

RESULTS

Association between NBI-ME findings and histological type

All of the depressed-type EGCs were macroscopically and histopathologically identified. The clinical characteristics of the patients enrolled in this study are summarized in Table 1. A total of 90 EGC lesions in 72 patients were analyzed. Histopathologically, 67 lesions (74.4%) were diagnosed as differentiated adenocarcinomas, and 23 lesions (25.6%) were undifferentiated adenocarcinomas. The depth of tumor invasion was mucosal in 93.3% (84/90) and submucosal in 6.7% (6/90). Previously reported[14] MV patterns include irregular, regular, and absent patterns (Figure 1). Table 2 shows that these regularity patterns were not associated with the differentiation of adenocarcinoma (P = 0.4174). Therefore, we used another previously reported[3] MV pattern classification for depressed-type EGCs in the present study: a fine-network pattern, a corkscrew pattern, and an unclassified pattern (Figure 2). Table 2 shows that the fine-network pattern indicated differentiated adenocarcinoma (25/25, 100%) and that the corkscrew pattern was likely to indicate undifferentiated adenocarcinoma (18/23, 78.3%). It was significantly useful for diagnosing the histological type (P < 0.001). However, 42 of the 90 (46.7%) lesions were not classifiable under any MV pattern by NBI-ME, and an unclassified pattern is incapable of predicting the histological type of depressed-type EGCs. Therefore, we considered it necessary to evaluate the unclassified MV patterns on NBI-ME images by additional methods.

Associations between ME and EME findings and histological type

To evaluate the different characteristics of unclassified MV patterns (42 cases), we investigated the associations between MS patterns evaluated by ME and EME and the differentiation of adenocarcinoma according to the following three categories: shape (Figure 3); width of crypt; and regularity. Table 3 shows that sessile barnacle (4/4, 100%) and villous type (21/22, 95.5%) shapes, narrow (16/16, 100%) and wide (9/10, 90%) crypts, and irregular (19/19, 100%) and regular (6/7, 85.7%) patterns indicated differentiated adenocarcinoma. However, there were no associations between MS patterns evaluated by ME and histological type because several lesions remained unclassified (16/42, 38.1%) by ME. Next, we used EME to evaluate MS patterns because EME is useful for visualizing the mucosal surface structure. Table 4 shows that sessile barnacle (10/10, 100%) and villous type (23/24, 95.8%) shapes, narrow (29/29, 100%) and wide (4/5, 80%) crypts, and irregular (31/31, 100%) and regular (2/3, 66.7%) patterns indicated differentiated adenocarcinoma of EGC. Thirty-four of the 42 (78.6%) lesions unclassified by NBI-ME were classified for MS patterns by EME. Therefore, 82 of the 90 (91.1%) lesions were classified into either MV or MS patterns, and 76 of the 90 (84.4%) lesions were matched with histological diagnoses. In 59 lesions, including 25 lesions classified into fine-network patterns by NBI-ME (Table 2) and 34 lesions classified into MS patterns by EME (Table 4), 58 lesions (98.3%) were adequately diagnosed as differentiated adenocarcinoma. With regard to the diagnosis of undifferentiated adenocarcinoma, there were no associations with MS patterns diagnosed by EME, although the corkscrew pattern using NBI-ME was an exception (18/23, 78.3%). With regard to the diagnosis of undifferentiated adenocarcinoma, only 4 of the 8 lesions (50%) unclassified by NBI-EME combination demonstrated undifferentiated adenocarcinoma.

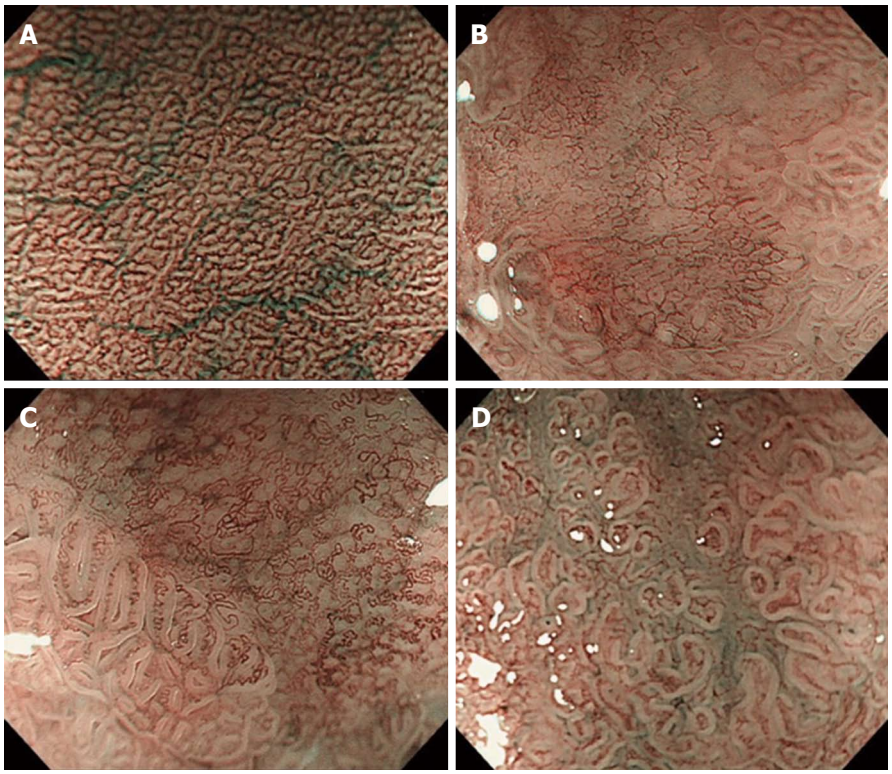

Figure 1 Representative endoscopic images of the microvascular pattern of depressed-type early gastric cancer, obtained using magnifying endoscopy combined with narrow-band imaging. A: Normal; B: Irregular pattern, C: Regular pattern, D: Absent.

Table 2 Associations between the microvascular pattern by magnifying endoscopy with narrow band imaging and histological type

	Differentiated	Undifferentiated	Total	*P* value
#1				
Irregular	50	17	67	0.4174
Regular	8	1	9	
Absent	9	5	14	
#2				
Fine network	25	0	25	< 0.001
Corkscrew	5	18	23	
Unclassified	37	5	42	

DISCUSSION

Recently, ESD treatment has been increasingly used to treat a subset of patients with EGC in Japan[15,16]. Accurate preoperative diagnosis, which includes the determination of the depth, spread of invasion, and histological findings, is critical for safe endoscopic therapy and for ensuring complete resection. Several endoscopic modalities have been developed to determine endoscopic pathology, resulting in a more accurate endoscopic diagnosis than with histological diagnosis. Early detection and accurate diagnosis of depressed-type gastric cancers have been effective in decreasing mortality because this morphological type is most predominant among all gastric cancers[17,18]. Moreover, the detection of EGCs measuring ⩽ 20 mm diameter is ideal because they are curable with minimally invasive treatment, such as endoscopic mucosal resection and ESD[19]. However, the conventional white-light imaging endoscopic approach alone is inadequate for determining an accurate diagnosis. NBI-ME is more reliable for characterizing gastric cancers and for evaluating the area of EGCs[14,20]. MV patterns, detected using standard ME and NBI-ME, are reportedly capable of predicting the histological type of gastric cancer[2,3]. Several studies have demonstrated a fine-network pattern and a corkscrew pattern specific to differentiated and undifferentiated adenocarcinoma, respectively, using NBI-ME[3,21]. The present study also demonstrated a fine-network pattern indicating differentiated adenocarcinoma (25/25, 100%) and a corkscrew pattern indicating undifferentiated adenocarcinoma (18/23, 78.3%). However, Nakayoshi *et al*[3] reported that 39 of the 165 (23.6%) lesions were not classifiable according to MV patterns. Because 46.7% of lesions remained unclassified in the present study, it was necessary to evaluate the MV patterns unclassifiable by NBI-ME using additional methods.

Several studies have reported associations between MS patterns and histological type[5,22]. Standard ME demonstrated that the depressed-type EGC had a finer-pit pattern, characterized by the destruction or disappearance of the mucosal microstructure[2]. Otsuka *et al*[22] classified MS patterns of gastric

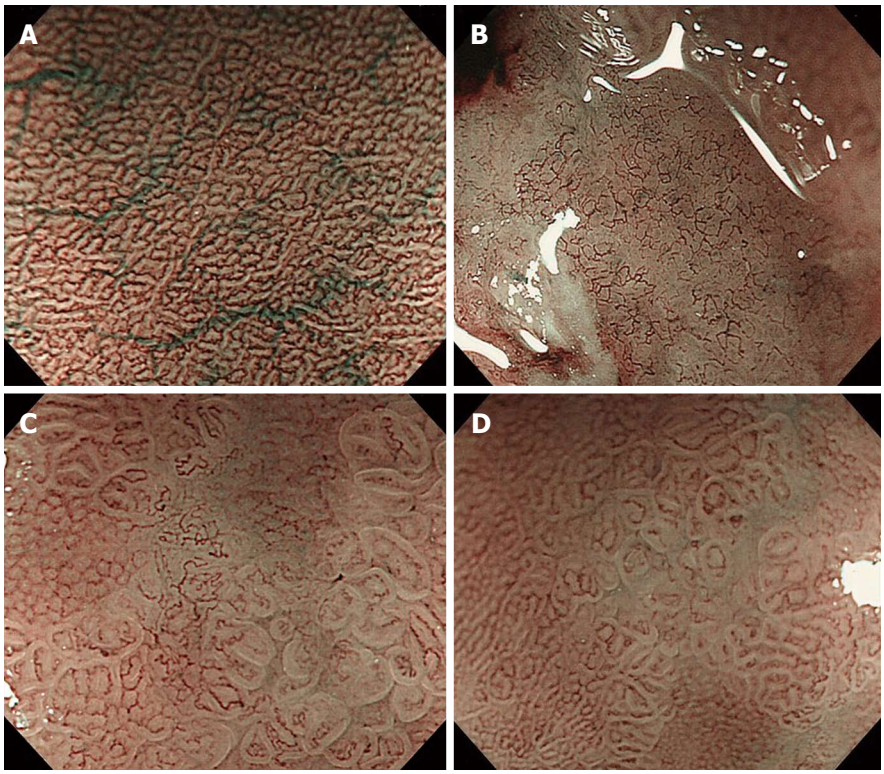

Figure 2 Representative endoscopic images of the microvascular pattern of depressed-type early gastric cancer, obtained using magnifying endoscopy combined with narrow-band imaging. A: Normal; B: Fine-network pattern; C: Corkscrew pattern; D: Unclassified pattern.

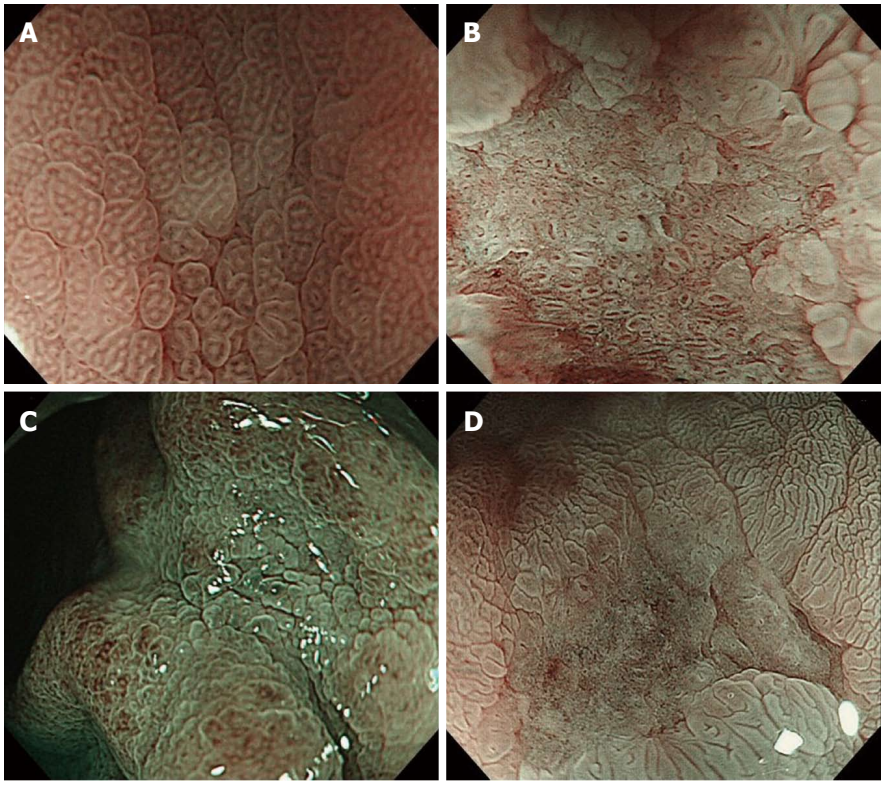

Figure 3 Representative endoscopic images of the mucosal surface pattern of depressed-type early gastric cancer, obtained with enhanced magnifying endoscopy using acetic acid staining. A: Normal; B: Sessile barnacle pattern; C: Villous pattern; D: Unclassified pattern.

Table 3 Associations between the findings of magnifying endoscopy and histological type

	Differentiated	Undifferentiated	Total	*P* value
Shape				
Sessile barnacle	4	0	4	0.1169
Villous	21	1	22	
Unclassified	12	4	16	
Width of Crypt				
Narrow	16	0	16	0.0901
Wide	9	1	10	
Unclassified	12	4	16	
Regularity				
Irregular	19	0	19	0.0735
Regular	6	1	7	
Unclassified	12	4	16	

Table 4 Associations between the findings of magnifying endoscopy with acetic acid staining and histological type

	Differentiated	Undifferentiated	Total	*P* value
Shape				
Sessile barnacle	10	0	10	0.0010
Villous	23	1	24	
Unclassified	4	4	8	
Width of Crypt				
Narrow	29	0	29	0.0005
Wide	4	1	5	
Unclassified	4	4	8	
Regularity				
Irregular	31	0	31	0.0003
Regular	2	1	3	
Unclassified	4	4	8	

cancers as evaluated by ME into the following three patterns: small and regular patterns of sulci and ridges; an irregular pattern of sulci and ridges; and a lack of visible structure. With regard to depressed-type EGCs, the latter two patterns were more frequently associated with undifferentiated adenocarcinoma (14/18), while the former pattern was more frequently associated with differentiated adenocarcinoma (22/45). However, ME was not sufficient for predicting the histological type, because 23 of 45 lesions were not adequately diagnosed by histological type. Furthermore, Tanaka *et al*[5] reported that the mucosal surface of gastric cancer, as evaluated by EME, could be characterized into five surface structure patterns: type Ⅰ (small round pits of uniform size and shape), type Ⅱ (slit-like pits), type Ⅲ (a fine villous or gyrus pattern), type Ⅳ (irregular arrangements and size of pattern types Ⅰ, Ⅱ, and Ⅲ), and type Ⅴ (destructive pattern of type Ⅰ, Ⅱ, and Ⅲ). Thirty lesions of depressed-type EGCs were characterized by two clearly recognizable surface patterns: type Ⅳ (70%, 21/30) and type Ⅴ (30%, 9/30). 8 (88.9%) of the 9 lesions with type Ⅴ patterns demonstrated undifferentiated adenocarcinoma. Although absent patterns of depressed-type EGCs were associated with undifferentiated adenocarcinoma, the diagnosis of differentiated adenocarcinoma remained unclear. In our study, 82 of the 90 (91.1%) lesions were classified into either MV or MS patterns. The decrease in unclassified lesions with the NBI-EME combination resulted in improvement in the diagnosis of differentiated adenocarcinoma. In fact, 76 of the 90 (84.4%) lesions were matched with histological diagnoses. The results of the present study suggested that the prediction of the histological type of depressed-type EGCs was more precise with the NBI-EME combination than with NBI-ME alone or EME alone.

Previous studies have reported the usefulness of NBI-ME and EME in predicting the histological type of EGC, although the diagnostic accuracy was low because many lesions remained unclassified in those studies. In the present study, most of the lesions were classified into MV and MS patterns using NBI-ME combined with EME, which showed increased diagnostic accuracy compared with that of NBI-ME alone or EME alone. Therefore, combination methods, *e.g.,* NBI-ME followed by EME, are more useful in identifying the histological type of depressed-type EGCs. Increased accuracy of the histological diagnosis of depressed-type EGCs using endoscopy is necessary for determining an appropriate therapeutic approach during the early phase of the disease.

COMMENTS

Background

The prediction of the histological diagnosis of early gastric cancer (EGC) using endoscopy is important for determining the appropriate therapeutic approach. However, an endoscopic technique for the histological diagnosis of EGC has not been completely established. In the present study, we combined magnifying endoscopy (ME) with narrow-band imaging (NBI) (NBI-ME) and enhanced ME (EME) to determine whether the microvascular (MV) and mucosal surface (MS) patterns of depressed-type EGCs could precisely predict the histological type.

Research frontiers

Previous studies reported associations between images obtained by NBI-ME and histological findings. This report concluded that the histological type of gastric cancer could be predicted using NBI-ME; however, endoscopic pathology by NBI-ME was insufficient to replace conventional histology. Furthermore, only a few studies have reported on the associations between EME findings and histological diagnosis of EGC. Research attention to this area could help to establish methods and techniques for predicting the histological type of EGC using endoscopy.

Innovations and breakthroughs

Previous studies have reported the usefulness of NBI-ME and EME in predicting the histological type of EGC, although the diagnostic accuracy was low because many lesions remained unclassified in those studies. In the present study, most of the lesions were classified into MV and MS patterns using NBI-ME combined with EME, which showed increased diagnostic accuracy compared to that of NBI-ME alone or EME alone. Therefore, combination methods, *e.g.,* NBI-ME followed by EME, were more useful in identifying the histological type of depressed-type EGC.

Applications

The study results suggested that the increased accuracy of histological diagnosis of depressed-type EGCs using the combination of NBI-ME and EME was necessary to determine the appropriate therapeutic approach during the early phases of gastric cancer.

Terminology

NBI is a video endoscopic imaging technique that enhances the display of the microstructures and capillaries in the superficial mucosal layer using narrow band filters that change the spectral features of the observation light. EME is a technique that combines magnification endoscopy with 1.5% acetic acid instillation. EME allows for the visualization of the actual villi and cryptal areas, which appear similar when observed with a stereoscopic microscope.

Peer review

Matsuo *et al* presented an interesting paper concerning the combination of NBI-ME and EME in the diagnosis of early gastric cancer. In fact, as we can see in previous reports, either of the methods could be applied to predict the histological type of lesion; however, both of them lacked sufficient accuracy when the samples were finally assessed by histological methods. In the current study, the authors attempted to increase the accuracy further by combining both of the above methods, and the results indicated that 84.4% of lesions were finally matched with histological diagnoses.

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ORIGINAL ARTICLE

Observational Study

Prognostic value of *KRAS* and *BRAF* mutations in curatively resected colorectal cancer

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Abstract

AIM: To investigate the prognostic role of *KRAS* and *BRAF* mutations after adjustment for microsatellite instability (MSI) status in Japanese colorectal cancer (CRC) population.

METHODS: We assessed *KRAS* and *BRAF* mutations and MSI status in 813 Japanese patients with curatively resected, stage Ⅰ-Ⅲ CRC and examined associations of these mutations with disease-free survival (DFS) and overall survival (OS) using uni- and multivariate Cox proportional hazards models.

RESULTS: *KRAS* and *BRAF* mutations were detected in 312 (38%) of 812 and 40 (5%) of 811 tumors, respectively. *KRAS* mutations occurred more frequently in females than in males ($P = 0.02$), while the presence of *BRAF* mutations was significantly associated with the female gender ($P = 0.006$), proximal tumor location ($P < 0.001$), mucinous or poorly differentiated histology ($P < 0.001$), and MSI-high tumors ($P < 0.001$). After adjusting for relevant variables, including MSI status, *KRAS* mutations were associated with poorer DFS (HR = 1.35; 95%CI: 1.03-1.75) and OS (HR = 1.46; 95%CI: 1.09-1.97). *BRAF* mutations were poor prognostic factors for DFS (HR = 2.20; 95%CI: 1.19-4.06) and OS (HR = 2.30; 95%CI: 1.15-4.71). Neither the *BRAF* by MSI interaction test nor the *KRAS* by MSI interaction test yielded statistically significant results for DFS and OS.

CONCLUSION: *KRAS* and *BRAF* mutations are associated with inferior survival, independent of MSI status, in

Japanese patients with curatively resected CRC.

Key words: Colorectal cancer; *KRAS*; *BRAF*; Microsatellite instability; Prognostic factor

Core tip: Although *KRAS* and *BRAF* mutations play a critical role in colorectal cancer development, little is known regarding the prognostic role of these genetic alterations after adjustment for microsatellite instability status in Asian populations. To the authors' knowledge, the current study is the first large-scale study to clarify the impact of *KRAS* and *BRAF* mutations on the survival outcomes of colorectal cancer in Asian populations. We found that *KRAS* and *BRAF* mutations were separately associated with inferior disease-free survival and overall survival, independent of microsatellite instability status, in patients with curatively resected colorectal cancer.

Kadowaki S, Kakuta M, Takahashi S, Takahashi A, Arai Y, Nishimura Y, Yatsuoka T, Ooki A, Yamaguchi K, Matsuo K, Muro K, Akagi K. Prognostic value of *KRAS* and *BRAF* mutations in curatively resected colorectal cancer. *World J Gastroenterol* 2015; 21(4): 1275-1283 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1275.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1275

INTRODUCTION

Colorectal cancer (CRC) develops through diverse mechanisms such as chromosomal instability (CIN), microsatellite instability (MSI), and epigenetic DNA promoter methylation [CpG island methylator phenotype (CIMP)][1]. CIMP and MSI-high (MSI-H) phenotypes are closely associated. Most sporadic MSI-H tumors develop through CIMP-associated methylation of *MLH1*, and *BRAF* mutations occur frequently in both phenotypes[2,3]. *KRAS* mutations mainly occur in CIN and are partly associated with intermediate CIMP epigenotype[4]. *KRAS* and *BRAF* mutations are mutually exclusive; both cause RAS/RAF/MAPK signaling pathway upregulation and are crucial in CRC development.

KRAS encodes a guanosine triphosphate/guanosine diphosphate binding protein; *KRAS* mutations are observed in approximately 30%-40% CRCs[5-8]. *KRAS* mutations are well known as predictive markers of resistance to epidermal growth factor receptor-targeted antibodies in metastatic CRC, but their prognostic value remains controversial. Some studies have shown that *KRAS* mutations are associated with poorer survival in CRC[8,9], while others found no association[6,7].

BRAF encodes a serine/threonine protein kinase, a downstream effector of the KRAS protein. Activating *BRAF* mutations occur in approximately 4%-20% CRCs[6,10-14], with the vast majority being the V600E hotspot mutation. Although some previous studies have shown that *BRAF* mutations confer poorer prognosis in CRC[10-12], others have not[6,13], probably because of associations with favorable MSI-H CRC prognosis[15-17].

Although genetic background and geographical factors may influence mutation frequency and prognosis, most reports are from Western countries; less data are available regarding the prognostic role of *KRAS* and *BRAF* mutations in Asian populations. Two independent studies from Taiwan and Japan have been published recently. However, both had a small sample size and heterogeneous cohorts including metastatic disease; the study from Taiwan did not examine MSI status[14,18]. Hence, a large homogenous cohort with MSI status is essential for assessing the prognostic value of various clinical or molecular variables in CRC. Here, we clarified associations of *KRAS* and *BRAF* mutations and MSI status with survival outcomes in a larger Japanese cohort of patients with curatively resected CRC.

MATERIALS AND METHODS

Patients and tissue samples

A total of 813 consecutive stage Ⅰ-Ⅲ CRC patients undergoing curative resection at Saitama Cancer Center between July 1999 and May 2006 were included. Written informed consent was obtained from all patients. Patients with the following conditions were excluded: (1) history of radiotherapy or chemotherapy preoperatively; (2) inflammatory bowel disease; or (3) history of familial adenomatous polyposis. Pathological staging was performed according to the tumor, node, and metastasis (TNM) classification system (6th edition)[19]. CRCs were typically divided into 3 types: rectum, distal colon (splenic flexure and descending and sigmoid colon), and proximal colon (cecum and ascending and transverse colon). Adjuvant chemotherapy was administered to 40% (129/322) and 76% (232/307) of stage Ⅱ and Ⅲ CRC patients, respectively. Among 361 patients treated with adjuvant chemotherapy, only 10 patients received combination chemotherapy with 5-fluorouracil, leucovorin, and oxaliplatin, while remaining were treated with single-agent fluoropyrimidines. Patients were followed-up until death or February 2012, whichever came first. We obtained approval from the Ethics Committee of Saitama Cancer Center.

Genomic DNA extraction and KRAS and BRAF mutation analysis

Primary CRCs and paired healthy colorectal mucosa obtained perioperatively were immediately frozen at -80 ℃ until analysis. Genomic DNA was extracted

from fresh frozen specimens using the standard phenol-chloroform extraction method. Exons 2 and 3 of *KRAS* were examined for mutations by denaturing gradient gel electrophoresis, as described previously[20]. The *BRAF* V600E mutation was detected using PCR and restriction enzyme digestion, as described previously[21].

MSI analysis

MSI analysis was performed using fluorescence-based PCR, as described previously[22]. Five Bethesda markers BAT25, BAT26, D5S346, D2S123, and D17S250 were used to classify tumor MSI status. MSI status was graded as MSI-H with 2 or more unstable markers, MSI-low (MSI-L) with only 1 unstable marker, and microsatellite-stable (MSS) with no unstable marker. MSI-positive markers were re-examined at least twice to confirm the result.

Statistical analysis

The aim of this study was to evaluate the impact of *KRAS*/*BRAF* mutations on prognosis in patients with resected CRC. Prognosis was evaluated according to 2 measures: overall survival (OS) and disease-free survival (DFS). OS was defined as the interval from the date of resection until death due to any cause or until the censor date of February 1, 2012. DFS was defined as the time from the date of resection to tumor recurrence, occurrence of a new primary colorectal tumor, or death due to any cause. Survival probability was estimated using the Kaplan-Meier method and compared using the log-rank test. Cox proportional hazards models were used to estimate uni- and multivariate adjusted hazard ratios for DFS and OS according to mutation status. Factors for which the multivariate models were adjusted are age (≥ 65 *vs* < 65), gender (male *vs* female), tumor stage (Ⅲ *vs* Ⅱ *vs* Ⅰ), adjuvant chemotherapy (Yes *vs* No), and status of MSI and *BRAF* or *KRAS* mutations (Yes *vs* No). To further evaluate the potential heterogeneity of the impact of *KRAS* and *BRAF* mutations according to MSI status and other covariates [age (≥ 65 *vs* < 65), gender (male *vs* female), tumor location (distal/rectum *vs* proximal), and stage (Ⅲ *vs* Ⅰ/Ⅱ)], we tested the models that included interaction terms, cross-products of gene mutation status, and another variable of interest in a multivariate Cox model. The likelihood ratio test was performed to determine the significance of the results.

Clinicopathological factor distribution according to gene mutation status was assessed using the χ^2 or Fisher's exact tests for categorical variables, when appropriate, and Student's *t*-test for continuous variables. All statistical analyses were performed using Dr. SPSS Ⅱ software (SPSS Japan Inc., Tokyo, Japan); 2-sided $P < 0.05$ was considered statistically significant.

RESULTS

Clinicopathological characteristics of KRAS and BRAF mutant tumors

Patient characteristics according to *KRAS* or *BRAF* status are summarized in Table 1. MSI status was determined in all cases, whereas mutation status was not determined in 1 case for *KRAS* and 2 for *BRAF*. *KRAS* or *BRAF* mutations were detected in 38% (312/812) and 5% (40/811) of cases, respectively. Only 1 patient harbored *KRAS* and *BRAF* mutations. *KRAS* mutations were more frequent in females than in males (43% *vs* 35%, $P = 0.02$). *BRAF* mutations were significantly more frequent in females than in males (7% *vs* 3%, $P = 0.006$), proximal than in distal or rectal tumors (13% *vs* 1% *vs* 2%, $P < 0.001$), mucinous or poorly differentiated tumors than in moderately or well-differentiated tumors (17% *vs* 4%, $P < 0.001$), and MSI-H tumors than in MSS/MSI-L tumors (36% *vs* 2%, $P < 0.001$).

Survival analysis

The median follow-up time was 87.7 mo (range: 13-148 mo). Based on univariate Cox proportional hazard analysis results (Table 2), greater age (≥ 65), male gender, advanced TNM stage, and presence of *KRAS* mutations were significantly associated with poor prognosis for DFS and OS. For *KRAS* mutant *vs KRAS* wild-type tumors, 5-year DFS was 71% *vs* 77% (log-rank $P = 0.02$; Figure 1A); 5-year OS was 80% *vs* 84%, respectively (log-rank $P = 0.01$; Figure 1B). Presence of *BRAF* mutations was not significantly associated with poorer DFS and OS in the entire cohort. For *BRAF* mutant *vs* wild-type tumors, 5-year DFS was 70% *vs* 75% (log-rank $P = 0.23$; Figure 1C); 5-year OS was 77% *vs* 83% (log-rank $P = 0.11$; Figure 1D), respectively.

In multivariate analysis, adjusting for potential prognostic variables, *KRAS* retained its prognostic impact on DFS (HR = 1.35; 95%CI: 1.03-1.75) and OS (HR = 1.46; 95%CI: 1.09-1.97; Table 3). Presence of *BRAF* mutations was significantly associated with poorer DFS (HR = 2.20; 95%CI: 1.19-4.06) and OS (HR = 2.30; 95%CI: 1.15-4.71) after adjustment (Table 3).

Survival analysis stratified by MSI status

Given the potential prognostic effect of MSI status, we evaluated interactions of *KRAS* or *BRAF* mutations with MSI status (Table 4). The effect of *KRAS* mutations on DFS and OS was limited to patients with MSS/MSI-L tumors (HR = 1.37; 95%CI: 1.05-1.80; HR = 1.49; 95%CI: 1.10-2.02, respectively); however, the *KRAS* by MSI interaction test was not significant ($P = 0.95$ and 0.70, respectively). *BRAF* mutations were significantly associated with reduced OS (HR = 2.74; 95%CI: 1.19-6.30) in MSS/MSI-L, but not MSI-H, tumors.

Table 1 Patient characteristics according to *BRAF* or *KRAS* status *n* (%)

Characteristics	KRAS status			BRAF status		
	Wild-type *n* = 500	Mutant *n* = 312	*P* value	Wild-type *n* = 771	Mutant *n* = 40	*P* value
Age (yr)			0.11			0.40
mean ± SD	63.5 ± 10.3	64.7 ± 10.3		63.9 ± 10.3	65.4 ± 11.6	
Gender			0.02			0.006
Male	308 (62)	166 (53)		459 (60)	15 (38)	
Female	192 (38)	146 (47)		312 (40)	25 (63)	
Tumor location			0.37			< 0.001
Proximal	134 (27)	98 (31)		201 (26)	31 (78)	
Distal	213 (43)	125 (40)		332 (43)	5 (13)	
Rectum	153 (31)	89 (29)		238 (31)	4 (10)	
Histological grade			0.24			< 0.001
Well/moderate	472 (94)	288 (92)		728 (94)	31 (78)	
Poor/mucinous	28 (6)	24 (8)		43 (6)	9 (23)	
T stage			0.12			0.89
1	52 (10)	31 (10)		79 (10)	4 (10)	
2	106 (21)	46 (15)		145 (19)	7 (18)	
3	286 (57)	200 (64)		462 (60)	23 (58)	
4	56 (11)	35 (11)		85 (11)	6 (15)	
LN metastasis			0.18			0.96
Yes	180 (36)	127 (41)		292 (38)	15 (38)	
No	320 (64)	185 (59)		479 (62)	25 (63)	
TNM stage			0.09			0.92
Ⅰ	125 (25)	58 (19)		173 (22)	10 (25)	
Ⅱ	195 (39)	127 (41)		306 (40)	15 (38)	
Ⅲ	180 (36)	127 (41)		292 (38)	15 (38)	
Adjuvant chemotherapy			0.44			0.57
Yes	217 (43)	144 (46)		344 (45)	16 (40)	
No	283 (57)	168 (54)		427 (55)	24 (60)	
MSI status			0.33			< 0.001
MSS/MSI-L	455 (91)	290 (93)		728 (94)	16 (40)	
MSI-H	45 (9)	22 (7)		43 (6)	24 (60)	

SD: Standard deviation; LN: Lymph node; TNM: Tumor-Node-Metastasis; MSI: Microsatellite instability; MSS: Microsatellite stable; MSI-L: Microsatellite instability-low; MSI-H: Microsatellite instability-high.

Table 2 Univariate prognostic analysis of disease-free survival and overall survival

Characteristics	Disease-free survival		Overall survival	
	HR	95%CI	HR	95%CI
Age (yr)				
< 65	1	Reference	1	Reference
⩾ 65	1.73	1.35-2.28	2.21	1.64-2.98
Gender				
Female	1	Reference	1	Reference
Male	1.57	1.20-2.06	1.57	1.16-2.13
Tumor location				
Proximal	1	Reference	1	Reference
Distal	0.92	0.67-1.25	0.9	0.64-1.26
Rectum	1.17	0.85-1.62	0.97	0.67-1.40
Histological grade				
Well/moderate	1	Reference	1	Reference
Poor/mucinous	1.53	0.97-2.42	1.43	0.84-2.42
AJCC stage				
Ⅰ	1	Reference	1	Reference
Ⅱ	2.6	1.61-4.19	2.26	1.36-3.75
Ⅲ	4.68	2.95-7.42	3.49	2.14-5.70
Adjuvant chemotherapy				
No	1	Reference	1	Reference
Yes	1.24	0.96-1.60	1.29	1.10-1.51
MSI				
MSS/MSI-L	1	Reference	1	Reference
MSI-H	0.71	0.42-1.20	0.92	0.54-1.59
KRAS				
Wild-type	1	Reference	1	Reference
Mutant	1.35	1.04-1.74	1.44	1.08-1.92
BRAF				
Wild-type	1	Reference	1	Reference
Mutant	1.38	0.82-2.32	1.57	0.90-2.76

HR: Hazard ratio; CI: Confidence interval; MSI: Microsatellite instability; MSS: Microsatellite stable; MSI-L: Microsatellite instability-low; MSI-H: Microsatellite instability-high.

However, the *BRAF* by MSI interaction test did not reach statistical significance ($P = 0.44$).

Survival analysis stratified by other potential variables

We also analyzed the prognostic value of *KRAS* and *BRAF* mutations for OS across strata of other potential prognostic factors (Figure 2). The prognostic effect of *KRAS* mutations appeared to be consistent across potential variables, and interactions between *KRAS* status and these factors were not significant. In contrast, *BRAF* mutations were significantly associated with poor OS in stage Ⅲ, but not stage Ⅰ-Ⅱ, disease. Interactions between *BRAF* status and TNM stage showed suggestive statistical significance

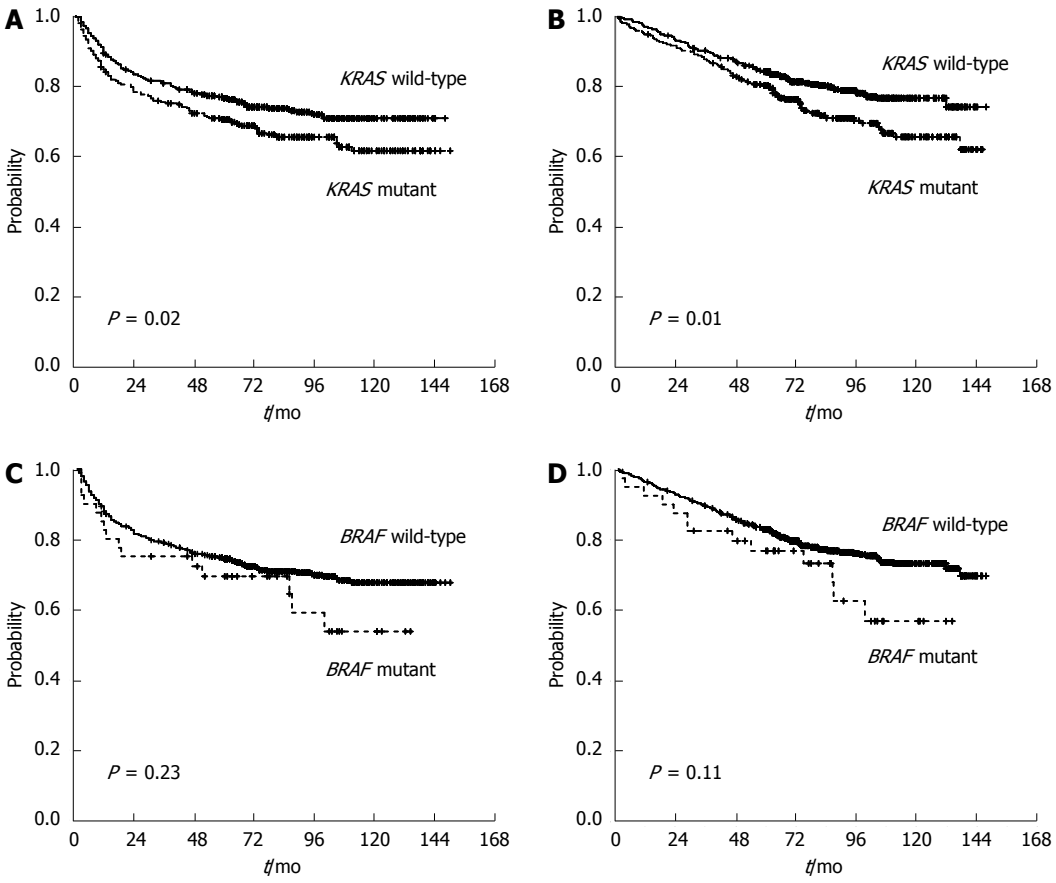

Figure 1 Kaplan-Meier curves for disease-free survival and overall survival according to *KRAS* or *BRAF* status. A: Disease-free survival (DFS) according to *KRAS* status; B: Overall survival (OS) according to *KRAS* status; C: DFS according to *BRAF* status; D: OS according to *BRAF* status.

Table 3 Prognostic effects of microsatellite instability, *KRAS*, and *BRAF* status in Cox proportional models

	Disease-free survival[1]		Overall survival[1]	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
MSI				
MSS/MSI-L	1 (reference)	0.14	1 (reference)	0.53
MSI-H	0.64 (0.35-1.16)		0.81 (0.42-1.56)	
KRAS				
Wild-type	1 (reference)	0.03	1 (reference)	0.01
Mutant	1.35 (1.03-1.75)		1.46 (1.09-1.97)	
BRAF				
Wild-type	1 (reference)	0.01	1 (reference)	0.02
Mutant	2.20 (1.19-4.06)		2.30 (1.15-4.71)	

[1]Covariates include age (< 65 or ≥ 65), gender, AJCC stage (Ⅰ/Ⅱ/Ⅲ), adjuvant chemotherapy (Yes/No), and MSI, *KRAS*, and *BRAF* status. CI: Confidence interval; MSI: Microsatellite instability; MSS: Microsatellite stable; MSI-L: Microsatellite instability-low; MSI-H: Microsatellite instability-high.

Table 4 Prognostic Effects of *KRAS* and *BRAF* mutations according to microsatellite instability status

	KRAS		*BRAF*	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
DFS[1]				
MSS/MSI-L	1.37 (1.05-1.80)	0.95	2.06 (0.96-4.43)	0.91
MSI-H	1.34 (0.34-5.24)		2.46 (0.49-12.4)	
OS[1]				
MSS/MSI-L	1.49 (1.10-2.02)	0.70	2.74 (1.19-6.30)	0.44
MSI-H	1.39 (0.33-5.78)		1.18 (0.23-6.02)	

[1]Covariates include age, gender, AJCC stage (Ⅰ-Ⅱ/Ⅲ), adjuvant chemotherapy, and *KRAS* and *BRAF* status. HR: Hazard ratio; CI: Confidence interval; DFS: Disease-free survival; OS: Overall survival; MSI: Microsatellite instability; MSS: Microsatellite stable; MSI-L: Microsatellite instability-low; MSI-H: Microsatellite instability-high.

(*P* = 0.10).

DISCUSSION

To our knowledge, this is the largest study to assess the prognostic value of *KRAS* and *BRAF* mutations for survival outcomes in CRC patients in Asian populations. Tumor specimens were prospectively collected from patients with curatively resected CRC (stage Ⅰ-Ⅲ); *KRAS* and *BRAF* mutations and MSI status were analyzed using a consistent methodology at a single institution. *KRAS* and *BRAF* mutations were associated with poor prognosis, independent of MSI status.

Many studies have examined associations of *KRAS* mutations with various clinical features, with no consistent results[5-8]. *KRAS* mutations were more frequent in females; however, these mutations were not associated with any other clinical variable.

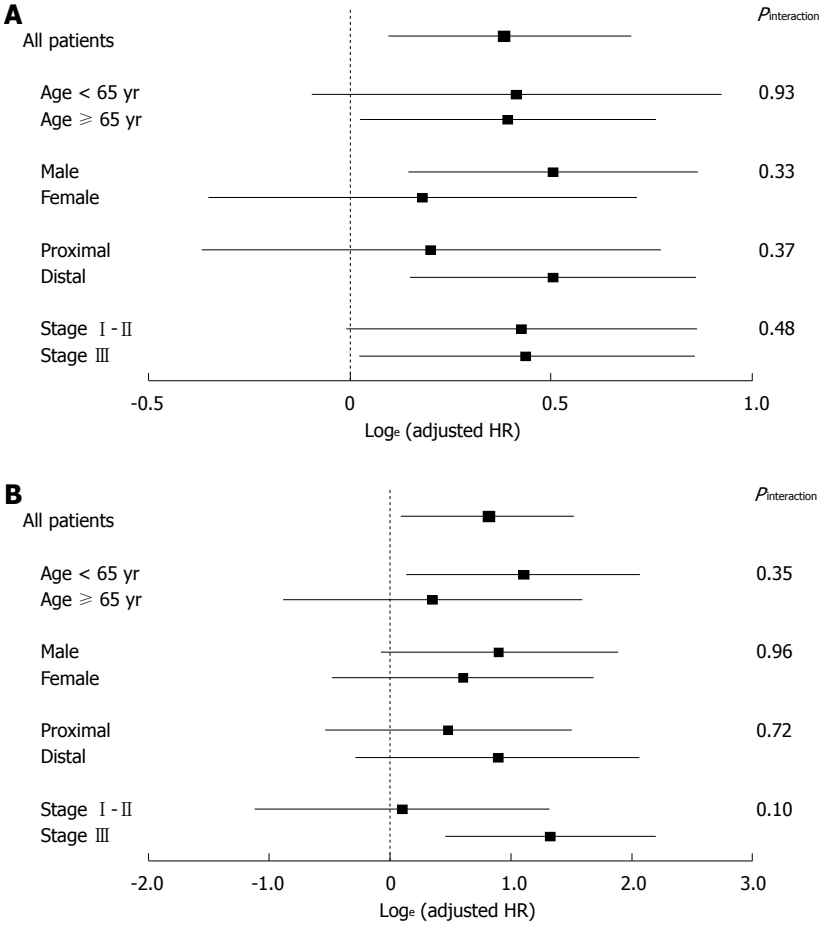

Figure 2 Stratified analysis of *KRAS* or *BRAF* status and overall survival. Loge [adjusted hazard ratio (HR)] and 95%CI for *BRAF* and *KRAS* mutant tumors (*vs* wild-type tumors) in various strata are shown. A: *KRAS* mutant tumors; B: *BRAF* mutant tumors.

Similarly, Watanabe *et al*[5] found relationships of *KRAS* mutations with the female gender and older age. In contrast, the Kirsten Ras Colorectal Cancer Collaborative Group study (RASCAL) demonstrated that *KRAS* mutations were associated with histological grade but no other variables[8]. In analysis of the PETACC-3 trial, Roth *et al*[6] reported associations of *KRAS* mutations with histological grade and tumor location but not gender. Such inconsistencies may be attributed to differences in the distribution of age, race, stage, or other factors among subject groups.

Currently, no convincing evidence exists that *KRAS* mutations are independent prognostic factors in CRC. In a Taiwanese study by Liou *et al*[14], KRAS mutations were not associated with inferior OS; however, the magnitude of multivariate HR (HR = 1.61; 95%CI: 0.91-2.84) was of the same order as that in the present study. A study from Japan revealed that the prognostic impact of *KRAS* mutations on recurrence-free survival was limited in patients with stage Ⅱ CRC, and the association of *KRAS* mutations with OS was not observed[18]. Both studies had a small sample size and heterogeneous cohorts, including stage Ⅳ disease. In the large homogeneous cohort in this study, we found significant association of *KRAS* mutations with inferior DFS and OS. Because we previously found no difference in survival outcomes among different *KRAS* mutations, including those in exons 2, 3, and 4[23], prognostic analyses of specific codons for these mutations were not performed in the present study. Similarly, the RASCAL study indicated that *KRAS* mutations resulted in overall poorer prognosis[8], whereas subsequent analysis (RASCAL Ⅱ) showed that only the glycine to valine substitution in codon 12 (G12V) was associated with poor prognosis in patients with Dukes' C disease[24]. Furthermore, recent randomized phase Ⅲ trial results supported *KRAS* mutations as prognostic factors; 3-year DFS ranged from 72% to 75% across treatments for *KRAS* wild-type tumors, with 65% to 67% for *KRAS* mutant tumors[25]. In contrast, in the PETACC-3 trial, no association was found between *KRAS* mutations and poorer relapse-free survival or OS[6]. Although further research of the prognostic effect of *KRAS* mutations is needed, the influence of these mutations seems to be mild across previously reported studies.

The frequency of *BRAF* mutations (5%) and

MSI-H (8%) in our cohort was lower than that in Western populations (*BRAF*: 8%-20%, MSI-H: 11%-17%)[6,9,11-13,15,16] and comparable with that in Asian populations (*BRAF*: 4%-7%, MSI-H: 6%-12%)[14,18,26]. Generally, *BRAF* mutations and MSI-H are frequently observed in females, proximal tumors, and poorly differentiated tumors. In a systematic review including 9885 CRC patients, a *BRAF* mutation was associated with a proximal tumor location, poor differentiation, and female sex[27]. Consistent with this observation, *BRAF* mutations were more frequent in proximal tumors, poorly differentiated tumors, and females. Previous Western cohorts showed more patients with proximal and poorly differentiated tumors compared with Asian cohorts, including the current cohort. Thus, the discrepancy in *BRAF* mutations and MSI-H status between Western and Asian populations may be attributed to the different distribution of patients' characteristics such as gender, tumor location, histological grade, or racial and/or environmental differences.

Most previous studies found associations of *BRAF* mutations with poorer survival[6,10-12]. In meta-analysis of 26 independent studies (11773 patients), *BRAF* mutations increased the risk of mortality in CRC patients (HR = 2.25; 95%CI: 1.82-2.83)[28]. However, this evidence is mainly based on studies in Western populations; little is known regarding the prognostic role of *BRAF* mutations in Asian populations. In a Taiwanese study[14], *BRAF* mutations were associated with reduced OS, but MSI status was not estimated. In a Japanese study, Nakanishi *et al*[18] found no such association because of the insufficient number of patients with *BRAF* mutations. In the present study with larger sample size and homogeneous cohorts, we found associations of *BRAF* mutations with poorer DFS and OS in CRC patients with stage I-Ⅲ disease, with the same order of magnitude of HR for OS as in the above meta-analysis. The prognostic effect of *BRAF* mutations on survival seems to be even stronger than that of *KRAS* mutations.

In contrast to previous reports[6,9,15-17], our analysis did not show that patients with MSI-H tumors exhibited better survival than those with MSS/MSI-L tumors. However, the number of patients with MSI-H tumors was too small to draw meaningful conclusions regarding the prognostic effect of MSI status. Therefore, additional larger studies are needed to clarify the prognostic impact of MSI status. Inconsistent results were reported regarding the prognostic effect of *BRAF* mutations according to MSI status[6,10,13]. Samowitz *et al*[10] found associations of BRAF mutations with poor survival in MSS/MSI-L, but not MSI-H tumors. Meanwhile, French *et al*[13] reported associations of *BRAF* mutations with poor survival in MSI-H tumors. In our analysis, associations of *BRAF* mutations with reduced OS were limited in MSS/MSI-L tumors. However, the *BRAF* by MSI interaction test was not significant; statistical power was considerably limited due to the small number of patients with MSI-H and *BRAF* mutant tumors. Larger studies are needed to clarify the modifying effect on the relation between *BRAF* mutations and survival outcome according to MSI status. Advantages of this study include comprehensive analysis of molecular markers using consistent methodology at a single institution, large sample size, and homogeneous cohort of Japanese patients. These results suggest that constitutive activation of the RAS/RAF/MAPK signaling pathway may be closely associated with clinical prognosis in CRC. Prognostic effects of *KRAS* and *BRAF* mutations seem to be consistent across most strata of clinical variables, while the adverse effect of *BRAF* mutations on OS may be attenuated in stage Ⅰ-Ⅱ CRC patients, with marginal statistical significance. The interaction of *BRAF* mutations with tumor stage warrants further research.

In conclusion, we found that Japanese CRC patients with *KRAS* or *BRAF* mutations have poorer survival, independent of MSI status. Additional investigations are warranted to clarify the interaction between these mutations and potential relevant factors, such as MSI status and tumor stage.

COMMENTS

Background

KRAS and *BRAF* mutations occur in 30%-40% and 4%-20% of colorectal cancers (CRCs), respectively. Microsatellite instability (MSI) is characterized by inactivation of the DNA mismatch repair system and is observed in 5%-15% of CRCs. MSI-high tumors are less likely to metastasize compared with the other phenotypes and have favorable survival outcomes. *KRAS* mutations are well known as predictive markers of resistance to epidermal growth factor receptor-targeted antibodies, and *BRAF* mutations are of current interest as a therapeutic target in metastatic CRCs. However, their prognostic value remains controversial for patients with curatively resected CRCs.

Research frontiers

Most previous studies investigating the prognostic role of *KRAS* and *BRAF* mutations in CRCs are from Western countries. Genetic background and geographical factors may influence mutation frequency and prognosis; however, few data are available regarding the prognostic role of these genetic alterations in Asian populations. Thus, clinical implications will be obtained by assessing the prognostic value of these mutations in a large cohort of CRCs in Japan, after adjustment for MSI status.

Innovations and breakthroughs

This study is the first large-scale study to demonstrate the prognostic impact of *KRAS* and *BRAF* mutations in Asian populations. After adjustment for relevant factors, including MSI, *KRAS* and *BRAF* mutations were independently associated with inferior disease-free survival and overall survival in patients with curatively resected CRCs. These findings will offer new insight into prognostic role of *KRAS* and *BRAF* mutations in CRCs.

Applications

BRAF and *KRAS* mutations may be useful as molecular markers for stratification of the clinical prognosis of curatively resected CRCs. Further investigation on whether the prognostic impact of *KRAS* and *BRAF* mutations could be modified by MSI status may provide more precise stratification of clinical outcomes in CRC.

Terminology

The protein product of the *KRAS* gene is a guanosine triphosphate/guanosine diphosphate-binding protein, and *KRAS* mutations play a key role in the development of various malignancies, including lung cancer, pancreatic cancer, and CRC. The protein product of the *BRAF* gene, a protein called B-Raf, is a serine/threonine protein kinase serving as downstream effector of the KRAS protein. *BRAF* mutations are involved in the development of many malignancies, *e.g.*, malignant melanoma, papillary thyroid cancer, and CRC.

Peer review

This is well written and illustrated paper. The authors investigate the prognostic role of *KRAS* and *BRAF* mutations after adjustment for MSI status. And they demonstrated that *KRAS* and *BRAF* mutations are associated with inferior survival, independent of MSI status in Asian colorectal cancer population. As the authors mentioned, in contrast to previous reports, their analysis did not show that patients with MSI-high tumors exhibited better survival than those with microsatellite-stable/MSI-low tumors.

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ORIGINAL ARTICLE

Prospective Study

Vitamin D status and viral response to therapy in hepatitis C infected children

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Abstract

AIM: To study the frequency of vitamin D deficiency in patients with hepatitis C virus (HCV) infection and to evaluate the role of vitamin D supplementation in improving antiviral therapy.

METHODS: Sixty-six children aged from 7-14 years (mean ± SD, 11.17 ± 2.293) diagnosed with HCV infection were matched to 28 healthy controls. Serum levels of 25 (OH) D3, calcium, phosphorus, alkaline phosphatase and plasma level of parathormone were measured. Quantitative PCR for HCV was performed Bone density was determined by dual energy X-ray absorptiometry. All cases received conventional therapy, and only 33 patients received vitamin D supplementation.

RESULTS: Children with HCV showed significantly increased levels of HCV RNA ($P < 0.001$), parathormone ($P < 0.01$) and decreased vitamin D levels ($P < 0.05$) (33.3% deficient and 43.3% insufficient) compared with controls. Abnormal bone status (Z score -1.98 ± 0.75) was found in ribs, L-spine, pelvis and total body. Cases treated with vitamin D showed significant higher early ($P < 0.04$) and sustained ($P < 0.05$) virological response. There was a high frequency of vitamin D deficiency among the Egyptian HCV children, with significant decrease in bone density. The vitamin D level should be assessed before the start of antiviral treatment with the correction of any detected deficiency.

CONCLUSION: Adding vitamin D to conventional Peg/RBV therapy significantly improved the virological response and helped to prevent the risk of emerging bone fragility.

Key words: Vitamin D; Viral response; Hepatitis C; Children

Core tip: Does vitamin D supplementation improves the viral response in hepatitis C infection? Previous studies raised the possibility that disease progression is associated with higher levels of vitamin D, and thus vitamin D supplementation does not have a role in chronic hepatitis C patients. This study aimed to investigate the frequency of vitamin D deficiency

among Egyptian hepatitis C virus-infected children, with assessment of bone status by measuring calcium, parathormone and alkaline phosphatase levels, and bone mineral density and to evaluate the role of vitamin D supplementation in improving the viral response of these patients.

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INTRODUCTION

Hepatitis C virus (HCV) infection remains an evolving cause of morbidity and mortality worldwide. Despite limited epidemiological data, a higher prevalence is found in Eastern Europe and in the Middle East[1]. Egypt has the highest prevalence of HCV infection; about 9% country wide and up to 50% in certain rural areas[2].

Vitamin Dis a potent immunomodulator that improves insulin sensitivity, suppresses proinflammatory cytokines, increases anti-inflammatory cytokines, and improves CD4 T cell hyper-responsiveness[3]. Recently, specific vitamin D receptors (VDRs) were observed to be expressed in liver cells and this expression of VDRs is reduced in chronic Hepatitis C patients. In addition, an inverse relationship between the liver VDR expression and inflammation severity was observed[4,5].

Abnormal bone metabolism, vitamin D axis, calcium (Ca) and parathormone (PTH) dysfunction have been reported in cholestatic children[6] with the disturbance in vitamin D metabolism, gonads, or chronic inflammation[7]. The role of chronic HCV infection in osteoporosis is supported with the decreased fracture risk in HCV children with successful antiviral treatment[8]. However, other studies demonstrated osteosclerosis in HCV infection[9].

The relation between vitamin D and the antiviral therapy response remains unclear, with previous studies raising the possibility of inconsistent results and failure of the reference methodology[10].

The present study aimed to investigate the frequency of vitamin D deficiency among Egyptian HCV infected children, and to assess bone status by measuring calcium, parathormone, alkaline phosphatase and bone mineral density. We also evaluated the role of vitamin D supplementation in improving the virological response of these patients.

MATERIALS AND METHODS

This prospective study included 66 cases (43 male and 23 female) aged from 7-14 years with HCV infection. They were admitted to the Gastroenterology and Hepatology unit at Assiut Children University Hospital from June 2010 to December 2012. The diagnosis of HCV was based on the quantitation of HCV RNA by quantitative real-time polymerase chain reaction[11].

Exclusion criteria

Patients with chronic liver disease other than HCV, patients with immunodeficiency, malignancy, decompensated liver cirrhosis, and patients on vitamin D or calcium therapy in the previous 3 mo were excluded.

Another 28 apparently healthy volunteer children drawn from Assiut Children University Hospital, of matched age and sex, were recruited as controls. The medical ethical committee of the Faculty of Medicine, Assuit University approved the study, and informed written consent was obtained from the parents or legal guardians.

A full history was taken and a thorough clinical examination was performed for all members of both study groups. All cases were subjected to the following investigations: (1) serum liver enzymes (AST and ALT), Ca, phosphorus, alkaline phosphatase, 25 (OH) D 3 and PTH levels; (2) PCR for HCV RNA; and (3) bone mineral density (BMD) as measured by the dual energy X-ray absorptiometry method (Hologic Model Delphi, CT, United States). BMD (g/cm^2 and Z-score) of the ribs, arms, head, lumbar spine, pelvis, legs and total body were measured. The BMD values were compared with those of the healthy controls. Osteoporosis was considered with a Z-score of -2 standard deviations (SDs) and osteopenia between -1.0 and 2.0 SDs in relation to the patient's age, as suggested by the World Health Organization[12].

The patients were classified randomly by a simple randomization method into two groups: Group A received PEG-alpha-2b interferon (60 μg/kg per week) SC and ribavirin (15 mg/kg per day) orally for 48 wk together with vitamin D3 2000 IU/d orally. Group B received the same therapy without vitamin D supplementation. Vitamin D insufficiency, deficiency and sufficiency were defined according to 25OHD levels as > 75 nmol/L, from 75-30 nmol/L, and < 30 nmol/L, respectively[13].

Follow up for all patients was performed at 12 and 24 wk from the beginning of the therapy and at 24 wk after cessation of therapy by measuring PCR for HCV RNA and liver functions. All complications and side effects during the course of treatment were recorded. Six cases (two from group A and four from group B) were excluded from the study after 12 wk of treatment because of a lack of response. Non-responders were defined as those who failed to clear HCV RNA from serum after 24 wk of therapy.

Discontinuation of therapy occurred if HCV RNA level after 12 wk < 2 log unit compared to baseline

or neutropenia < 500/cm, platelets < 5000 mm³ and Hb < 8 gm/dL. Sustained virological response (SVR) was considered when an undetectable HCV-RNA was found at 24 wk after therapy.

Sample collection and laboratory investigations

Venous blood samples were collected from patients under standardized conditions. Samples were centrifuged (3000 × *g* for 10 min); serum and plasma samples were divided and stored in aliquots at -20 ℃ until analysis. Serum calcium, phosphorus and alkaline phosphatase were measured by conventional methods using a COBAS INTEGRA 400 autoanalyzer. Serum 25-OH Vitamin D measurement was performed by Immundiagnostik AG kit (Bensheim; Germany; Ref.K2110), using an enzyme immunoassay technique, based on competition of 25 (OH) D present in the sample with 20(OH) D tracer for the binding pocket of vitamin D binding protein. Assay of plasma levels of parathyroid hormone using MAGLUMI Intact PTH (Shenzhen; China) Ref. 13330211001M (Sandwich immunoluminometric assay) Quantitative PCR for HCV was performed using an AB Applied Biosystems 7500 Fast Real-Time PCR System.

Statistical analysis

Statistical analysis was carried out using SPSS (version 16, SPSS Inc., Chicago, IL, United States). Quantitative data were expressed as mean ± SD and categorical data were presented in the form of frequency and percent (%), as appropriate. Student's *t*-test was used for parametric data and non-parametric χ^2 was used for independent variables when comparing the two groups. Multiple groups were compared using the one-way ANOVA test. Linear correlations were performed by Pearson's test. For all tests, a difference was considered significant if the probability (*P*) was < 0.05.

RESULTS

Positive family history of HCV was significantly different between group A and B ($P < 0.05$) (Table 1).

Complications during the course of treatment were detected in 10 cases, with mild symptoms in the form of fever, nausea, itching, headache, anemia < 9 gm/dL, neutropenia < 5000/cm³ or thrombocytopenia < 100000 mm³, where the antiviral dose were reduced to half the dose.

Significant decrease in serum vitamin D levels ($P < 0.05$) and significant increases in plasma PTH ($P < 0.01$) and HCV RNA ($P < 0.001$) were detected in the studied cases compared with the controls, despite no significant differences being found regarding liver enzymes, albumin, Phosphorus and ALP between the two groups (Table 2).

Table 3 represents the mean values of Bone density parameters in the studied groups with HCV infection. There were significant differences in Z- score regarding ribs ($P < 0.04$), pelvis ($P < 0.04$), L-spine ($P < 0.05$) and total BMD ($P < 0.03$) between the two groups (Table 3).

Vitamin D sufficiency was present in 23.3%, insufficiency in 43.3% and deficiency in 33.3% of cases. There were significant decreases in serum calcium levels in the deficient group vs the sufficient group. Serum vitamin D showed a statistically significant difference between the sufficient, insufficient and deficient groups compared with the control. PCR for HCV RNA was significantly different in the sufficient and insufficient versus the deficient groups and also compared with control (Table 4).

There were significant difference in ribs ($P < 0.04$), pelvis ($P < 0.05$), L spine ($P < 0.05$) and head ($P < 0.005$) in BMD between the sufficient and deficient groups (Table 5).

At 12 wk, there were no significance in the early virological response (EVR) between the two groups, where 10/31 patients (32.3%) from group A and 6/29 (20.6%) from group B were HCV-RNA negative. At 24 wk, there were significant differences in virological response (*P* value < 0.04),where 24/31 patients (77.4%) from group A and 17/29 (58.6%) from group B were negative for HCV-RNA. A significant difference in SVR was detected at 24 wk after treatment, between group A and B ($P < 0.05$). 22/31 patients (70.9%) from group A and 15/29 (51.7%) from group B were HCV-RNA negative.

DISCUSSION

Egypt has the highest worldwide prevalence of HCV infection, about 9% countrywide and up to 50% in rural areas[2]. This goes with our results where 15% of patients had positive family history of HCV, with a significant higher proportion in group A than in group B.

The baseline of PCR HCV RNA (> 400000 IU/mL) was significantly higher compared with the controls in this study, with no significance between group A and B. Assy *et al*[13] showed that patients supplemented with vitamin D had a significantly higher baseline of HCV RNA (60%) than those treated without vitamin D (40%). While Abu-Mouch *et al*[14], found that the baseline of HCV RNA with high viral load (> 800000 IU/mL) in patients treated with vitamin D (50%) showed higher significant results than those without vitamin D treatment (42%).

In this study, vitamin D sufficiency was present in 23.3%, insufficiency in 43.3% and deficiency in 33.3% of cases with no significant difference between the two groups. This agrees with the results of previous studies[13,14], which found no significant difference regarding the baseline vitamin D

Table 1 Demographic and clinical data for the studied groups and control

	A (*n* = 31)	B (*n* = 29)	Total (*n* = 60)	Control (*n* = 28)	*P* value
Age (yr)	11.1 ± 2.178	11.043 ± 2.882	11.17 ± 2.293	9.35 ± 3.24	0.445
Duration of illness (yr)	3.4 ± 1.984	3.123 ± 1.688	3.262 ± 1.832	-	0.563
BMI (kg/m^2)	20.81 ± 4.29	20.28 ± 3.13	20.54 ± 3.73	21.21 ± 2.34	0.588
Male/female	23/8 (74.1%/25.8%)	20/9 (68.9%/31.1%)	43/17 (71.6%/28.3 %)	17/13 (60.7%/46.4%)	0.284
Blood transfusion, *n* (%)	16 (51.6)	16 (55.1)	32 (53.3)	-	0.602
Positive family history of HCV	8 (25.8)	1 (3.4)	9 (15.0)	-	0.05[1]
Jaundice, *n* (%)	19 (61.2)	13 (44.8)	32 (53.3)	-	0.098
Fever, *n* (%)	9 (29.0)	9 (31.0)	18 (30.0)	-	0.611
History of bleeding, *n* (%)	17 (54.8)	16 (55.1)	33 (55.0)	-	0.501

[1]Significance total *vs* control. No significant difference between group A and B. Data are presented as the mean ± SD or number (%) as appropriate. BMI: Body mass index.

Table 2 Mean ± SD values of laboratory parameters in the studied groups and control

	Group A (*n* = 31)	Group B (*n* = 29)	Total (*n* = 60)	Control (*n* = 28)	*P* value
ALT (IU/L)	75.46 ± 18.18	87.96 ± 14.11	81.71 ± 18.25	21.35 ± 6.21	0.148
AST (IU/L)	75.5 ± 24.95	104 ± 11.204	89.75 ± 23.41	35.74 ± 4.92	0.24
Calcium (mg/dL)	6.733 ± 1.52	6.657 ± 1.24	6.69 ± 1.38	8.2 ± 0.91	0.83
Phosphorus (mg/dL)	4.24 ± 0.42	4.21 ± 0.39	4.25 ± 0.41	3.1 ± 0.65	0.68
Alkaline phos (IU/L)	140.97 ± 10.98	99.12 ± 24.94	120.05 ± 27.66	78.48 ± 21.10	0.20
HCV RNA (IU/mL)	1393290 ± 52580	967371 ± 56534	1180334 ± 79910	10.21 ± 0.74	0.001[1]
Vitamin D (nmol/L)	65.26 ± 22.71	57.9 ± 16.17	61.58 ± 17.05	98.31 ± 3.50	0.05[1]
PTH (pg/mL)	179.10 ± 10.25	188.03 ± 14.96	186.56 ± 25.67	65.71 ± 12.05	0.01[1]

[1]Significance total *vs* control. No significant difference between group A and B. AST: Aspartate transaminase; ALT: Alanine transaminase; PTH: Parathormone.

Table 3 Mean ± SD values of bone density parameters in the studied groups with hepatitis C virus infection

	A (*n* = 31)	B (*n* = 29)	Total (*n* = 60)	*Z*- score	*P* value
Head	1.226 ± 0.394	1.12 ± 0.383	1.173 ± 0.389	-0.16 ± 0.42	0.297
Arm	1.063 ± 1.223	0.89 ± 0.277	0.976 ± 0.883	-0.916 ± 1.2	0.453
Leg	0.912 ± 0.225	0.969 ± 0.38	0.94 ± 0.311	-1.13 ± 1.34	0.482
Rib	0.688 ± 0.248	0.869 ± 0.411	0.778 ± 0.349	-1.31 ± 0.78	0.043[1]
Pelvis	0.839 ± 0.187	0.92 ± 0.238	0.879 ± 0.216	-2.8 ± 1.53	0.05[1]
L Spine	0.77 ± 0.207	0.901 ± 0.294	0.835 ± 0.261	-2.43 ± 1.7	0.051[1]
Total	0.834 ± 0.281	0.913 ± 0.381	0.874 ± 0.334	-1.98 ± 0.75	0.036[1]

[1]Significance between total BMD of HCV patients *vs* control. BMD: Bone mineral density (g/cm^2). HCV: Hepatitis C virus.

Table 4 Mean ± SD values of laboratory parameters in relation to Vitamin D status in the studied groups

	Vitamin D status			Control (*n* = 28)	Suff *vs* Insuff	Suff *vs* Def	Insuff *vs* Def	Total *P* value
	Sufficient (*n* = 14)	Insufficient (*n* = 26)	Deficient (*n* = 20)					
ALT (IU/L)	97.643 ± 18.06	75.654 ± 15.954	78.45 ± 17.824	21.35 ± 6.21	0.327	0.475	0.885	0.610
AST (IU/L)	97.786 ± 24.215	94.923 ± 13.151	77.4 ± 13.363	35.74 ± 4.92	0.934	0.453	0.551	0.772
Calcium (mg/dL)	7.993 ± 1.183	7.023 ± 1.396	6.13 ± 1.185	8.2 ± 0.91	0.769	0.03[1]	0.090	0.077
Phosphorus (mg/dL)	3.31 ± 0.25	4.21 ± 0.31	4.24 ± 0.61	3.1 ± 0.65	0.354	0.412	0.614	0.456
Alkaline phosphatase (IU/L)	82.92 ± 15.62	109.34 ± 13.08	159.96 ± 14.64	78.48 ± 21.10	0.493	0.068	0.233	0.192
HCV RNA(IU/mL)	1300824 ± 664010	1417943 ± 137873	787100 ± 65915	10.21 ± 0.74	0.705	0.03[1]	0.022[1]	0.043[2]
Vitamin D (nmol/L)	84.21 ± 17.821	49.23 ± 15.07	22.6 ± 3.858	98.31 ± 3.50	0.000[1]	0.00[1]	0.000[1]	0.001[2]
PTH (pg/mL)	128.53 ± 20.37	164.05 ± 12.63	179.46 ± 19.24	65.71 ± 12.05	0.181	0.322	0.607	0.296

[1]Significance between groups *vs* control; [2]Significance total *vs* control. AST: Aspartate transaminase; ALT: Alanine transaminase; PTH: Parathormone; Suff: sufficient; Insuff: Insufficient; Def: Deficient.

Table 5 Mean ± SD values of bone mineral density in relation to Vitamin D status in the studied groups

	Vitamin D status			Suff *vs* Insuff	Suff *vs* Def	Insuff *vs* Def	Total
	Sufficient (n = 14)	Insufficient (n = 26)	Deficient (n = 20)				P value
Head	0.921 ± 0.343	1.237 ± 0.415	1.267 ± 0.317	0.020[1]	0.005[1]	0.790	0.018[2]
Arm	1.03 ± 0.901	0.879 ± 0.259	1.065 ± 1.325	0.428	0.931	0.487	0.759
Leg	0.873 ± 0.28	1.001 ± 0.321	0.978 ± 0.291	0.341	0.428	0.798	0.067
Rib	0.856 ± 0.34	0.749 ± 0.358	0.662 ± 0.353	0.365	0.043[1]	0.910	0.637
Pelvis	0.996 ± 0.234	0.895 ± 0.219	0.748 ± 0.107	0.990	0.05[1]	0.469	0.738
L- Spine	0.959 ± 0.115	0.838 ± 0.24	0.716 ± 0.258	0.808	0.059[1]	0.766	0.892
Total	0.918 ± 0.444	0.897 ± 0.11	0.812 ± 0.28	0.863	0.398	0.340	0.596
Z score	1.121 ± 0.577	0.927 ± 0.25	-1.875 ± 0.441	0.619	0.042[1]	0.431	0.493

[1]Significance between groups; [2]Significance total *vs* control. BMD: Bone mineral density (g/cm^2). Suff: Sufficient; Insuff: Insufficient; Def: Deficient.

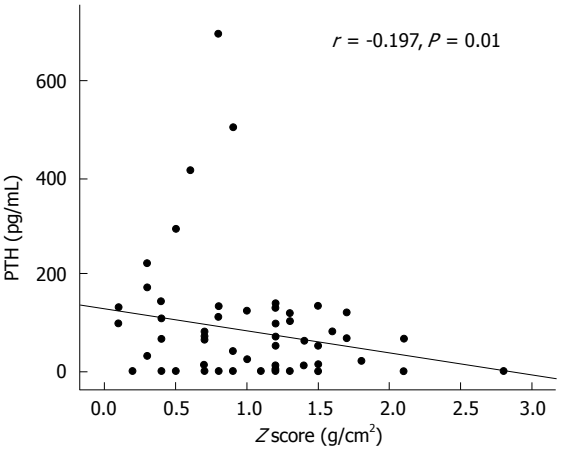

Figure 1 Correlation between plasma parathormone levels and the Z-score of the total body.

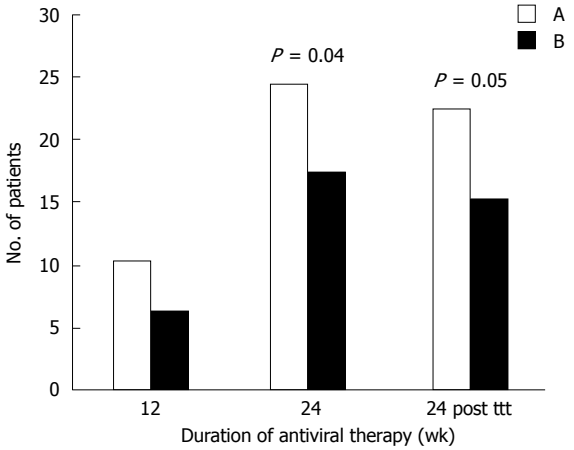

Figure 2 Early and sustained viral response of hepatitis C virus RNA in group A and B. PTH: Parathormone.

between cases supplemented with vitamin D and those without. Highleyman[15] stated the importance of vitamin D in the immune response against HCV infection: 84% of adult patients with HCV infection have low vitamin D levels and 1/3 of patients had severe deficiency.

Mandorfer *et al*[16] also found sufficient levels of vitamin D in 1/5 of their participants and 57% were insufficient and 23% were deficient. Ladero *et al*[17], found vitamin D insufficiency in 40% and deficiency in 36% of adult patients.

Vitamin D status may differ across different geographical locations and environments[13]. Consequently, its influence on disease pathogenesis is likely to vary from one location to the other. Vitamin. D deficiency could be related to lack of exposure to sunlight, although Africa is sunny, and/or to the lack of vitamin D in the diet[13].

Vitamin D is an important modulator of inflammatory responses and fibrosis in HCV infection by inhibiting TNF-α, which regulates the immune response and inhibits the fibrosis process by suppressing TGF-β, which affects fibrosis progression[18]. Ladero *et al*[17], concluded that vitamin D deficiency is more common in Spanish patients with HCV infection, but it is neither related to biochemical and virological variations, nor to fibrosis stage or IL 28B polymorphisms.

On the contrary, other studies[19], raised the possibility that the disease progression is associated with higher levels of vitamin D, and thus vitamin D supplementation does not have a role in chronic hepatitis C patients. Terrier *et al*[20], showed that were no associations between serum 25 OH D3 levels and viral response to therapy or the severity of immunodeficiency in HCV patients.

The present study observed significantly higher plasma PTH levels in HCV patients compared with controls. This may be related to the functioning feedback mechanisms of low serum calcium and 25(OH)D levels. Malabanan *et al*[21] stated that when the 25(OH)D concentration reaches < 50 nmol/L (20 ng/mL), the PTH concentration increases.

In the present study, plasma PTH levels were negatively associated with BMD and the Z-score of the total body (Figure 1). The increased PTH levels found in our patients may lead to increased bone resorption and decreased BMD. The decrease in sensitivity of the PTH effect on bone resorption[22] or the anabolism of the osteoblasts to PTH could be an explanation[23]. Similarly, Choudhary *et al*[24] found a negative correlation between PTH and BMD in viral

cirrhosis. By contrast, other studies[25,26] revealed normal or low PTH levels in HCV infection. This discrepancy could be explained by the increased prevalence of 25(OH)D deficiency or insufficiency in Egyptian children with HCV infection.

In partial agreement with other studies[27], we observed that the peripheral skeleton was less influenced than the axial skeleton in our HCV patients. The accelerated growth in the peripheral skeleton in children could explain this, while during puberty, it occurs in the axial skeleton[27]. In addition, most of our patients had been diagnosed before puberty. Therefore, the highest bone mass was obtained during the disease process, which affects the bone status in these patients. The protective role of puberty on bone tends to be opposite to temporary bone loss, which seems to be a complication of the disease[28].

Regarding bone density in the present study, the *Z* score was significantly decreased and showed significant difference between the two groups. This is agrees with the results of El Karmouty *et al*[29], who revealed that BMD, T score and *Z* score were significantly lower in children with HBV and HCV than HAV infection. The severity of osteoporosis increases with the severity of liver disease. However, no significant correlation could be detected between serum Ca, Phosphorus, vitamin D and the degree of bone loss, which is higher in osteoporosis than in osteomalacia[29]. Kryskiewicz *et al*[6] observed some risk factors of bone tissue pathology, which included hepatocyte dysfunction, disorders of vitamin D, Ca and Phosphorus metabolism, immunosuppressive therapy and malnutrition. Theoretically disturbance in the endocrine Ca - PTH- vitamin D axis seems to play a role in the pathogenesis of osteo metabolic disturbances.

López-Larramona *et al*[30] stated that the origin of hepatocyte osteodystrophy is unclear; it may be multifactorial and its etiology and severity vary in accordance with the underlying liver disease. Bone loss occurs as a result of increased bone turnover and/or remodeling in balance, the latter being caused by reduced formation, and increased resorption or a combination of both. Vitamin D metabolism in severe HCV infection and deficiency may cause hyperparathyroidism, increase bone turnover and accelerate the loss of BMD. This regulation of the RANKL/OPG system is activated by cytokines involved in the pathogenesis of chronic liver disease (IL 1, 11, 6, TNF-α1). Previous studies[24,31] found that patients with chronic liver disease have low BMD in 93% of cases. Other factors, including decreased physical activity, decreased body mass, deficiency of vitamin D and low IGF level were considered.

Yurci *et al*[32] stated that all anti osteoporotic agents in hepatic osteodystrophy seems to be safe and effective. Oral bisphosphonate (anti resorptive drug) was the most effective in preventing both cortical and trabecular bone loss in patients with chronic viral hepatitis, but only limited data are available.

By contrast, Yenice *et al*[25], stated that hepatitis B and C infection do not pose a risk factor for osteoporosis and bone loss, and that the diagnosis of BMD should be based on multiple parameters. It should be kept in mind that the key osteomalacia is rare and requires bone biopsy. The differences observed between reports may be related to the study design, different methods used for measuring BMD and selection of patients.

Regarding the use of vitamin D in this study, cases in group A showed significantly higher rates of EVR, a greater response after 24 wk and higher SVRs than group B (Figure 2). This agrees with a previous study[14] that found that patients treated with vitamin D in addition to conventional antiviral therapy had significantly higher responses at 4 wk than those treated without vitamin D (44% *vs* 17%), at 12 wk (94% *vs* 43%), and at 24 wk after stoppage of therapy (86% *vs* 42%) with a small relapse rate (8%). Previous studies[13,14] concluded that the addition of vitamin D to the therapy in HCV patients increases the rate of rapid and early SVR. Therefore, assessment of vitamin D levels before combined therapy and correction during the course of therapy may be needed.

Bitetto *et al*[33] concluded that unfavorable responses to antiviral therapy are predicted in vitamin D deficient patients with RHC. Supplementing vitamin D improves the possibility of achieving an SVR after antiviral treatment. In addition, low levels of vitamin D are associated with significant poor rate of EVR and SVR in co-infection of HIV[16], and this decreased vitamin D is limited to fibrosis and low SVR in interferon-based therapy[34]. The exact mechanism of vitamin D supplementation action on EVR, RVR and SVR is unclear. It may reflect the fact that 1, 25 dihydroxy vitamin D appears to be an immunomodulator via regulation of T cell function through its effect on T cell antigen[35]. T helper cell type I action is intensified when vitamin D is insufficient or when VDR signals are weak[36]. Vitamin D increases the expression of VDR proteins and inhibits viral replication[37].

Gal-Tanamy *et al*[38], stated that the interferon sparing effect of vitamin D improves the antiviral treatment in patients with HCV infection. 25(OH) D3 is a novel antiviral agent and a better therapeutic option to reduce the enzyme activity in patients with HCV[39].

In conclusion, there was a high frequency of Vitamin D deficiency among the Egyptian HCV children studied, with significant decrease in bone density. Vitamin D levels should be assessed before the start of antiviral treatment, with correction of any detected deficiency. Adding vitamin D to

conventional Peg/RBV therapy significantly improved viral response and helped to prevent the risk of emerging bone fragility.

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COMMENTS

Background

Hepatitis C virus (HCV) infection remains an evolving cause of morbidity and mortality worldwide. Despite limited epidemiological data, a higher prevalence is found in Eastern Europe and the Middle East. Abnormal bone metabolism, vitamin D axis, calcium and parathormone dysfunction have been reported in cholestatic children. The relation between vitamin D and antiviral therapy response remains unclear, with previous studies raising the possibility of inconsistent results.

Research frontiers

To investigate the frequency of vitamin D deficiency among Egyptian HCV-infected children, with assessment of bone status by measuring calcium, parathormone and alkaline phosphatase levels, and bone mineral density. We also to evaluated the role of vitamin D supplementation in improving the viral response of these patients.

Innovations and breakthroughs

Previous studies raised the possibility that disease progression is associated with higher levels of vitamin D, and thus vitamin D supplementation does not have a role in chronic hepatitis C patients. In this study, cases supplemented with vitamin D showed a significantly higher percentage of early virological response (EVR), higher response after 24 wk and a higher sustained virological response (SVR) than those without supplementation.

Applications

The addition of vitamin D to the therapy in HCV patients increases the rate of rapid and early SVR. Cases supplemented with vitamin D showed a significantly higher percentage of EVR, higher response after 24 wk and a higher SVR than those without supplementation.

Terminology

The addition of vitamin D to the therapy in HCV patients increases the rate of rapid and early SVR. Assessment of Vitamin D levels before combined therapy and correction during the course of therapy is required.

Peer review

The study investigated the effect of vitamin D in the response to antiviral treatment in chronic infected pediatric patients with HCV. There are several works published in the literature exploring this issue, however the relation between vitamin D status and the antiviral response to therapy remains unclear.

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ORIGINAL ARTICLE

Prospective Study

Impact of discontinuing non-steroidal antiinflammatory drugs on long-term recurrence in colonic diverticular bleeding

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Abstract

AIM: To determine the effect of discontinuing non-steroidal antiinflammatory drugs (NSAIDs) on recurrence in long-term follow-up patients with colonic diverticular bleeding (CDB).

METHODS: A cohort of 132 patients hospitalized for CDB examined by colonoscopy was prospectively enrolled. Comorbidities, lifestyle, and medications (NSAIDs, low-dose aspirin, antiplatelet agents, anticoagulants, acetaminophen, and corticosteroids) were assessed. After discharge, patients were requested to visit the hospital on scheduled days during the follow-up period. The Kaplan-Meier method was used to estimate recurrence.

RESULTS: Median follow-up was 15 mo. The probability of recurrence at 1, 6, 12, and 24 mo was 3.1%, 19%, 27%, and 38%, respectively. Of the 41 NSAID users on admission, 26 (63%) discontinued NSAID use at discharge. Many of the patients who could discontinue NSAIDs were intermittent users, and could be switched to alternative therapies, such as acetaminophen or an antiinflammatory analgesic plaster. The probability of recurrence at 12 mo was 9.4% in discontinuing NSAID users compared with 77% in continuing users ($P < 0.01$, log-rank test). The hazard ratio for recurrence in the discontinuing NSAIDs users was 0.06 after adjusting for age > 70 years, right-sided diverticula, history of hypertension, and hemodialysis. No patients developed cerebrocardiovascular events during follow-up.

CONCLUSION: There is a substantial recurrence rate after discharge among patients hospitalized for diverticular bleeding. Discontinuation of NSAIDs is an effective preventive measure against recurrence. This study provides new information on risk reduction strategies for diverticular bleeding.

Key words: Non-steroidal anti-inflammatories; Drug withdrawal; Diverticular hemorrhage; Hemodialysis; Antithrombotic drugs

Core tip: The probability of recurrence of diverticular bleeding at 1, 12, and 24 mo was 3.1%, 27%, and 38%, respectively. Of the 41 non-steroidal anti-inflammatory drugs (NSAIDs) users on admission, 26 (63%) discontinued NSAID use at discharge. The probability of recurrence at 12 mo was 9.4% in discontinuing NSAID users compared with 77% in continuing users (P < 0.01, log-rank test). The hazard ratio for recurrence in the discontinuing NSAIDs users was 0.06 after adjusting for age > 70 years, right-sided diverticula, history of hypertension, and hemodialysis. No patients developed cerebrocardiovascular events during follow-up. This study provides new information on risk reduction strategies for diverticular bleeding.

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INTRODUCTION

Colonic diverticular bleeding (CDB) is the most common cause of acute lower gastrointestinal bleeding (LGIB), representing approximately 40% of cases[1]. A significant proportion of patients with CDB experience severe bleeding and receive emergency treatment with resuscitative measures, possibly because of their advanced age, use of antithrombotic agents, or comorbidities[2-4]. Even though intensive management of CDB is successful during the hospital stay, following discharge, patients are at high risk of recurrent bleeding, which has been reported at a rate of 14%-43%[3,5-7]. As a result, patients often undergo frequent examinations, rehospitalization, and a consequent decrease in quality of life.

Risk reduction is an important clinical issue because no preventive therapy for recurrence is currently available for LGIB, such as proton-pump inhibitors used for the prevention of upper gastrointestinal bleeding (UGIB)[8]. Withdrawal of aspirin was recently reported to significantly reduce UGIB recurrence[9]. In LGIB, particularly CDB, the use of non-steroidal anti-inflammatory drugs (NSAIDs) is a significant risk factor for recurrence[2,10,11], but the risks and benefits of their discontinuation on CDB have not been examined.

We previously conducted a study on the recurrence risk of CDB[5], but the retrospective investigation included biases that likely led to exposure variables being missed. Furthermore, it was unknown whether antithrombotic agents were continued, and follow-up was not conducted in a systematic manner. The present study builds upon our previous work by focusing on multiple risk factors for CDB, prospective follow-up of newly diagnosed cases, and detailed assessment of medications. The objectives of this study were to identify the risk factors associated with the recurrence of CDB after discharge, and to evaluate the effect of discontinuing NSAIDs on rebleeding risk over long-term follow-up.

MATERIALS AND METHODS

Subjects

Patients admitted to our hospital for overt LGIB between September 2009 and October 2013 and examined at the endoscopy unit of the National Center for Global Health and Medicine (NCGM) were enrolled. Patients from a previous study cohort[12], a newly diagnosed cohort (approval No. 750), and a randomized trial cohort (approval No. 765) were prospectively followed after discharge. Inclusion criteria were: (1) > 18 years old; (2) Japanese nationality; (3) overt LGIB examined within 1 wk of onset; and (4) being managed in hospital. Exclusion criteria were: (1) no informed consent provided; (2) not independent in activities of daily living; (3) not undergoing colonoscopy; (4) LGIB due to causes other than CDB; and (5) barium impaction therapy in the randomized controlled trial (approval No. 765). All inclusion and exclusion criteria were fulfilled before patients were enrolled. The NCGM is a large emergency hospital, with 900 beds, located in metropolitan Tokyo, Japan. All patients provided written informed consent prior to enrollment. The institutional review board at the NCGM approved this study (approval No. 810), and all clinical procedures conformed to Japanese and International ethical guidelines (Declaration of Helsinki).

Exposure variables

Patients were asked about their lifestyle habits, medications, and comorbidities in a face-to-face interview on the pre-colonoscopy day.

Medication: The questionnaire survey form asked about the use of 9 kinds of NSAIDs, 2 kinds of low-

dose aspirin, 10 other antiplatelet agents, 3 anticoagulants, acetaminophen, and oral corticosteroids. The questionnaire included photographs of all of these oral drugs, which are approved in Japan. Use of a medication was defined as oral administration within 1 mo of the interview. During hospitalization, low-dose aspirin, other antiplatelet drugs, and anticoagulants were temporarily stopped or other bridging drugs given whenever possible after consulting a cerebrocardiovascular specialist. Low-dose aspirin and other antiplatelet agents were resumed after hemostasis before discharge. Also, NSAIDs were temporarily stopped on admission and prescribed during hospitalization only on the advice of an orthopedist or rheumatology specialist. We suggested NSAID withdrawal to those who were intermittent users and to those who were suitable for alternative therapy such as acetaminophen, an antiinflammatory analgesic plaster, antigout drugs, antirheumatic drugs, or corticosteroids.

Comorbidities: Hypertension, diabetes mellitus, dyslipidemia, cerebrocardiovascular disease, chronic liver disease, and chronic kidney disease (CKD) were assessed. A history of cerebral infarction, cerebral hemorrhage, myocardial infarction, or angina pectoris was considered cerebrocardiovascular disease. CKD was considered present in patients on hemodialysis. Chronic liver disease included chronic viral hepatitis and alcoholic liver disease.

Diagnostic criteria of colonic diverticular bleeding

An electronic high-resolution video endoscope (model CFH260; Olympus Optical, Tokyo, Japan) was used after spontaneous cessation of bleeding. CDB was defined as either definite or presumptive on the basis of colonoscopy with multidetector computed tomography (MDCT)[13,14]. Definitive diagnosis was based on colonoscopic visualization of colonic diverticula with stigmata of recent hemorrhage such as active bleeding, adherent clot, or visible vessel[13,14]. A presumptive diagnosis was based on MDCT visualization of the extravasation of contrast medium in colonic diverticula and colonoscopy showing (1) a potential bleeding site in an area of positive MDCT findings; (2) fresh blood localized to colonic diverticula in the presence of a potential bleeding source on complete colonoscopy; or (3) bright red blood in the rectum confirmed by objective color testing and colonoscopy demonstrating a single potential bleeding source in the colon, complemented by negative upper endoscopy, or negative capsule endoscopy[13,14]. Patients in whom the bleeding source was identified received endoscopic treatment such as clipping or endoscopic ligation.

Follow-up and rebleeding

After discharge, all patients were requested to visit the hospital in the case of bloody stools or on a scheduled day within the first month and then every three months during the observation and follow-up periods. Telephone interviews were conducted with patients who did not visit the hospital on scheduled days, and a hospital visit was recommended. Rebleeding was defined as a significant amount of fresh bloody or wine-colored stools (> 200 mL) during the follow-up period and was evaluated by both anoscopy and MDCT within 12 h of onset. Clinically suspected rebleeding should prompt further colonoscopy when possible, but no routine second-look colonoscopy was performed when rebleeding occurred during hospitalization or within 1 mo of discharge. Colonoscopy was performed to confirm rebleeding and determine the need for intervention when frequent or massive bleeding occurred along with unstable vital signs, systolic blood pressure $\leqslant$ 90 mmHg or pulse $\geqslant$ 110 beats/min, and a non-response to $\geqslant$ 2 units of transfused blood during a 24-h period. We distinguished between rebleeding and remaining blood from the index bleeding episode.

Statistical analysis

The primary outcome was rebleeding due to CDB. The secondary outcome was a cerebrocardiovascular or thrombotic event during the follow-up period. Patients lost to follow-up or death were censored at the time of their last visit. The Kaplan-Meier method was used to estimate the recurrence of CDB at 1, 6, 12, and 24 mo after discharge. Risk factors and the effect of NSAID withdrawal on rebleeding were analyzed using the log-rank test. Cox proportional hazards regression was used to analyze the independent risk factors for rebleeding by considering factors significant ($P < 0.1$) in the log-rank test and adjusting for factors such as age > 70 years and hypertension, as previously reported[5-7].

A value of $P < 0.05$ was considered significant. All statistical analysis was performed using Stata version 10 software (StataCorp, College Station, TX, United States).

RESULTS

Participants

During the study period, 337 patients were admitted to our hospital for acute LGIB. After exclusion, 132 patients with CDB were enrolled in this study. Baseline characteristics at discharge are shown in Table 1. Diverticula were located predominantly in the bilateral colon in 48%, in the right side in 32%, and in the left side in 20% of cases. Of these, 17% (23/132) had colonoscopic evidence of stigmata of recent hemorrhage diverticula and were treated by endoscopic procedures during hospitalization, in which 37 patients (28%) received a mean transfusion of 8 units of packed red blood cells.

Among the patients using NSAIDs on admission

Table 1 Baseline characteristics of the study cohort (*n* = 132) *n* (%)

Characteristic	Value
Mean age (mean ± SD), yr	70 ± 12
Age > 70 yr	70 (53)
Sex, male	87 (66)
Anatomical distribution of diverticula	
Right-sided type	42 (32)
Left-sided typ	27 (20)
Bilateral type	63 (48)
Definite diagnosis (stigmata of recent hemorrhage)	23 (17)
Presumptive diagnosis	109 (83)
Transfusion requirement during hospitalization	37 (28)
Mean units of transfused blood per patient[1] ± SD	8.0 ± 6.0
Current drinker	126 (95)
Current smoker	36 (27)
NSAID[2] users on admission	41 (27)
Discontinuing NSAID users at discharge	26 (20)
Continuing NSAID users at discharge	15 (11)
Low-dose aspirin[2] users on admission	29 (22)
Low-dose aspirin users at discharge	29 (22)
Non-aspirin antiplatelet[2] users on admission	27 (21)
Non-aspirin antiplatelet users at discharge	27 (21)
Anticoagulant[2] users on admission	9 (6.8)
Anticoagulant users at discharge	9 (6.8)
Acetaminophen users on admission	2 (1.5)
Acetaminophen users at discharge	13 (9.9)
Corticosteroid users on admission	4 (3.0)
Corticosteroid users at discharge	5 (3.3)
Hypertension	83 (63)
Diabetes mellitus	27 (20)
Dyslipidemia	29 (22)
Cardiovascular disease	33 (25)
Cerebrovascular disease	2 (1.5)
Chronic liver disease	5 (3.8)
Hemodialysis	7 (5.3)

[1]Analysis of patients who received transfusion. [2]Non-selective NSAIDs included loxoprofen, diclofenac, naproxen, etodolac, zaltoprofen, meloxicam, lornoxicam, and celecoxib (*n* = 3). Distribution type was defined as follows: right-sided, involving the transverse or proximal colon; left-sided, involving the descending or distal colon; or bilateral, involving the entire colon. Low-dose aspirin included enteric-coated aspirin (100 mg) and buffered aspirin (81 mg). Non-aspirin antiplatelet agents included ticlopidine, clopidogrel, cilostazol, dipyridamole, sarpogrelate hydrochloride, ethyl icosapentate, dilazep, limaprost, and beraprost. Anticoagulants included warfarin and dabigatran etexilate. NSAIDs: Non-steroidal anti-inflammatory drugs.

(*n* = 41), 26 (63%) discontinued NSAID use. Many patients who could withdraw NSAIDs were intermittent users (*n* = 23), and could be switched to alternative therapies included acetaminophen (*n* = 11), an antiinflammatory analgesic plaster (*n* = 10), narcotic analgesics (*n* = 2), antigout drugs (*n* = 3), anti-rheumatic drugs (*n* = 1), and corticosteroids (*n* = 1), while the remaining patients (*n* = 7) took nothing. Some of these 26 patients took more than one of these drugs. No patients were taking NSAIDs for cardiovascular disease. Fifteen patients (11%) on NSAIDs and 13 patients (8.5%) on acetaminophen continued to take them after discharge. No patients on low-dose aspirin, non-aspirin antiplatelet agents, or corticosteroids discontinued use or started taking them during the follow-up period (Table 1).

The most common comorbidities were hypertension

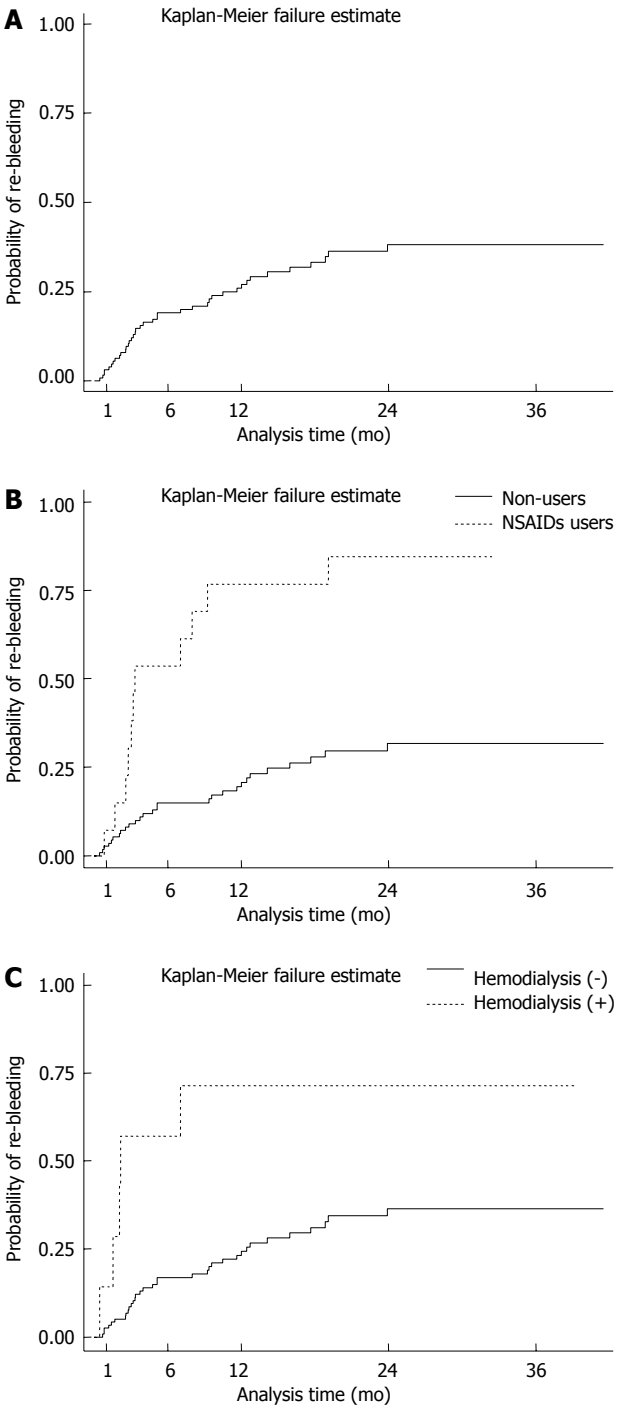

Figure 1 Probability of bleeding recurrence during the follow-up period (*n* = 132). A: Incidence of rebleeding with a mean follow-up period of 15 mo; B: Probability of rebleeding was significantly higher in the [non-steroidal antiinflammatory drug (NSAID) group than in the non-NSAID group ($P < 0.01$)]; C: Probability of rebleeding was significantly higher in the hemodialysis group than in the non-hemodialysis group ($P < 0.01$).

(63%), cardiovascular disease (25%), dyslipidemia (22%), diabetes mellitus (20%), hemodialysis (5.3%), chronic liver disease (3.8%), and cerebrovascular disease (1.5%) (Table 1).

Rebleeding outcomes

After discharge, 39 patients (30%) experienced rebleeding with a mean follow-up of 15 ± 13 mo. The

Table 2 Risk factors for rebleeding after discharge on univariate analysis (*n* = 132)

Factor	Non-rebleeding/ rebleeding	Hazard ratio (95%CI)	*P*
Age > 70 yr	45 (48)/25 (64)	1.6 (0.83-3.1)	0.15
Sex, male	59 (63)/28 (72)	1.1 (0.57-2.3)	0.70
Anatomical distribution of diverticula			
Right-sided diverticula	34 (37)/8 (21)	0.49 (0.22-1.1)	0.06
Left-sided diverticula	19 (20)/8 (21)	1.3 (0.60-2.8)	0.51
Bilateral diverticula	40 (43)/23 (59)	1.5 (0.79-2.8)	0.21
Endoscopic procedure	20 (22)/3 (7.7)	0.43 (0.13-1.4)	0.16
Transfusion requirement	25 (27)/12 (31)	1.2 (0.63-2.5)	0.53
Current drinker	90 (97)/36 (92)	0.78 (0.24-2.5)	0.68
Current smoker	26 (28)/10 (26)	0.80 (0.39-1.7)	0.55
NSAID users	4 (4.3)/11 (28)	5.1 (2.5-10)	< 0.01
Low-dose aspirin[1] users	20 (22)/9 (23)	0.88 (0.42-1.9)	0.74
Non-aspirin antiplatelet[1] users	16 (17)/11 (28)	1.6 (0.78-3.1)	0.21
Anticoagulant[1] users	7 (7.5)/2 (5.1)	0.54 (0.13-2.2)	0.38
Acetaminophen users	10 (11)/3 (7.7)	0.72 (0.22-2.3)	0.58
Corticosteroid users	3 (3.2)/2 (5.1)	1.4 (0.35-6.0)	0.61
Hypertension	55 (60)/28 (72)	1.4 (0.69-2.8)	0.35
Diabetes mellitus	22 (24)/5 (13)	0.52 (0.20-1.3)	0.16
Dyslipidemia	20 (22)/9 (23)	0.88 (0.42-1.8)	0.73
Cardiovascular disease	25 (27)/8 (21)	0.55 (0.25-1.2)	0.13
Cerebrovascular disease	1 (1.1)/1 (2.6)	1.4 (0.19-10)	0.76
Chronic liver disease	3 (3.2)/2 (5.1)	1.4 (0.34-5.9)	0.62
Hemodialysis	2 (2.2)/5 (13)	4.0 (1.5-10)	< 0.01

[1]Non-selective NSAIDs included loxoprofen, diclofenac, naproxen, etodolac, zaltoprofen, meloxicam, lornoxicam, and celecoxib (*n* = 3). Distribution type was defined as follows: right-sided, involving the transverse or proximal colon; left-sided, involving the descending or distal colon; or bilateral, involving the entire colon. Low-dose aspirin included enteric-coated aspirin (100 mg) and buffered aspirin (81 mg). Non-aspirin antiplatelets included ticlopidine, clopidogrel, cilostazol, dipyridamole, sarpogrelate hydrochloride, ethyl icosapentate, dilazep, limaprost, and beraprost. Anticoagulants included warfarin and dabigatran etexilate. CI: Confidence interval; NSAIDs: Non-steroidal anti-inflammatory drugs.

probability of rebleeding was 3.1% at 1 mo, 19% at 6 mo, 27% at 12 mo, and 38% at 24 mo (Figure 1A). No patients required angiographic embolization or surgical resection within the follow-up period.

Log-rank tests revealed NSAID use and hemodialysis as significant risk factors for rebleeding, while the other factors were not found to be significant (Table 2). Patients without right-sided diverticula had a risk of rebleeding, but this did not reach statistical significance (Table 2). The probability of rebleeding was 2.6% (95%CI: 0.86%-8.0%) at 1 mo and 21% (95%CI: 14%-30%) at 12 mo in non-NSAID users, and 7.1% (95%CI: 1.0-41) at 1 mo and 77% (95%CI: 52-94) at 12 mo in NSAID users (Figure 1B). The probability of rebleeding was 2.5% (95%CI: 1.0-7.6) at 1 mo and 24% (95%CI: 17-34) at 12 mo in non-hemodialysis patients, and 14% (95%CI: 2.1%-67%) at 1 mo and 71% (95%CI: 39%-96%) at 12 mo in hemodialysis patients (Figure 1C). Multivariate analysis revealed NSAID use and hemodialysis as independent risk factors for rebleeding (Table 3).

Discontinuation of NSAIDs and rebleeding

Of the NSAID users (*n* = 41), 15 continued and 26 discontinued NSAID use after discharge. During the follow-up period, no patient developed a thrombotic event, such as pulmonary embolism, deep vein thrombosis, or a cerebrocardiovascular event. No patient who discontinued NSAID use on discharge resumed NSAID use during the follow-up period. The probability of rebleeding was 0% at 1 mo and only 9.4% (95%CI: 2.4%-33%) at 12 mo in discontinuing NSAID users compared with 7.1% (95%CI 1.0%-41%) at 1 mo and 77% (95%CI: 52%-94%) at 12 mo in the continuing users (P < 0.01 by the log-rank test; Figure 2). The hazard ratio (HR) for rebleeding in the discontinuing NSAIDs group was 0.06 (95%CI: 0.01%-0.31%) after adjusting for age > 70 years, right-sided diverticula, history of hypertension, and hemodialysis in the Cox proportional hazards model.

Table 3 Risk factors for rebleeding after discharge on multivariate analysis (*n* = 132)

Factor	Hazard ratio (95%CI)	*P*
Age > 70 yr	1.2 (0.6-2.3)	0.68
Right-sided diverticula	0.6 (0.3-1.5)	0.29
Continued NSAID use	4.6 (2.2-9.4)	< 0.01
Hypertension	1.3 (0.6-2.8)	0.43
Hemodialysis	3.0 (1.1-7.8)	0.03

CI: Confidence interval; NSAIDs: Non-steroidal anti-inflammatory drugs.

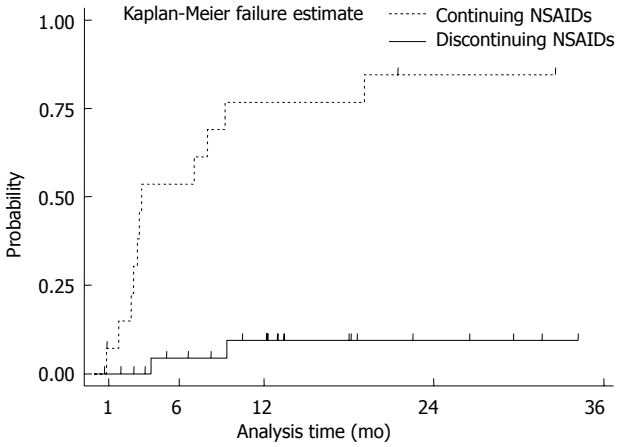

Figure 2 Probability of recurrent bleeding in patients with non-steroidal antiinflammatory drugs on admission (*n* = 41). The probability of rebleeding was higher in subjects continuing non-steroidal antiinflammatory drugs (NSAIDs) than in those discontinuing NSAIDs (P < 0.01).

DISCUSSION

To our knowledge, this is the first report showing that discontinuation of NSAIDs at discharge significantly reduced rebleeding compared with continuous NSAID use. Although, the number of NSAIDs users was small, this finding is useful in the long-term management of CDB. Moreover, no patients developed a worsening condition of underlying disease, thrombotic events, or cerebrocardiovascular events after cessation of

NSAIDs.

In agreement with previous studies, this study showed that patients using NSAIDs had a risk of rebleeding during follow-up (often within the first year) approximately 5-fold greater than those without. A previous large cohort study found that NSAID users had a 1.7-fold higher risk of CDB[10], and other case-control studies have reported increased bleeding risks of 4-15-fold[6,11,15] and re-bleeding risks of 3-6-fold[5,6]. Tsuruoka *et al*[16] conducted CDB case-control (patients hospitalized for other diseases during the same period) investigations and showed that only NSAID use was an independent risk factor for bleeding (OR = 9.9) and rebleeding (OR = 5.4). Another report by Wilcox *et al*[17] compared 105 patients with LGIB and 1895 non-bleeding controls, and found that NSAID users had a 3.4-fold higher risk of CDB. Although a wide range of risk ratios have been reported, likely due to differences in study design or sample size, it is clear that NSAID use is an unequivocal risk factor for CDB.

In contrast to this greater risk of rebleeding, withdrawal of NSAIDs significantly reduced the risk of rebleeding during follow-up compared with continued use (HR = 0.06). In UGIB, protonpump inhibitors reduce recurrence[8], but there is no preventive therapy for LGIB. It is thus all the more critical to validate factors associated with risk reduction in LGIB. A few studies have examined the risk reduction of GI bleeding associated with drug withdrawal, but not non-aspirin NSAIDs. Witt *et al*[18] found that patients with GI bleeding who resumed warfarin use had a higher GI re-bleeding risk and fewer thrombotic events than those who discontinued use. Sung *et al*[9] conducted a randomized controlled trial in patients with peptic ulcer bleeding and found that continuous low-dose aspirin increased the risk of re-bleeding, but potentially reduced mortality, while prolonged discontinuation of aspirin led to a lower risk of rebleeding, but higher mortality. One of the reasons we could withdraw NSAIDs in many of our patients (26/41) was that most were not regular users and could be switched to other drugs, such as acetaminophen, an antiinflammatory analgesic plaster, antigout drugs, antirheumatic drugs, or corticosteroids. Furthermore, we could closely monitor these patients and confirm no exacerbation of pain. However, one randomized controlled trial on the treatment of painful knee osteoarthritis found that discontinuation of non-aspirin NSAIDs exacerbated pain and negatively impacted patient quality of life[19]. Thus, the decision to terminate NSAIDs should be carefully considered.

In contrast, the cessation of NSAIDs may cause cardiovascular events. Fischer *et al*[20] conducted a large case-control study and suggested that the risk of developing acute myocardial infarction (AMI) is increased for a period of several weeks after discontinuing NSAID use, particularly in patients using NSAIDs on a long-term basis. Their study indicated that the risk of AMI was not increased in patients currently using NSAIDs or in users who stopped using NSAIDs more than 3 mo before. In this study, no patients had a high risk of AMI such as long-term internal users or regular users. Therefore, caution should be exercised in such patients when discontinuing NSAIDs.

Hemodialysis was associated with rebleeding in the present study. Intermittent use of heparin for dialysis treatment and the presence of uremia-induced platelet dysfunction are associated with GI bleeding[21,22]. In addition, patients with chronic kidney disease demonstrate abnormalities in blood coagulation and bleeding predisposition, resulting in consistent anemia in UGIB cases[22,23]. Thus, hemodialysis patients with CDB should be carefully managed after discharge.

In this study, we were able to prospectively collect detailed information on comorbidities and medication, and to conduct long-term follow-up of patients hospitalized for CDB. Nonetheless, there are several limitations to this study. First, the number of patients who discontinued NSAIDs was relatively small. Second, no data on hemodynamic instability, hematocrit, and abnormal white blood cell count were collected. Third, the study had a non-randomized controlled design.

In conclusion, discontinuation of NSAIDs is an effective preventive measure against rebleeding in CDB. Patient education about the reduction in the risk of bleeding is important, but medication withdrawal should be considered in view of its risks and benefits, particularly in patients of advanced age or with comorbidities.

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COMMENTS

Background

Even though intensive management for diverticular bleeding is successful during the hospital stay, patients are at high risk of recurrent bleeding following discharge. Unlike upper gastrointestinal (GI) bleeding, there are no preventive therapies for lower GI bleeding. Thus, preventing recurrence requires risk factors to be identified.

Research frontiers

The use of non-steroidal anti-inflammatory drugs (NSAIDs) is a significant risk factor for recurrence, but the risks and benefits of their discontinuation on diverticular bleeding have not been examined.

Innovations and breakthroughs

In total, 39 patients (30%) had rebleeding during a median follow-up of 15 mo. Of the 41 NSAID users on admission, 26 (63%) discontinued NSAID use at discharge. Multivariate analysis revealed continued NSAID use (HR = 4.6, $P < 0.01$) and hemodialysis (HR = 3.0, $P < 0.01$) as independent risk factors for rebleeding. Discontinuation of NSAIDs significantly reduced rebleeding risk

compared with continued use (log-rank test, $P < 0.01$).

Applications

Discontinuation of NSAIDs is an effective preventive measure against rebleeding in diverticular bleeding. However, medication withdrawal should be considered in view of its risks and benefits, particularly in patients of advanced age or with comorbidities.

Terminology

The precise mechanisms of NSAID-induced diverticular bleeding has been suggested to be due to the inhibition of platelets, cyclooxygenase-1, and prostaglandin synthesis in the lower GI tract. Mucosal injury due to the inhibition of intestinal prostaglandin synthesis might lead to the development of intestinal erosion and ulcers. When mucosal ulceration induced by NSAIDs occurs at the neck or dome of the diverticula, nutrient-providing arteries rupture into the colonic lumen with the potential to cause sudden bleeding.

Peer review

The manuscript investigates the impact of discontinuing NSAIDs and the long-term recurrence in colonic diverticular bleeding. The authors tried to bring new ideas about preventive measure against colonic diverticular re-bleeding.

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ORIGINAL ARTICLE

Prospective Study

Impact of enteral nutrition on energy metabolism in patients with Crohn's disease

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Jie Zhao, Jian-Ning Dong, Jian-Feng Gong, Hong-Gang Wang, Yi Li, Liang Zhang, Lu-Gen Zuo, Yun Feng, Li-Li Gu, Ning Li, Jie-Shou Li, Wei-Ming Zhu, Department of General Surgery, Jinling Hospital, Medical School of Nanjing University, Nanjing 210002, Jiangsu Province, China

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Abstract

AIM: To investigate the impact of enteral nutrition (EN) on the body composition and metabolism in patients with Crohn's disease (CD).

METHODS: Sixty-one patients diagnosed with CD were enrolled in this study. They were given only EN (enteral nutritional suspension, TPF, non-elemental diet) support for 4 wk, without any treatment with corticosteroids, immunosuppressive drugs, infliximab or by surgical operation. Body composition statistics such as weight, body mass index, skeletal muscle mass (SMM), fat mass, protein mass and inflammation indexes such as C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and CD activity index (CDAI) were recorded before and after EN support.

RESULTS: The 61 patients were divided into three groups according to CDAI before and after EN support: A (active phase into remission *via* EN, n = 21), B (remained in active phase before and after EN, n = 19) and C (in remission before and after EN, n = 21). Patients in group A had a significant increase in SMM (22.11 ± 4.77 kg *vs* 23.23 ± 4.49 kg, P = 0.044), protein mass (8.01 ± 1.57 kg *vs* 8.44 ± 1.45 kg, P = 0.019) and decrease in resting energy expenditure (REE) per kilogram (27.42 ± 5.01 kcal/kg per day *vs* 22.62 ± 5.45 kcal/kg per day, $P < 0.05$). There was no significant difference between predicted and measured REE in active CD patients according to the Harris-Benedict equation. There was no linear correlation between the measured REE and CRP, ESR or CDAI in active CD patients.

CONCLUSION: EN could decrease the hypermetabolism in active CD patients by reducing the inflammatory response.

Key words: Crohn's disease; Enteral nutrition; Body composition; Metabolism

Core tip: Unlike traditional research that uses normal volunteers or ulcerative colitis patients as the control group, this study aimed to observe the same patient in different phases of Crohn's disease (CD), and in this study, several confounding factors, such as height, age, gender and race, were removed. This study showed that enteral nutrition could decrease the hypermetabolism in active CD patients by reducing the inflammatory response.

Zhao J, Dong JN, Gong JF, Wang HG, Li Y, Zhang L, Zuo LG, Feng Y, Gu LL, Li N, Li JS, Zhu WM. Impact of enteral nutrition on energy metabolism in patients with Crohn's disease. *World J Gastroenterol* 2015; 21(4): 1299-1304 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1299.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1299

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing-remitting inflammatory bowel disease (IBD) of unknown etiology[1]. Nutritional deficits are very common in IBD, particularly in CD, which are attributed to many reasons including anorexia, active inflammation, and increased intestinal nutrient losses. Some researchers have indicated that approximately 65% to 75% of CD patients are malnourished[2]. Therefore, nutritional support can be important to medical therapy in the management of CD[3]. In Western countries, the role of enteral nutrition (EN) in CD is controversial[4,5]; however, many studies in Japan have shown that EN was effective not only in maintaining remission but also in inducing remission of CD. EN can also decrease the hospitalization rate in patients with CD[6]. In Japan, EN has been advocated as the primary therapy for both active and quiescent CD in accord with the guidelines of the Japan Ministry of Health, Labor and Welfare[7-10].

Many studies have found that the disease activity has a close relationship with body composition in CD patients. However, so far, no researchers can have definitively described the relationship between them. Some researchers have proposed that CD patients in active phase had significant deficits in lean mass but preserved fat mass compared with patients in remission[11-13]. However, others shown that fat mass decreased in active phase, to the same extent as muscle mass[14].

Resting energy expenditure (REE) was also closely related to disease activity. Nevertheless, some studies that investigated REE in CD have suggested that energy expenditure is raised, particularly in the active phase[15,16], while others have suggested that REE is unchanged. The inflammatory process associated with the active disease is more than capable of increasing REE above what would be expected[17]. The exact relationship between body composition, metabolism and disease activity in CD patients requires well-designed trials in large cohorts of patients. The impact of EN support on body composition and REE in CD patients is poorly understood in the therapeutic course.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee of Jinling Hospital, and informed consent was given to each patient.

Study design

This study was aimed at finding out the impact of EN on body composition and metabolism in CD patients.

This was a prospective, single-center study undertaken at the Jinling Hospital.

Patients

Inclusion criteria for patients were (1) age between 18 and 60 years; (2) endoscopic and histological diagnosis of CD; (3) no operations over the past six months; (4) can tolerate total EN; (5) no systemic diseases that greatly influence metabolism, such as diabetes mellitus and hyperthyroidism; (6) nutritional deficiencies; or (7) no severe symptoms (such as acute strangulated intestinal obstruction). Exclusion criteria were (1) cannot tolerate EN or malabsorption syndrome; (2) medication use such as corticosteroids; and (3) surgery. Sixty-one consecutive patients (43 males and 18 females; mean age, 33.4 years) who met the criteria were included.

Treatment

All of the patients included were fasted and given only EN (enteral nutritional suspension, TPF, non-elemental diet) support for 4 wk, without any treatment with corticosteroids, immunosuppressive drugs, infliximab or by surgical operation. The patients were naso-gastrically fed TPF, a type of intact-protein nutrition (bottled preparations, net content of one 500 mL bottle: 20 g of protein, 19.5 g of fat, 61.5 g of carbohydrates; 1 mL of TPF provides 1 kcal of energy) as for the quantity of daily enteral formula designed by the measured REE before EN support calculated by indirect calorimetry. An appropriate amount of exercise was also required for the patients.

Assessment

The clinical disease activity was determined as CD activity index (CDAI). The active phase was defined as CDAI $\geqslant$ 150 and remission as CDAI < 150. The included patients were divided into three groups according to CDAI before and after

Table 1 General information of patients among the three groups

	Group A (n = 21)	Group B (n = 19)	Group C (n = 21)	P value
Male (n)	15	12	16	
Female (n)	6	7	5	0.83
Age (yr)	40.3 ± 12.6	39.6 ± 11.7	40.6 ± 14.0	0.97
BMI (kg/m^2)	16.68 ± 2.21	17.96 ± 3.37	17.74 ± 2.90	0.31
CD type (n)				
Ileal	8	6	9	
Ileo-colonic	5	6	6	
Colonic	8	7	6	0.88
Duration (yr)	2.41 ± 1.01	2.28 ± 1.22	2.33 ± 1.25	0.94

P-values were calculated by χ^2 test or one-way analysis of variance. Group A: Active phase in remission *via* enteral nutrition (EN); group B: Remained in active phase before and after EN; group C: In remission before and after EN. BMI: Body mass index; CD: Crohn's disease.

EN support; group A (active phase in remission *via* EN, n = 21), group B (remained in active phase before and after EN, n = 19) and group C (in remission before and after EN, n = 21). The data collected before and after EN support were the blood inflammation indexes [hs-C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and CDAI], the body composition [weight, body mass index (BMI), skeletal muscle mass, fat mass and protein mass] and REE measured by professional and technical personnel using indirect calorimetry. The measurement of REE requires adherence to strict conditions including environmental temperature, fasting, reclining supine for a 30-min period (in our study), and rest to obtain repeatable values (the temperature difference before and after EN was less than 0.3 ℃, excluding the impact of temperature on the REE). Predicted REE was commonly calculated using the Harris-Benedict equation (variables: age, weight, height and gender), the Schofield equation (variables: age, weight and gender) or just from previous experimental data (some Chinese studies have declared that 25 kcal/kg per day can be used to conveniently evaluate the REE of Chinese people). The correlations between REE and inflammation markers were also evaluated by linear analysis.

Statistical analysis

The data gathered were analyzed using SPSS 19.0. Statistical analysis between groups was performed using paired-samples t-test and independent-samples t-test, and linear analysis was used to evaluate the relations between REE and markers of inflammation markers. Significance was set at P < 0.05.

RESULTS

This was a prospective study. In total 61 patients were evaluated in our cohort. The majority of the patients were men (43 out of 61, mean age 36.5 ± 12.5 years) and women accounted for the rest (mean age 42.3 ± 14.2 years). Among all of the patients in our cohort, 23 had ileal CD, 17 had ileo-colonic and 21 colonic. The 61 patients were divided into the following three groups according to the efficiency of EN support for 4 wk: A (active phase into remission *via* EN, n = 21), B (remained in the active phase *via* EN, n = 19) and C (in remission before and after EN, n = 21). Table 1 shows that the ileal involvement distribution and duration of the disease among the three groups showed no statistical difference (P > 0.05).

Body composition

Body composition data in each group before and after EN support are shown in Table 2. The BMI of all of the enrolled patients increased significantly (P = 0.017). In group A, the protein mass (P = 0.019) and skeletal muscle mass (P = 0.044) had a statistical increase after EN support that was not observed for fat mass (P = 0.263). The minerals of patients among three groups remained the same before and after EN (P > 0.05), while the level of 25(OH)D$_3$ of patients in groups A and B had a significant increase (P < 0.05). Nevertheless, as indicated, no significant differences were found between the body composition before and after EN support in either group B or C (P > 0.05).

Markers of inflammation

In our cohort, the rate of EN induced remission of CD was 52.5% (group A/group A + B, 21/40). The objective of the study was to assess the inflammation indexes of patients in active phase before EN support (groups A and B). When the active groups, groups A and B, were analyzed, significant differences were consistently observed in CRP (P < 0.05), ESR (P < 0.05) and CDAI (P < 0.05) after EN support for 4 wk compared to the level before EN support, all of which are explicitly demonstrated in Table 3.

Energy metabolism

As shown in Figure 1, REE per kilogram in group A had a significant decrease *via* EN support (P = 0.025), different from groups B and C (P = 0.091 and 0.309, respectively).

The results showed that the experimental 25 cal/kg would undoubtedly underestimate the REE of CD patients (Figure 2A; P = 0.025), but there were no significant differences between the actual REE and predicted REE using the Harris-Benedict equation (P = 0.888) (Figure 2B). In addition, no positive linear correlation between REE (baseline values) and CRP (r = -0.511, P = 0.21), ESR (r = -0.395, P = 0.085) or CDAI (r = 0.185, P = 0.435) was found, as we expected.

Table 2 Comparison of body composition of patients with Crohn's disease before and after enteral nutrition support for 4 wk (mean ± SD)

Index	Group A (n = 21)			Group B (n = 19)			Group C (n = 21)		
	Before EN	After EN	P value	Before EN	After EN	P value	Before EN	After EN	P value
BMI (kg/m^2)	16.68 ± 2.61	17.31 ± 2.55	0.013	17.96 ± 3.37	18.06 ± 2.61	0.92	17.74 ± 2.90	18.11 ± 2.76	0.67
SMM (kg)	22.11 ± 4.77	23.23 ± 4.49	0.044	21.62 ± 4.15	21.79 ± 4.36	0.90	22.29 ± 5.84	23.38 ± 4.52	0.50
Fat mass (kg)	4.80 ± 4.31	4.14 ± 3.35	0.260	8.53 ± 3.81	9.64 ± 3.92	0.38	8.06 ± 1.92	8.14 ± 1.75	0.89
Protein (kg)	8.01 ± 1.57	8.44 ± 1.45	0.019	7.87 ± 1.35	7.85 ± 1.47	0.96	7.21 ± 4.72	8.34 ± 4.66	0.44
Minerals(kg)	3.56 ± 2.12	3.61 ± 1.98	0.860	3.39 ± 2.50	3.31 ± 2.01	0.79	3.43 ± 3.10	3.49 ± 2.44	0.65
25(OH)D$_3$ (ng/mL)	10.8 ± 4.8	12.3 ± 4.1	0.014	10.6 ± 3.9	12.0 ± 4.2	0.02	11.7 ± 4.3	12.2 ± 5.0	0.10

P-values were calculated by paired-samples t test. Group A: Active phase in remission *via* enteral nutrition (EN); group B: Remained in active phase before and after EN; group C: In remission before and after EN. BMI: Body mass index; SMM: Skeletal muscle mass.

Table 3 Comparison of inflammation indexes of patients with Crohn's disease in active phase (groups A and B) before and after enteral nutrition support for 4 wk (mean ± SD)

Index	Group A (n = 21)			Group B (n = 19)			Active groups (A + B, n = 40)		
	Before EN	After EN	P value	Before EN	After EN	P value	Before EN	After EN	P value
CRP (mg/L)	27.17 ± 31.60	10.37 ± 14.42	0.019	28.75 ± 16.29	16.30 ± 14.70	0.031	28.09 ± 31.21	10.00 ± 14.56	0.024
ESR (mm/h)	29.14 ± 15.12	16.25 ± 12.41	0.020	27.96 ± 16.88	20.12 ± 14.01	0.042	28.65 ± 17.35	18.30 ± 18.73	0.030
CDAI	239.21 ± 52.60	126.10 ± 33.21	0.013	226.18 ± 60.24	188.02 ± 49.33	0.045	230.93 ± 61.69	174.32 ± 68.52	0.044

P-values were calculated by paired-samples t test. CRP: C-reactive protein; ESR: Erythrocyte sedimentation rate; CDAI: Crohn's disease activity index; EN: Enteral nutrition.

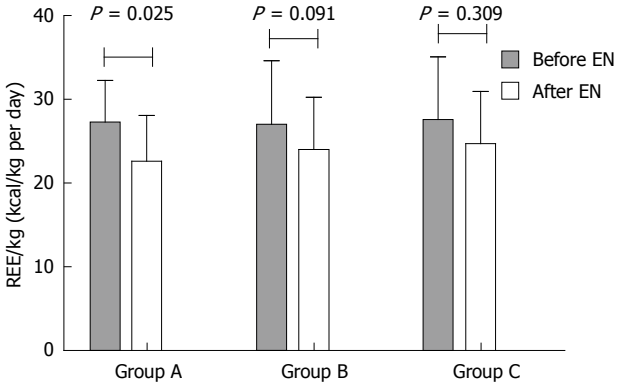

Figure 1 Difference of the measured resting energy expenditure before and after enteral nutrition support in each group. Metabolism was measured by resting energy expenditure (REE) per kilogram. P-values were calculated by paired-samples t-test. EN: Enteral nutrition.

DISCUSSION

Some researchers have proposed that CD patients in the active phase have significant deficits in lean mass but preserved fat mass compared with patients in remission[11-13]. However, others believe that fat mass decreases in the active phase, along with muscle mass[14]. EN can improve the BMI, skeletal muscle and protein mass in active CD patients while inducing CD remission, most likely by correcting the state of negative nitrogen balance, increasing the storage and decreasing the expenditure of the muscle and protein mass. Increased REE further increases REE, most likely in response to the increase of organ mass which is more metabolically active than skeletal muscle[15,16].

Studies have shown that the measured REE in CD patients is significantly higher than that of healthy controls[17], though no linear correlation between the measured REE and CRP, ESR or CDAI has been observed. Predicting the REE with the experimental 25 cal/kg would underestimate REE of CD patients, which is shown in Figure 2A. No significant difference between the actual REE and REE predicted using the Harris-Benedict equation was found. The Harris-Benedict equation was suitable for patients in Western countries. Chinese studies have shown that predicting the REE by the Harris-Benedict equation was usually 20% to 30% higher than the measured REE in normal people. Therefore, the REE in active CD patients was much higher than that in normal people, as proved by many previous studies[18]. The REE predicted by the Harris-Benedict equation was roughly the same as the measured REE of active CD patients in this study with a restricted number of patients. The opinion that the REE likely decreases with increasing disease activity has been recently proposed[16]. Some studies have suggested that the increased REE in CD patients is related to the increased lean tissue instead of the hypermetabolism in CD patients[19,20]. However, this study demonstrated that for the same patient REE in the active phase of CD was significantly elevated compared with the REE in remission with an increase of lean tissue. The result proves that the state of hypermetabolism actually exists in active

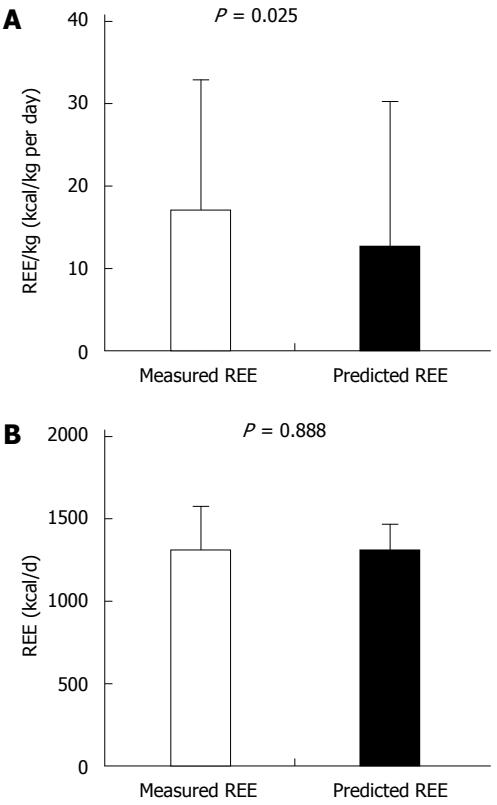

Figure 2 Measured resting energy expenditure *vs* predicted resting energy expenditure. Measured resting energy expenditure (REE) *vs* predicted REE using 25 kcal/kg (A) and the Harris-Benedict (H-B) equation (B). The comparison was aimed at patients in the active phase before enteral nutrition support (group A + B). *P*-values were calculated by using a paired-samples *t*-test.

CD patients. Increased REE with unmatched dietary intake is amongst the many proposed mechanisms for the poor nutritional status of patients with CD. During nutritional therapy for CD patients, the REE variation with disease activity should be a primary consideration, but whether they had the accurate correlation still confused researchers. Some studies have examined the effect of disease activity on children with CD and have shown either no change in REE with disease activity[16,21,22] or increased REE at times of active disease[23].

Routine energy supplements for patients with active CD cannot be justified on the basis of predicted REE or just by experience. Individual management plans are essential and emphasis should be placed on the assessment of total energy needs (including the hypermetabolism and activity level) and titrating intake against weight gain to optimize energy balance and thereby promote body composition[24,25]. The IBD study group of the Japanese Ministry of Health, Labor and Welfare recommended that the total energy of TPN or EN should be 40-45 kcal/body weight/d in active CD patients. In contrast, European guidelines recommend that 25-30 kcal/body weight/d is optimal for active CD[26,27]. The energy metabolism status of active CD patients varied while they took EN in the remission induction therapy. Therefore, timing detection of the REE is recommended to appropriately assess the nutritional requirements of CD patients.

COMMENTS

Background

Nutritional deficits are very common in inflammatory bowel disease (IBD), particularly in Crohn's disease (CD), in which nutritional deficits are attributed to many causes, including anorexia, active inflammation, and increased intestinal nutrient losses. Enteral nutrition (EN) was not only effective in maintaining remission but also in inducing remission of CD. EN has been advocated as a primary therapy for both active and quiescent CD. The impact of EN on body composition and metabolism in CD patients remains inconclusive when EN induced CD remission.

Research frontiers

EN support plays an important role in the treatment of IBD, particularly CD. The quantity, time, type and appropriate use of EN for CD patients attracted much attention from IBD researchers.

Innovations and breakthroughs

Unlike traditional research that used normal volunteers or ulcerative colitis patients as the control group, this study aimed at observing the same patient in different phases of CD and eliminated several confounding factors, such as height, age, gender and race. The results showed that EN could decrease the hypermetabolism in active CD patients by reducing the inflammatory response.

Applications

This study provided new information about the proper quantity of EN for CD patients. To appropriately assess the nutritional requirements of CD patients, dynamic monitoring of resting energy expenditure (REE) is recommended.

Terminology

Metabolism was measured by REE per kilogram. The inflammatory status of CD patients was evaluated with C-reactive protein, erythrocyte sedimentation rate and CD activity index.

Peer review

It is an interesting study, although the number of the patients enrolled is small. I think this article is a good study about EN that is effective in remission induction of active CD and also has an impact on the body metabolism and composition of CD patients.

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META-ANALYSIS

Accuracy of urea breath test in *Helicobacter pylori* infection: Meta-analysis

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Abstract

AIM: To quantitatively summarize and appraise the available evidence of urea breath test (UBT) use to diagnose *Helicobacter pylori* (*H. pylori*) infection in patients with dyspepsia and provide pooled diagnostic accuracy measures.

METHODS: We searched MEDLINE, EMBASE, Cochrane library and other databases for studies addressing the value of UBT in the diagnosis of *H. pylori* infection. We included cross-sectional studies that evaluated the diagnostic accuracy of UBT in adult patients with dyspeptic symptoms. Risk of bias was assessed using QUADAS (Quality Assessment of Diagnostic Accuracy Studies)-2 tool. Diagnostic accuracy measures were pooled using the random-effects model. Subgroup analysis was conducted by UBT type (^{13}C *vs* ^{14}C) and by measurement technique (Infrared spectrometry *vs* Isotope Ratio Mass Spectrometry).

RESULTS: Out of 1380 studies identified, only 23 met the eligibility criteria. Fourteen studies (61%) evaluated ^{13}C UBT and 9 studies (39%) evaluated ^{14}C UBT. There was significant variation in the type of reference standard tests used across studies.Pooled sensitivity was 0.96 (95%CI: 0.95-0.97) andpooled specificity was 0.93 (95%CI: 0.91-0.94). Likelihood ratio for a positive test was 12 and for a negative test was 0.05 with an area under thecurve of 0.985. Meta-analyses were associated with a significant statistical heterogeneity that remained unexplained after subgroup analysis. The included studies had a moderate risk of bias.

CONCLUSION: UBT has high diagnostic accuracy for detecting *H. pylori* infection in patients with dyspepsia. The reliability of diagnostic meta-analytic estimates however is limited by significant heterogeneity.

Key words: *Helicobacter pylori*; Dyspepsia; Breath tests; Urea/analysis; Diagnosis; Sensitivity; Specificity; Gastritis; Positive predictive value; Negative predictive value

Core tip: Urea breath test (UBT) is a commonly used non-invasive test to diagnose *Helicobacter pylori* (*H. pylori*) infection in patients with dyspepsia. Multiple trials are available in literature, but they reported different diagnostic accuracy estimates. We conducted systemic review and meta-analysis to explore the available evidence and provide pooled diagnostic accuracy measures. Our meta-analysis showed that UBT has high diagnostic accuracy for detecting *H. pylori* infection in patients with dyspepsia. Given the potentially preventable diseases associated with chronic, untreated *H. pylori* infection, more widespread adoption of UBT testing may be indicated.

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium found on the luminal surface of the gastric epithelium. It was first isolated by Warren and Marshall in 1983. It induces chronic inflammation of the underlying mucosa. The infection is usually contracted in the first few years of life and tends to persist indefinitely unless treated. At least 50% of the world's population is thought to carry *H. pylori*. The organism can survive in the acidic environment of the stomach partly owing to its remarkably high urease activity. Urease converts the urea present in gastric juice to alkaline ammonia and carbon dioxide[1].

Although the full spectrum of pathogenesis is currently unknown, *H. pylori* has been linked to a variety of upper gastrointestinal disorders. Reported symptoms of *H. pylori* infection are relatively non-specific, such as epigastric pain, postprandial fullness, bloating, nausea, and vomiting, along with signs of acid hypersecretion and delayed gastric emptying[2,3]. In addition, infection with *H. pylori* is linked to three important upper gastrointestinal diseases: duodenal or gastric ulcers, gastric cancer, and gastric mucosa-associated lymphoid-tissue lymphoma.

Many invasive and non-invasive methods can be used to diagnose *H. pylori* infection, including endoscopy with biopsy, serology for immunoglobulin titers, stool antigen analysis, and the urea breath test (UBT). Given the user-friendly, non-invasive features of UBT, this detection method may be preferred in many clinical settings. However, to date, the performance characteristics of UBT have been inconsistently described and remain incompletely defined.

UBT can play a useful role in the diagnostic evaluation of dyspeptic patients who have comorbidities that increase their risk of upper endoscopy, are intolerant to upper endoscopy, or have known or suspected gastric atrophy. Stool antigen testing can also be used to non-invasively detect active *H. pylori* infection, and the choice of diagnostic modality depends on factors such as cost, laboratory infrastructure, and concomitant use of medications such as proton pump inhibitors or antibiotics that may influence test results. Serum antibody test results can vary by geographic region, and may stay positive for a prolonged period following *H. pylori* eradication, thereby limiting the clinical utility for determining the presence or absence of current infection[4].

There are two UBTs available and gained Food and Drug Administration approval: ^{13}C and ^{14}C tests. Both tests are affordable and can provide real-time results. Some physicians may prefer the ^{13}C test as it is non-radioactive compared to ^{14}C which uses a radioactive isotope, especially in young children and pregnant women, though dose of radianis very minimal (about 1 microCi)[5]; the dose of radiation is the dose of ^{14}C-UBT with the mini dose equals to 1 microCi (37 kbq) which has a high diagnostic accuracy[6]. UBT is indicated to confirm *H. pylori* colonization and to monitor its eradication. Positive UBT indicates an active *H. pylori* infection which require treatment or further confirmation with invasive procedures. Initial treatment for *H. Pylori* consist of either triple, quadruple, or sequential therapy regimens, which all of them includes a proton pump inhibitor plus various antibiotic regimen; treatment periods generally varied from 7 to 14 d[4].

In this systematic review and meta-analysis, we aimed at summarizing data and appraising the relevant articles of UBT for diagnosis of *H. pylori* infection in dyspeptic patients and provide pooled diagnostic accuracy measures.

MATERIALS AND METHODS

Search and analysis methods, eligibility criteria, and the outcomes of interest were specified in advance in a protocol developed by study investigators.

Inclusion criteria

We included cross-sectional studies with consecutive patients that evaluated the diagnostic accuracy of UBT in adult patients with dyspeptic symptoms. We included articles that compare ^{13}C-UBT or ^{14}C-UBT

H. pylori test with a reference standard which is *H. pylori* (culture and/or histological examination) and/or not (serologic test either blood or stool).

We excluded studies that enrolled children or adolescents under 18 year of age, subjects who presented for reasons other than dyspeptic symptoms, bleeding peptic ulcer, complicated dyspeptic cases that need surgery, those who received previous therapy for *H. pylori* within the last 3 mo, or long term use of corticosteroids and immunosuppressant drugs and screening studies. Only articles presenting true positive, true negative, false positive and false negative data were included in the present study. Studies where data was missing and studies with high risk of bias were excluded.

UBT variants

There was no inclusion restriction on the type of UBT performed. Both ^{13}C and ^{14}C types where included. Studies where the UBT was performed through an invasive method were excluded.

Search strategy

A librarian searched electronic databases for published and in-press studies from 1990 (the date where UBT became available) through November 2013 including PubMed, EMBASE, LILACS and Cochrane databases. The search terms used were "*H. pylori*", "*Helicobacter pylori*", "*Helicobacter* infection", "gastritis", "dyspepsia", "breath test", "urea breath test", "UBT", "^{13}C-UBT" and "^{14}C-UBT" with its MeSH terms (Medical Subject Headings) and keywords. We used Boolean operator (OR) to combine synonyms and (AND) to combine the cases with tests. No language restriction was applied. Reference lists were also scanned.

Study and data selection

Two authors (MF, WM) screened titles and abstracts for inclusion criteria. Full text articles were retrieved for relevant articles. An abstraction format developed by authors that includes: study citation, author name and year of publication, patients' mean age and other baseline characteristics, UBT variant, gold standard used, time between the test and gold standard, description of the cases, and diagnostic study data (numbers of true positive, false positive, false negative, and true negative test results). Disagreement was resolved by consensus.

Quality assessment

Two reviewers (MF and IY) independently assessed the quality of the included studies using the QUADAS (Quality Assessment of Diagnostic Accuracy Studies)-2 instrument[7]. This tool is designed to assess the quality of primary diagnostic accuracy studies for inclusion in the systematic review. It consists of four key domains covering patient selection, index test, reference standard, and flow of patients through the study and timing of the index test(s) and reference standard. Each domain is assessed in terms of the risk of bias and the first three are also assessed in terms of concerns regarding applicability.

Risk of bias is judged as "low", "high", or "unclear". If all signaling questions for a domain are answered "yes" then risk of bias can be judged "low". If any signaling question is answered "no" this flags the potential for bias.

We considered low risk of bias in different domains as follows: Patient selection if non-complicated dyspeptic patients were enrolled in consecutively. Index test, where it was interpreted independent from the reference standard. Reference standard, when it correctly classifies *H. pylori* and non-*H. pylori*. Flow and time, the appropriate interval between index test and the reference standard is within 7 d, and breathing samples were collected within 30 min.

Meta-analysis

The meta-analysis was conducted using Meta-Disc 1.4[8]. Random effect model was followed in all analyses. The diagnostic accuracy measures used in the analysis were sensitivity, specificity, likelihood ratio for positive and negative test (LR+ and LR-), receiver operating characteristics (ROC) curve, and diagnostic odds ratio. We assessed heterogeneity using the I-squared statistic and *Q* test. Publication bias was conducted using the Deeks' funnel plot asymmetry test, with *P*-value < 0.05 for the slope coefficient indicating significant asymmetry[9].

Subgroup and sensitivity analyses

To explore the robustness of our results and evaluate for potential causes of heterogeneity, we conducted several a priori determined analyses. We tested the bivariate mixed effects regression model to determine if results were robust to the correlation between sensitivity and specificity. Bivariate analysis were conducted as implemented in STATA version 12.0 (StataCorp, College Station, TX, United States)[10].

We also conducted subgroup analyses based on the risk of bias in the included studies as it pertains to the various domains of QUADAS-2 tool (such as for the index test and the gold standard test). We evaluated if the type of UBT test (^{13}C *vs* ^{14}C) or measurement technique (isotope ration mass spectrometry *vs* infrared mass spectrometry) affected the pooled estimates. We conducted an interaction test for subgroup analyses as suggested by Altman and Bland[11] and there was no statistically significant difference to suggest a subgroup effect.

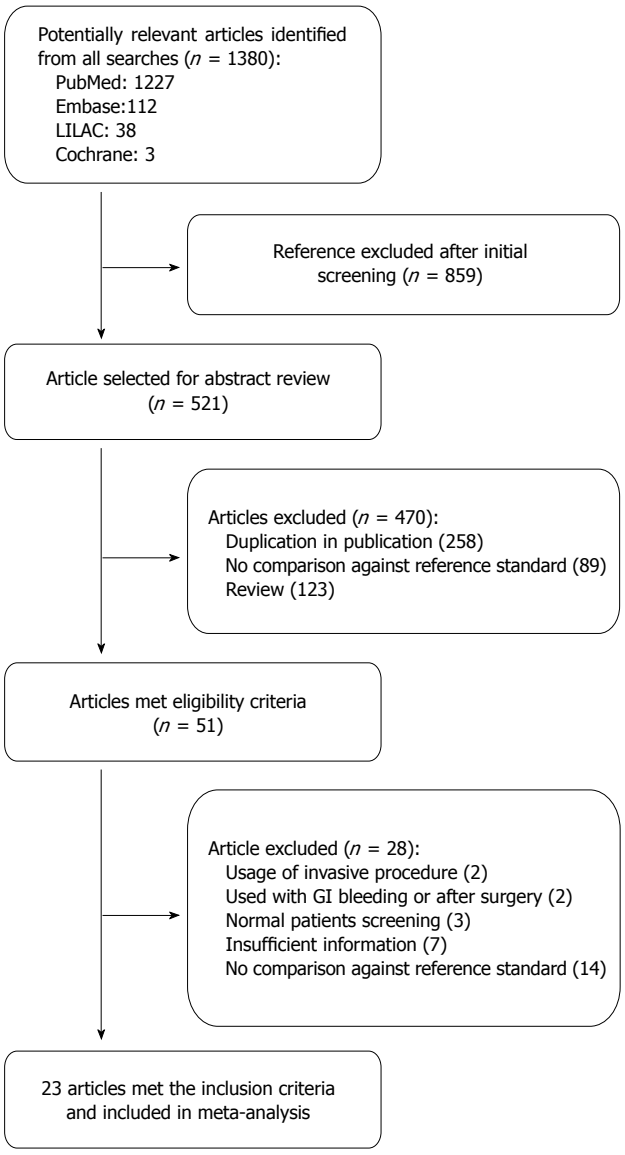


Figure 1 Study selection process.

RESULTS

Search results

The initial search yielded 1380 studies that were potentially relevant; of which, 23 studies that enrolled a total of 3999 participants were finally included. The study selection process is depicted in Figure 1 including causes of exclusion. More than 50% of quality assessment items articles have low risk of bias of all domains. The agreement between risk of bias assessment between reviewers were 70%, disagreement was resolved by discussion and consensus. Figure 2 visually summarizes the risk of biasin the included studies.

Characteristics of included studies

Table 1 shows the characteristics of all included studies. Of the 23 studies, 14 studies (61%) compared ^{13}C UBT with a reference standard, while 9 studies (39%) used ^{14}C UBT. The included studies were conducted in 16 countries, however all but one were published in English (Spanish)[12]. The mean age across studies was (40-59 year) and female gender distribution was (13%-74%).

There was variation (10 folds) in the type of reference standard tests used by different studies (Table 2). Seven studies (30.4%) used one reference standard starting with either histopathology or culture at first and only used subsequent tests if the first test was negative (histopathology in three studies[13-15], and culture in four studies[16-19]). Two studies (8.7%)[20,21] used histopathology or culture, nine studies (39.1%)[12,22-24] used two combined tests (histopathology and rapid urease test "RUT" in four studies, histopathology and serology in one study[25], histopathology and culture in one study[26], and any two tests in three studies[27-29]. Four studies (17.4%)[30-33] used three combined tests, and one study (4.3%)[3] used four combined tests as reference standard. Histopathology is the most common approach when combined tests were used. In three studies[3,27,31], UBT was part of combined reference standards.

Pooled estimate for UBT (Combined ^{13}C and ^{14}C)

UBT had high sensitivity and specificity 0.96 (95%CI: 0.95-0.97) and 0.93 (95%CI: 0.91-0.94); respectively. LR+ and LR- were 12.32 (95%CI: 8.38-18.1) and 0.05 (95%CI: 0.03-0.07) respectively. The AUC was 0.985. Forest plots are depicted in Figure 3. There was no evidence of publication bias (P > 0.05 using Deeks' asymmetry test).

Test of heterogeneity

Inconsistency between results for sensitivity and specificity among studies were 72.9% and 72% respectively with statistically significant Q test (P < 0.05). Heterogeneity could be explained by either clinical or methodological variation; the performed subgroup analyses could not explain the difference.

Subgroup analysis

^{13}C UBT *vs* ^{14}C UBT: Of the total studies recruited in this systematic review, 14 were conducted using ^{13}C UBT *vs* 9 using ^{14}C UBT (Table 3). Both versions of the test showed high performance against the Gold standard test without a significant difference. Figures are shown in online supplement materials (Figure 3). Interaction test for subgroup analyses as suggested by Altman and Bland[11] showed no statistically significant difference to suggest a subgroup effect (P = 0.87).

Use of infrared in UBT: Out of total 23 studies, 6 studies used infrared technique in measuring urea level. Both methods showed high performance against the gold standard test without a significant difference. Subgroup analysis based on the risk

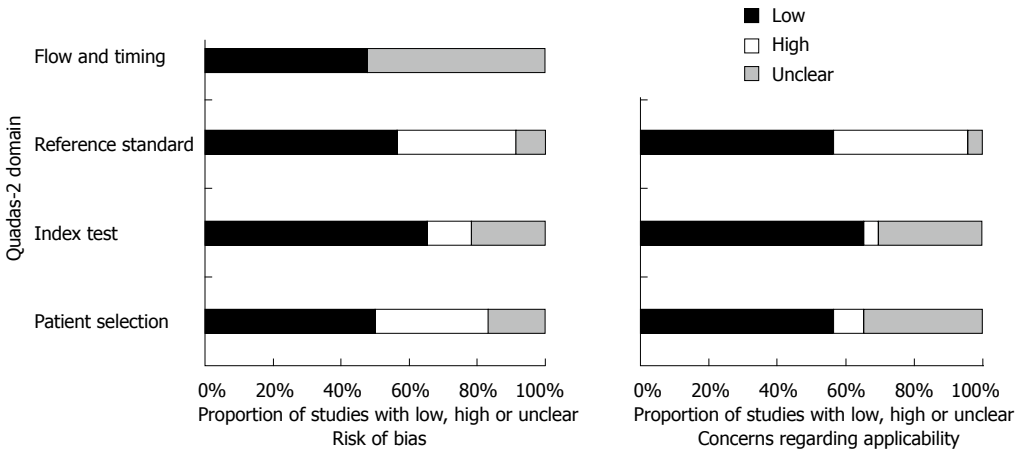


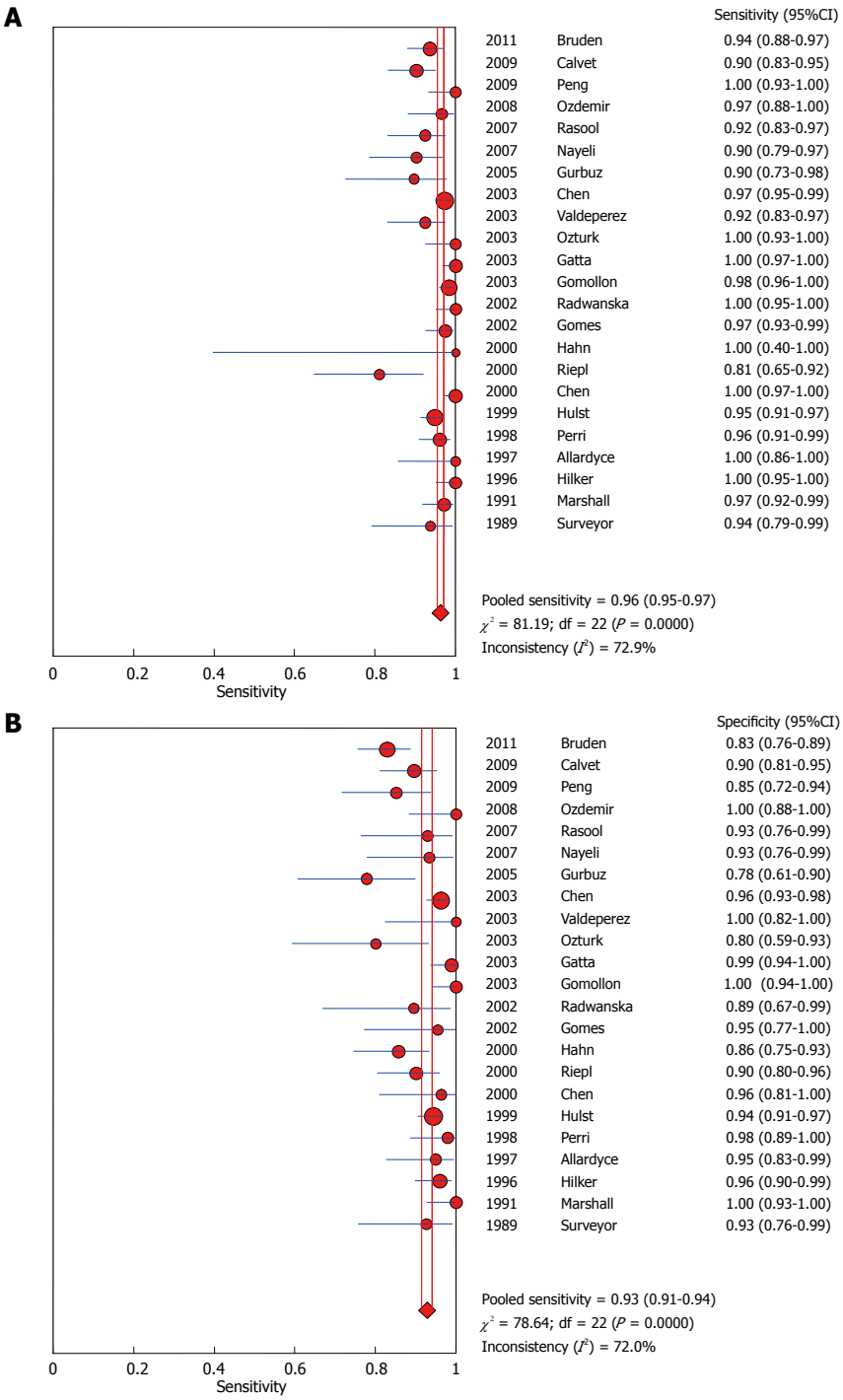
Figure 2 Risk of bias assessment.

Table 1 Baseline characteristics of the included studies

Ref.	Country	Year	No. of patients	Study design	UBT ($^{13}C/^{14}C$)	Infrared assisted	Reference standard	Mean age (mean ± SD)	Females	UBT threshold	Time
Allardyce *et al*[13]	New Zealand	1997	63	Cross-sectional	^{14}C	No	Histo or (Biopsy and rapid urea test)	56.5	26 41%	82% DPM	30 min and 60 min post ingestion
Bruden *et al*[16]	Estonia	2011	280	Cross-sectional	^{13}C	No	Culture or (Histo and RUT)	53.5	185 66%	≥ 5%	NA
Calvet *et al*[27]	Spain	2009	199	Cross-sectional	^{13}C	Yes	Any two positive (Histopathology, RUT, UBT, and fecal serology)	48.2 ± 14.2	107 53%	8.5%	20 min after drinking solution
Chen *et al*[29]	Taiwan	2003	586	Cross-sectional	^{13}C	Yes	Culture alone or RUT	45.7 ± 13.3	280 46.6%	≥ 2%	20 min after drinking solution
Chen *et al*[25]	Japan	2000	169	Cross-sectional	^{13}C	No	Combined (Histo and serology)	53.9 ± 15.7	68 40%	2.5%	20 min after normal respiration
Gatta *et al*[30]	Italy	2003	200	Cross-sectional	^{13}C	No	Combined (Histology and rapid urease) and/or culture	53 ± 13	113 56%	NA	30 min post ingestion
Gomes *et al*[22]	Brazil	2002	137	Cross-sectional	^{14}C	No	Combined (Histo and RUT)	46.7 ± 16.6	67 45%	1000-2000 CPM	30 min post ingestion
Gomollon *et al*[17]	Spain	2003	314	Cross-sectional	^{13}C	No	Culture and/or Combined (Histo and RUT)	54.1 ± 18	168 53.5%	≥ 5%	30 min post ingestion
Gurbuz *et al*[23]	Turkey	2005	65	Cross-sectional	^{14}C	No	Combind tests (Histo and RUT)	42.4 ± 15.5	46 67.7%	> 50 CPM	10 min after drinking solution
Hahn *et al*[31]	United States	2000	100	Cross-sectional	^{13}C	No	Combined (Histo, UBT and serology)	58.8 ± 14	9 13.4%	> 2.3%	30 min after administration
Hilker *et al*[14]	Germany	1996	174	Cross-sectional	^{13}C	No	Histo	46	106 60.9%	> 250	30 min after administration
van der Hulst *et al*[26]	Italy	1999	544	Cross-sectional	^{13}C	Yes	Histo and culture	46.5	379 62.7%	> 5%	30 min after administration
Marshall *et al*[32]	United States	1990	153	Cross-sectional	^{14}C	No	Combined (Culture, RUT and histo)	--	77 50%	> 6%	30 min after administration
Ortiz-Olvera Nayeli *et al*[18]	Mexico	2007	88	Cross-sectional	^{13}C	No	Culture and/or combined (Histo and RUT)	45 ± 15	49 55.6%	> 4.22%	30 min after administration
Ozdemir *et al*[28]	Turkey	2008	89	Cross-sectional	^{14}C	No	Combined; any 2 positive (RUT, PCR and histo)	45 ± 13	59 66%	> 25 CPM as Heliprobe	10 min after drinking solution
Oztürk *et al*[15]	Turkey	2003	75	Cross-sectional	^{14}C	No	Histology	41 ± 14	56 74.6%	100 DPM	NA
Peng *et al*[19]	Taiwan	2009	100	Cross-sectional	^{13}C	Yes	Culture or combind (Histo and RUT)	55	44 55%	4.8%	15 min after drinking solution
Perri *et al*[20]	Belgium	1998	172	Cross-sectional	^{13}C	No	Histo and/or culture	39.7 ± 14.1	81 47%	3.3%	Every 15 min for 1 h after ingestion of the urea solution

Kopański *et al*[3]	Poland	2002	92	Cross-sectional	^{14}C	No	Combined (Culture, serology, UBT and urine test for C-urea)	45.5	36	39%	> 5%	30 min after administration
Rasool *et al*[24]	Pakistan	2007	94	Cross-sectional	^{14}C	No	Two reference tests. Patient did both separately: (1) Histo; (2) RUT	40.8 ± 12.8	34	36%	> 50 CPM	After 10 min
Riepl *et al*[33]	Austria	2000	100	Cross-sectional	^{13}C	Yes	Combined 3 tests (Histo, UAT and culture)	51.6 ± 1.4	49	49%	> 4%	NA
Surveyor *et al*[21]	Australia	1989	63	Cross-sectional	^{14}C	No	Histo and/or culture	58.8 ± 14.5	30	47%	NA	Every 5 min for 30 min
Valdeperez *et al*[12]	Spain	2003	85	Cross-sectional	^{13}C	No	Histo and RUT	41.6	44	50.5%	NA	30 min after administration

Histo: Histopathology; RUT: Rapid urea test; UAT: Urea antigen; CLO: The CLOtest™ (Ballard Medical Products, Draper, UT, United States) was used for RUT; PCR: Polymerase chain reaction; NA: Not available; CPM: Counts per min; UBT: Urea breath test; DPM: Disintegrations per minute.



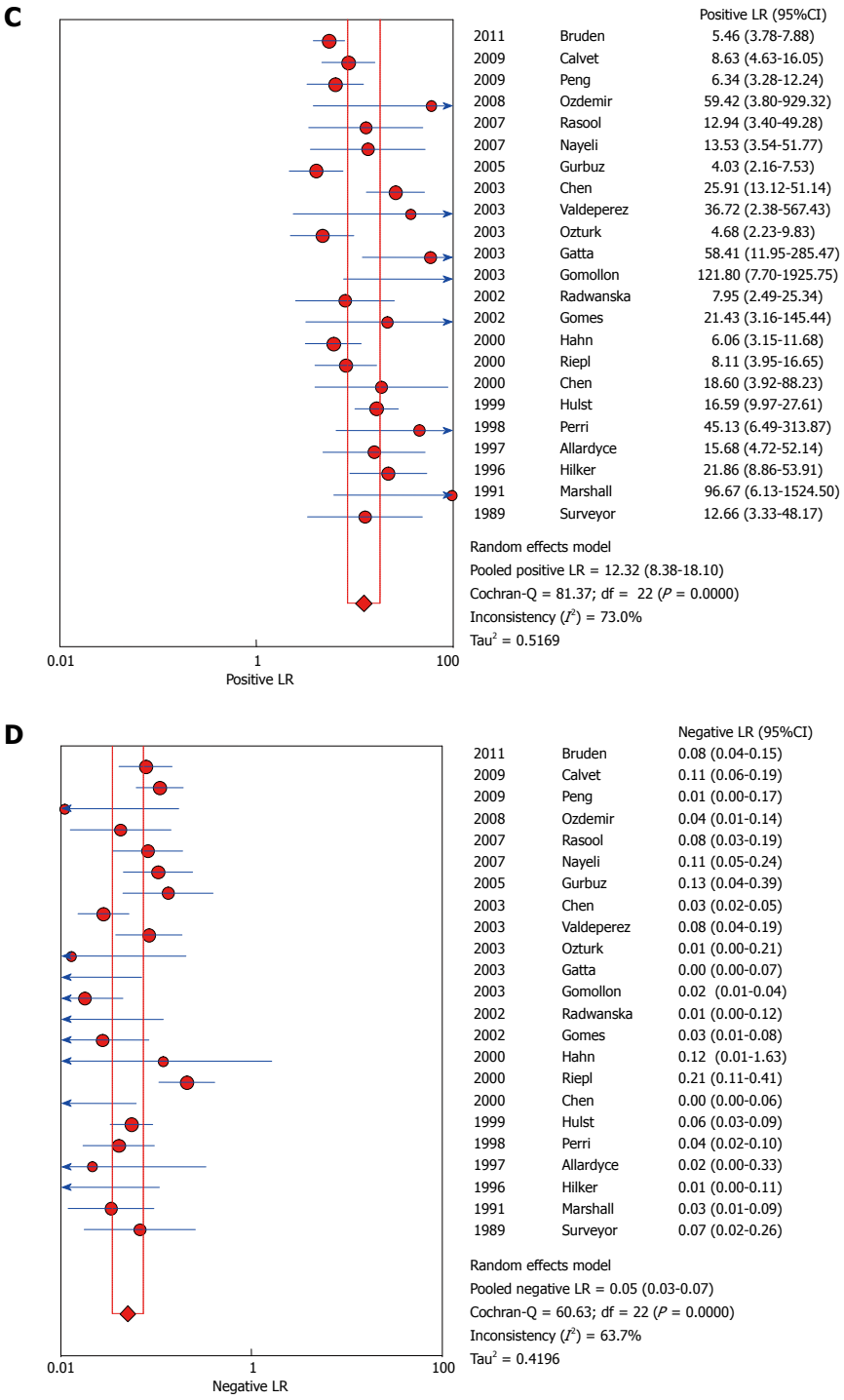


Figure 3 Pooled urea breath test result. A: Overall sensitivity; B: Overall specificity; C: Overall likelihood ratio for positive test; D: Overall likelihood ratio for negative test.

of bias. Figures are shown in online supplement materials (Figure 2).

There was no significant difference in diagnostic accuracy measures based on the risk of bias in terms of the key domains of patient selection, index test, reference standard, and flow of patients through the study and timing of the index test and reference standard. Interaction test for subgroup analyses showed no statistically significant difference to suggest a subgroup effect (P = 0.23).

Sensitivity analysis using bivariate model: Diagnostic accuracy measures were similar under the bivariate model and meta-analysis results appeared robust to the choice of model.

DISCUSSION

UBT is a noninvasive test for diagnosis of gastric *H. pylori* infection. Twenty-three studies for both UBT ^{13}C and ^{14}C for detection of *H. pylori* infection in

Table 2 Test values of included studies

Ref.	TP	FP	FN	TN	Total
Allardyce *et al*[13]	24	2	0	37	63
Bruden *et al*[16]	131	24	9	116	280
Calvet *et al*[27]	102	9	11	77	199
Chen *et al*[29]	361	8	10	205	584
Chen *et al*[25]	135	1	0	26	162
Gatta *et al*[30]	113	1	0	86	200
Gomes *et al*[22]	112	1	3	21	137
Gomollon *et al*[17]	249	0	4	61	314
Gurbuz *et al*[23]	26	8	3	28	65
Hahn *et al*[31]	4	9	0	54	67
Hilker *et al*[14]	76	4	0	94	174
van der Hulst *et al*[26] part 1	255	14	14	231	514
van der Hulst *et al*[26] part 2	161	3	12	72	248
Marshall *et al*[32]	101	0	3	49	153
Ortiz-Olvera Nayeli *et al*[18]	46	2	5	28	81
Ozdemir *et al*[28]	57	0	2	30	89
Oztürk *et al*[15]	48	5	0	20	73
Peng *et al*[19]	53	7	0	40	100
Perri *et al*[20]	121	1	5	46	173
Kopański *et al*[3]	75	2	0	17	94
Rasool *et al*[24]	61	2	5	26	94
Riepl *et al*[33]	30	7	7	63	107
Surveyor *et al*[21]	30	2	2	25	59
Valdeperez *et al*[12]	61	0	5	19	85

TP: True positive; FP: False positive; FN: False negative; TN: True negative.

adults were included. The result of the meta-analysis showed that the test performance was high and the test has significant discrimination power between those who have the infection and those who haven't.

The quality of this evidence is considered moderate due to the presence of heterogeneity, which may be explained by using different types of reference standards, timing between ingestion of the capsule and testand may be due to the variation in the methodological quality of the included studies It is very likely that the test performance is different across patients with varying pre-test risk although our analysis could not detect such difference. This analysis, focused on adults, shows similar diagnostic accuracy measures to those found in a different meta-analysis in children (sensitivity of 0.95 and specificity of 0.94 in children)[34].

In addition to the non-invasive nature of UBT, it offers the advantage of providing a comprehensive assessment that is not reliant upon the possible sampling error associated with endoscopic biopsy, due to patchy distribution of *H. pylori*[15]. Other limitations of the biopsy-based tests relate to their dependency on the pathologist skill and experience with studies documenting intern observer variability[35,36]. On the other hand, there are some limitations for UBT. For example, UBT results can be affected by exposure to *H. pylori* therapy such as, antibiotics, proton pump inhibitors or bismuth. It requires specialized equipment for carbon dioxide measurement and infrastructure to manage radioactive materials, and it is an expensive test.

Table 3 Subgroup analysis

Subgroup	No. of studies	Sensitivity	Specificity
UBT ^{13}C	14	0.96 (0.95-0.97)	0.94 (0.92-0.95)
UBT ^{14}C	9	0.97 (0.95-0.98)	0.91 (0.87-0.94)
Infrared assisted UBT	5	0.95 (0.93-0.96)	0.93 (0.91-0.95)
Infrared not assisted UBT	18	0.97 (0.96-0.98)	0.93 (0.91-0.95)

UBT: Urea breath test.

Strengths and limitations

The primary strength of this study relates to the search of electronic databases for relevant articles and the careful appraisal of study quality. The limitations mainly relate to dealing with aggregate data that limits our ability to provide estimates based on patient-level characteristics and pre-test risk level. Another significant limitation relates to heterogeneity that was unexplained despite multiple subgroup analyses. The observed heterogeneity can be attributed to several factors. The urease activity of the oral flora can affect the reading of the UBT; this can be accounted for by asking the patient to wash the mouth before conducting the test. Other authors suggested the use of Nasogastric tube. The cut off value and the time to take the reading after the meal ingestion was not clearly stated in many of the studies involved. The nature of the radioisotope meal and individual patient characteristics such as anthropometric measures, sex and age might have also contributed to within as well as between studies variability. All these factors could have contributed to the persistence of heterogeneity even after adjusting for UBT type (^{13}C *vs* ^{14}C) and technique of measurement (radioisotope mass spectrometry *vs* infrared spectrometry) in subgroup analysis.

In conclusion, UBT has high diagnostic accuracy for detecting *H. pylori* infection in patients with dyspepsia. Given the clinically significant, potentially preventable diseases associated with chronic, untreated *H. pylori* infection (such as gastric adenocarcinoma), more widespread adoption of UBT testing may be indicated to simultaneously improve public health and reduce treatment expense. The reliability of diagnostic meta-analytic estimates however is limited by significant heterogeneity, and the findings from this study should therefore be interpreted with appropriate caution.

COMMENTS

Background

Helicobacter pylori (*H. pylori*) is a gram-negative bacterium found on the luminal surface of the gastric epithelium and induces chronic inflammation of the underlying mucosa. The organism can survive in the acidic environment of the stomach partly owing to its remarkably high urease activity. Urease converts the urea present in gastric juice to alkaline ammonia and carbon dioxide. Urea

breath test (UBT) is a commonly used non-invasive test to diagnose *H. pylori* infection in patients with dyspepsia.

Research frontiers

There are two UBTs available and gained Food and Drug Administration approval: ^{13}C and ^{14}C tests. Both tests are affordable and can provide real-time results. UBT is indicated to confirm *H. pylori* colonization and to monitor its eradication.

Innovations and breakthroughs

Many invasive and non-invasive methods can be used to diagnose *H. pylori* infection, including endoscopy with biopsy, serology for immunoglobulin titers, stool antigen analysis, and the UBT. Given the user-friendly, non-invasive features of UBT, this detection method may be preferred in many clinical settings.

Applications

UBT can play a useful role in the diagnostic evaluation of dyspeptic patients who have comorbidities that increase their risk of upper endoscopy, are intolerant to upper endoscopy, or have known or suspected gastric atrophy. The study results suggest that UBT has high diagnostic accuracy for detecting *H. pylori* infection in patients with dyspepsia.

Peer review

This systematic review has been well performed; with a well expressed objective, precise criteria for the studies included and the relevant studies which have been selected for further evaluation. The quality of each included study has been properly evaluated. Its main drawback is the heterogeneity of the included studies; this, however, is not the fault of the authors of the meta-analysis.

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META-ANALYSIS

Clinical characteristics of incidental or unsuspected gallbladder cancers diagnosed during or after cholecystectomy: A systematic review and meta-analysis

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Abstract

AIM: To perform a systematic review of incidental or unsuspected gallbladder (GB) cancer diagnosed during or after cholecystectomy.

METHODS: Data in PubMed, EMBASE, and Cochrane Library were reviewed and 26 publications were included in the meta-analysis. The inclusion criterion for incidental GB cancer was GB cancer diagnosed during or after cholecystectomy that was not suspected at a preoperative stage. Pooled proportions of the incidence, distribution of T stage, and revisional surgery of incidental GB cancer were analyzed.

RESULTS: The final pooled population comprised 2145 patients with incidental GB cancers. Incidental GB cancers were found in 0.7% of cholecystectomies performed for benign gallbladder diseases on preoperative diagnosis (95%CI: 0.004-0.012). Nearly 50% of the incidental GB cancers were stage T2 with a pooled proportion of 47.0% (95%CI: 0.421-0.519). T1 and T3 GB cancers were found at a similar frequency, with pooled proportions of 23.0% (95%CI: 0.178-0.291) and 25.1% (95%CI: 0.195-0.317), respectively. The pooled proportion that completed revisional surgery for curative intent was 40.9% (95%CI: 0.329-0.494). The proportion of patients with unresectable disease upon revisional surgery was 23.0% (95%CI: 0.177-0.294).

CONCLUSION: A large proportion of incidental GB cancers were T2 and T3 lesions. Revisional surgery for radical cholecystectomy is warranted in T2 and more advanced cancers.

Key words: Gallbladder cancer; Laparoscopic surgery; Cholecystectomy; Revisional surgery; Incidental diagnosis

Core tip: A low incidence of gallbladder (GB) cancer was diagnosed incidentally during or after cholecystectomy. In incidental GB cancers, revisional surgery for radical resection is inevitable. This systematic review provides clinical information of incidental GB cancers based on

a relatively large number of patients. Approximately three-quarters of incidental GB cancers were T2 and more advanced cancers. Therefore, a large proportion of the patients with incidental GB cancers required revisional surgery to achieve R0 resection. However, more than 20% of patients demonstrated unresectable disease when revisional surgery was attempted. Therefore, additional imaging studies are necessary in patients with GB cancers diagnosed following cholecystectomy.

Choi KS, Choi SB, Park P, Kim WB, Choi SY. Clinical characteristics of incidental or unsuspected gallbladder cancers diagnosed during or after cholecystectomy: A systematic review and meta-analysis. *World J Gastroenterol* 2015; 21(4): 1315-1323 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1315.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1315

INTRODUCTION

The prognosis of gallbladder (GB) cancer is poor, and a high proportion of patients are diagnosed at an advanced stage[1,2]. Laparoscopic cholecystectomy (LC) is the gold standard for the surgical treatment of benign GB diseases. Although benign GB disease can be diagnosed preoperatively, GB cancer is diagnosed during or after cholecystectomy at a low incidence. If GB cancer is suspected during LC, conversion to open surgery to perform radical resection after confirmation of the cancer by intraoperative frozen biopsy is considered. When GB cancer is diagnosed after cholecystectomy, reoperation for radical resection according to the depth of invasion of the cancer (T stage) is inevitable[3]. However, reoperation with radical surgery is not performed in all patients for several reasons including refusal to undergo radical surgery, poor medical condition, or cancer progression suggesting unresectability.

The diagnosis of advanced GB cancer by computed tomography (CT) is accurate and reliable, but the ability to identify early-stage cancer on CT remains disappointing. Therefore, preoperative staging using CT has an overall accuracy ranging from 83%-86%[4]. Diagnostic features of GB include wall thickening suggesting that the GB cancer area is heterogeneously enhanced, a thick one-layer pattern or a strongly enhanced thick inner layer with a weakly enhanced (or non-enhanced) thin outer layer; these features were found to be highly sensitive and specific for GB cancer in one study[5]. The diagnosis (or suspicion) of cancer can be missed preoperatively when combined with cholecystitis. Although cholecystectomy is a suitable treatment for early GB cancer, the diagnostic rate is low[6]. Most published studies on incidentally diagnosed GB cancer are based on a single-center experience with a relatively small number of patients compared with the clinical significance of incidental GB cancer. The aim of this study was to perform a systematic review of incidental or unsuspected GB cancer diagnosed during or after cholecystectomy (laparoscopic or open). The incidence and clinical characteristics of the incidentally found GB cancers were investigated.

MATERIALS AND METHODS

Search strategy

Published literature in PubMed, EMBASE, and the Cochrane Library was searched using the following keywords and MeSH terms: "gallbladder neoplasm(s)", "gallbladder cancer(s)", "unsuspected", "incidental", "cholecystectomy", "laparoscopic". Language limitation was not applied during the initial search, but was restricted to English language literature in the last step of the selection process. Studies were limited to those on humans. All retrieved articles were manually screened to ensure a satisfactory study design.

Selection and exclusion criteria

The inclusion criterion for incidental GB cancer was GB cancer diagnosed during or after cholecystectomy that was not suspected at the preoperative stage. Therefore, studies including patients who had suspected GB cancer at preoperative evaluation were excluded, even if laparoscopic cholecystectomy was performed. Studies that included patients with both suspected and unsuspected GB cancers were enrolled in this study if the clinicopathologic characteristics of the unsuspected (incidental) GB cancers were available exclusively. If data on the incidental GB cancer were insufficient, the study was excluded. Case series and studies that included fewer than 20 patients with incidental GB cancers were excluded from this systematic review.

Data extraction

Two authors (CSB, CKS) independently extracted information using retrieved abstracts. After determining inclusion of the studies, the following details were investigated: study period, country of the study, number of patients with incidental GB cancer, overall number of patients who underwent cholecystectomy during the same period, number of reoperations for radical surgery, reason not to perform reoperation, operative procedures, pathologic characteristics focusing on the depth of invasion (T stage) and lymph node metastasis, and residual disease after revisional surgery. Any discrepancies in data collection between the two authors were solved by consensus.

We focused on the incidence of incidentally diagnosed GB cancer and the clinical characteristics associated with reoperation for radical surgery by pooled analysis. The primary outcomes were

Table 1 Summary of the publications included

Ref.	Year	Study period	Country	Study setting	No. of IGC	Revisional surgery	T stage					Study quality
							Tis	T1	T2	T3	T4	
Z'graggen *et al*[8]	1998	1992-1995	Swiss	Swiss registry	37	6	0	9	16	8	4	4
Sarli *et al*[9]	2000	1986-1995	Italy	Single center	20	6	1	6	4	9	0	5
Suzuki et al[10]	2000	1992-1998	Japan	Multicenter	41	11	1	25	14	1	0	5
Wakai *et al*[11]	2002	1992-1999	Japan	Single center	28	10	0	15	13	0	0	5
Toyonaga *et al*[12]	2003	1982-2000	Japan	Multicenter	73	21	0	23	43	7	0	6
de Aretxabala *et al*[13]	2004	Unavailable	Chile	Single center	64	26	2	5	39	18	0	4
Yildirim *et al*[14]	2005	1990-2003	Turkey	Single center	65	28	0	13	34	18	0	4
Lam *et al*[15]	2005	1998-2002	Hong Kong	Multicenter	63	4	1	4	23	26	7	6
Xu *et al*[16]	2007	1990-2005	China	Single center	23	6	0	11	7	5	0	3
Pawlik *et al*[17]	2007	1984-2006	United States, Brazil, Italy, Germany	Multicenter	148	109	0	18	85	41	4	5
Shih *et al*[18]	2007	1995-2004	United States	Single center	53[5]	39						5
Shukla *et al*[19]	2008	2003-2007	India	Single center	90 (76[1])	54	0	23	33	20	0	4
Kwon *et al*[20]	2008	1992-2004	Japan	Single center	38	14	0	20	17	1	0	5
Zhang *et al*[21]	2009	1999-2007	China	Single center	20	7	4	4	6	4	2	5
Choi *et al*[22]	2009	2002-2007	Korea	Single center	33	7	2	10	17	4	0	5
Butte *et al*[23]	2010	2000-2008	Chile	Single center	49	20	0	8	32	9	0	4
Glauser *et al*[24]	2010	1994-2004	Swiss	Swiss registry	89 (69[1])	19	2	14	34	14	5	6
Kim *et al*[25]	2010	1997-2008	Korea	Single center	26	2	1	6	17	2	0	4
de Aretxabala *et al*[26]	2010	2005-2009	Chile	Single center	23	15	0	3	15	5	0	3
Goetze *et al*[27]	2010	1997-	German	German registry	624[2]	231	22	118	300	143	30	6
Fuks *et al*[28]	2011	1998-2008	France	Multicenter	218	148	0	24	84	81	29	6
Clemente *et al*[29]	2012	1998-2009	Italy	Single center	44[3]	34	0	5	19	10	0	4
Maker *et al*[30]	2012	1992-2009	United States	Single center	162[6]	162	0	12	71	79	0	5
Lendoire *et al*[31]	2012	1999-2010	Argentina	Single center	40[4]	24	0	1	12	11	0	4
Yi *et al*[32]	2013	1992-2009	China	Single center	38	10	0	14	4	12	8	6
Xu *et al*[33]	2013	1993-2011	China	Single center	36	20	0	16	11	9	0	5

[1]Number of available data; [2]11 patients were Tx (unknown T stage); [3]The exact T stages of 10 patients were not described; [4]The exact T stages of 16 patients were not described; [5]Exact T stages were not described. Instead, AJCC TNM stages were shown; [6]All patients included in this publication received revisional surgery. IGC: Incidental gallbladder cancer.

the incidence of incidental GB cancers following cholecystectomy and the distribution of T stage (depth of invasion) of the GB cancers. Secondary outcomes were the proportion of patients who underwent reoperation after cholecystectomy or conversion to open surgery during operation for radical surgery, the proportion of patients with unresectable disease even though radical surgery was attempted, and the proportion of patients with residual malignant disease after radical surgery for GB cancers. The quality of all publications was assessed using the Newcastle-Ottawa Scale[7]. Of the three categories used in the Newcastle-Ottawa Scale (Selection, Comparability, and Outcome), we used the following for study assessment: "Selection," (1) representativeness of the exposed cohort; (2) selection of the non-exposed cohort; and (3) ascertainment of exposure; and "Outcome" (1) assessment of outcome; (2) sufficiency of length of follow up; and (3) adequacy of follow-up of cohorts. A study was given one star for each question. The numbers of stars and characteristics of the included studies are presented in Table 1.

Statistical analysis

Data and outcomes extracted from each study were pooled and analyzed using Comprehensive Meta-Analysis software Version 2 (Biostat, New Jersey, United States). A single weight-adjusted proportion for each variable was computed for each study. The random effect model was used to derive pooled estimates of proportion with 95%CI for the outcomes explored.

RESULTS

Study characteristics and incidence of incidental (unsuspected) GB cancers

A total of 986 publications were initially identified and 26 were finally included in this systematic review (Figure 1). These 26 studies[8-33] were observational cohort studies based on data from national registries (n = 3), multicenter studies (n = 5), and single center surgical experiences (n = 18). In total, 2145 patients with incidental GB cancers (diagnosed during or after cholecystectomy) were included in this systematic review. The characteristics of the publications are shown in Table 1. Ten publications[8-10,15,20-22,24,25,33] reported the incidence of incidentally found GB cancers and the total number of cholecystectomies performed during the same study period. Among the ten publications[8-10,15,20-22,24,25,33], 403 incidental GB cancers were detected in the 80228 cholecystectomies. The pooled proportion of incidental

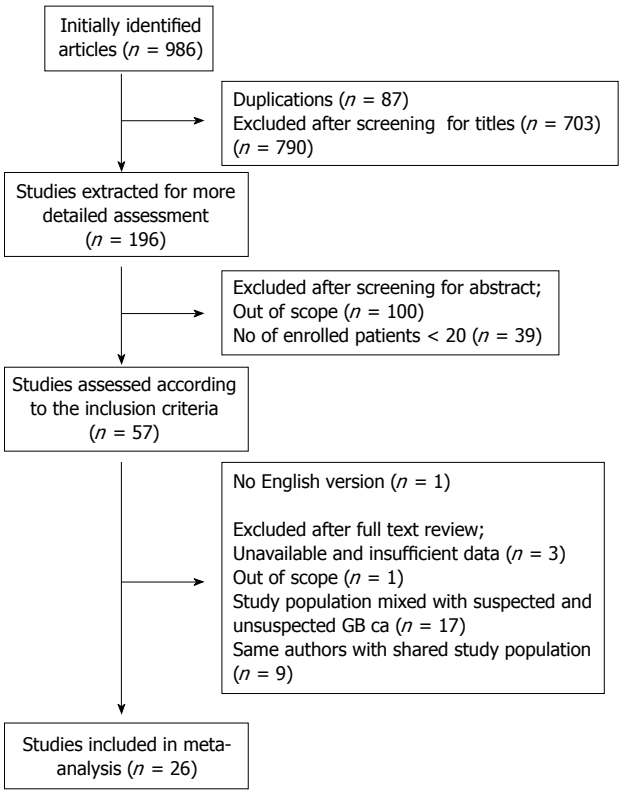

Figure 1 Selection of the publications.

GB cancers among the cholecystectomies performed for benign gallbladder diseases was 0.7% (95%CI: 0.004-0.012).

Distribution of T stage and presence of lymph node metastasis in incidental GB cancers

The versions of cancer stage according to the American Joint Committee on Cancer (AJCC) used were different according to the study period: 3rd edition[8,34], 4th edition[13,35], 5th edition[10-12,36], 6th edition[14,15,17,18,20-23,25,27,37], 7th edition[24,28,29,31,32,38], and Nevin staging[16,39]. In five studies[8,16,19,26,30], the exact version of the staging system used was not clearly defined. For T stage, Tis, T1, and T2 are the same in the 3rd to 7th editions of AJCC stage. Tis is carcinoma in situ. T1a tumor invades mucosa and T1b invades muscle layer. T2 invades perimuscular connective tissue, without extension beyond the serosa or into the liver[34-38]. T3 tumors are those that perforate the serosa, or directly invade one adjacent organ, or both (extension 2 cm or less into the liver), whereas T4 tumors extend more than 2 cm into the liver and/or into two or more adjacent organs in the 4th and 5th editions[35,36]. In the 6th and 7th editions of AJCC[37,38], T3 tumors perforate the serosa or directly invade the liver and/or one other adjacent organ or structure, and T4 tumors invade the main portal vein or hepatic artery, or two or more extrahepatic structures. Although T3 and T4 stage are somewhat different among the versions of AJCC stage, we regarded T3, T4 in each edition as the same T3, and T4, respectively, throughout the editions.

Table 2 Distribution of T stage in incidental gallbladder cancer in 25 studies[8-17,19-33]

T stage	Range of proportion reported by primary studies	Pooled proportion	95%CI
Tis	0%-20.0%	2.4%	1.5%-3.8%
T1	4.2%-61.0%	23.0%	17.8%-29.1%
T2	13.2%-75.0%	47.0%	42.1%-51.9%
T3	0.0%-69.8%	25.1%	19.5%-31.7%
T4	0.0%-21.1%	4.2%	2.6%-6.5%

Table 2 shows the pooled proportion of T stages among the incidental GB cancers in 25 studies[8-17,19-33]. Nearly 50% of the incidental GB cancers were T2 stage, with a pooled proportion of 47.0% (95%CI: 0.421-0.519). T1 and T3 GB cancers were found at a similar frequency, with pooled proportions of 23.0% (95%CI: 0.178-0.291%) and 25.1% (95%CI: 0.195-0.317), respectively.

Patients with incidental GB cancers tended to undergo less aggressive surgery than those with suspected (diagnosed preoperatively) GB cancers as some patients did not undergo revisional surgery for incidental GB cancer. Therefore, information on the lymph node status of incidental GB cancers was limited as not all patients underwent lymph node dissection. Thirteen publications[11,14-17,20,22,23,25,28-31] reported the presence of lymph node metastasis. In principle, lymph node status is confirmed by pathologic examination after lymph node dissection. However, a review of the publications revealed that lymph node dissection was not performed for GB cancers, but was usually performed at the discretion of the surgeon according to the T stage. Moreover, the extent of dissection was not homogeneous. Considering this limitation, the reported rate of lymph node metastasis might be underestimated compared with the actual lymph node status. Nonetheless, the pooled proportion of detected lymph node metastasis among the patients with incidental GB cancers was 14.2% (95%CI: 0.107-0.185) with a range of 7.9%-26.5%.

Revisional surgery for radical cholecystectomy

If the GB cancer is found during or after operation, proceeding with revisional surgery for R0 resection is necessary. Twenty-four publications reported performing revisional surgery for curative intent[8-29,31-33]. We analyzed the proportion of patients in which the revisional surgery was completed, excluding patients who underwent only exploration. The pooled proportion that had complete revisional surgery was 40.9% (95%CI: 0.329-0.494) (Figure 2). The revisional surgery consisted of liver resection and/or bile duct resection and/or lymph node dissection. The extent of liver resection was somewhat different among the studies; however, most of the liver re-section procedures involved wedge resection of the

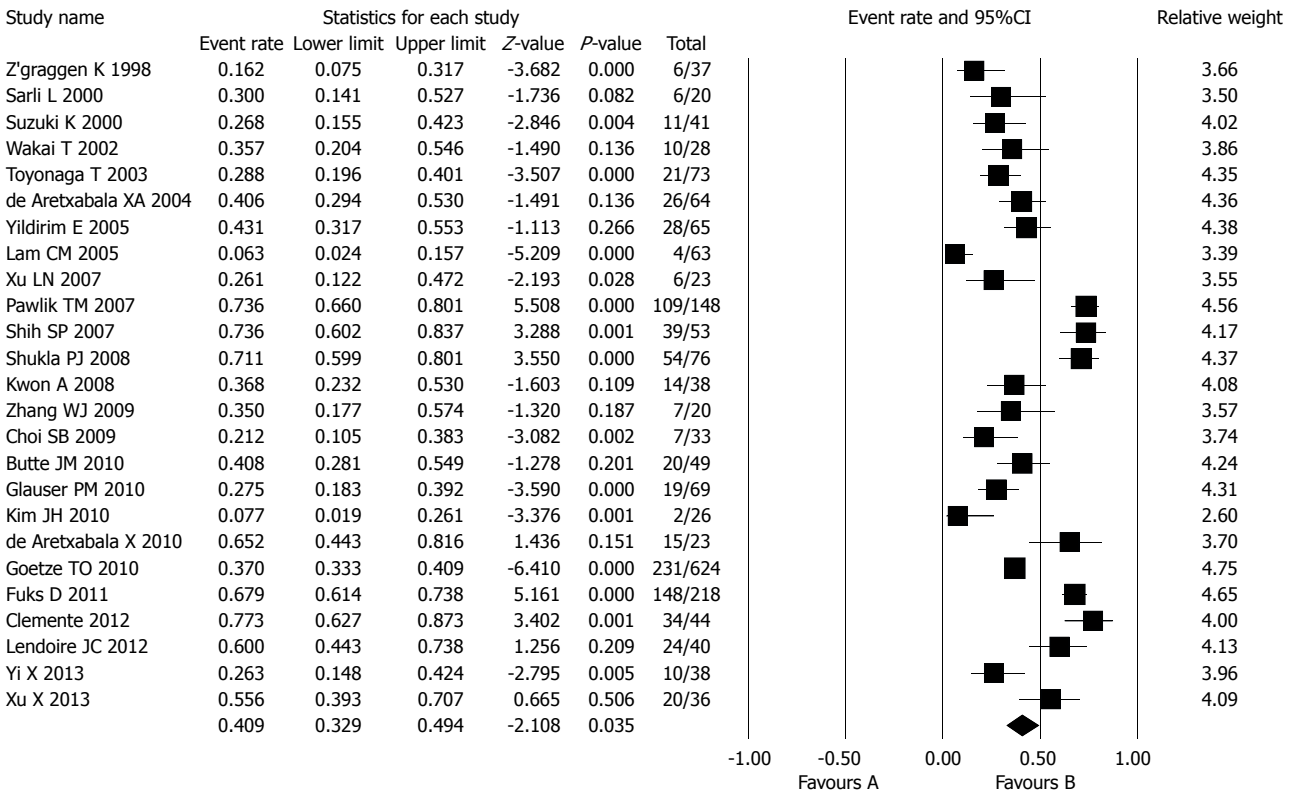

Figure 2 Pooled proportion to complete revisional surgery.

liver and bisegmentectomy of segment ⅣB and Ⅴ.

Another clinical problem in patients who undergo laparoscopic cholecystectomy for incidental GB cancer is whether port site resection should be included in the revisional surgery. Nine publications[10,11,17,19,28-31,33] discussed whether port site excision should be performed. Two studies[19,33] did not report the total number of patients who underwent port site excision; however, two patients in each study demonstrated a residual cancerous lesion in the pathologic exam after revisional surgery. Two studies[10,11] included whether port site excision was performed and five studies[17,28-31] reported the total number of port site excisions and the positive rate for cancer cells in pathologic examination of the port site. The pooled proportion of patients with positive cancer cells at the port site was 8.1% (95%CI: 0.03-0.202).

Proportion of unresectable GB cancers when revisional surgery was attempted and the presence of residual cancerous lesions after revisional surgery

Although the failure to detect incidental GB cancers at preoperative evaluation infers the presence of early cancers that might be missed, the proportion of advanced incidental cancers is too serious to be ignored. When revisional surgery was attempted (intraoperative conversion or reoperation after initial surgery) some patients were confirmed to have unresectable/inoperable diseases and underwent only exploration. Twenty-one publications[8-13,15,17-26,28,31-33] reported the proportion of unresectable disease when revisional surgery was attempted, and the pooled proportion of patients with unresectable disease was 23.0% (95%CI: 0.177-0.294) (Figure 3).

The aim of revisional surgery is to achieve an adequate resection margin and to perform lymph node dissection for locoregional control. The proportion of patients in which residual cancerous lesions were found after revisional surgery was reported in 14 publications[9-12,14,17,19,20,22,23,25,28,29,31] and the pooled proportion with residual disease was 38.7% (95%CI: 0.316-0.462). The most common locations of residual disease were the liver (GB bed) and lymph nodes; less common sites were the bile duct and port site. Two studies[22,31] did not report the location of residual disease in detail.

DISCUSSION

From a prognostic point of view, R0 resection is the most important positive factor for overall survival of GB cancers[1,2]. The extent of surgery is different according to the depth of invasion (T stage) of the tumors. For a T1a tumor, cholecystectomy is the standard procedure, whereas for a T1b tumor, cholecystectomy with lymph node dissection has been performed[40]. For T2 and more advanced tumors, liver resection including the gallbladder bed and lymph node dissection are recommended. Extrahepatic bile duct resection is not performed uniformly, and is

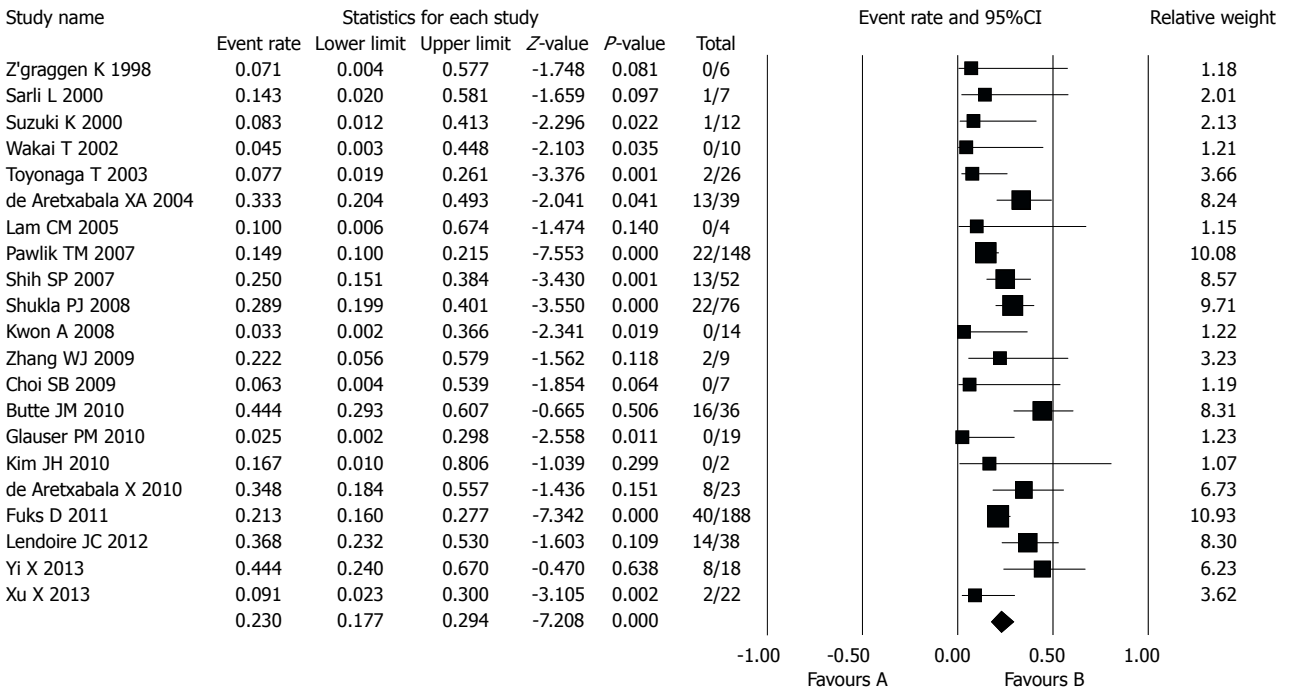

Figure 3 Pooled proportion of patients with unresectable disease when revisional surgery attempted.

somewhat controversial in the surgical treatment of GB cancers[41,42]. According to our study, approximately one-quarter of the patients did not require revisional surgery because they presented with Tis or T1 disease. Therefore, approximately three-quarters of patients with incidental GB cancers were ultimately candidates for revisional surgery.

The most important clinical problem related to incidentally found GB cancers is the decision of whether to proceed with revisional surgery for radical cholecystectomy. If the GB cancer is found during the operation, conversion to radical surgery is relatively easy. However, if GB cancers are found after the operation, reoperation for revisional surgery is both necessary and critical. Although R0 resection is the treatment of choice, some patients with incidental GB cancers diagnosed following cholecystectomy refused reoperation for revisional surgery. As most of the publications were based on the retrospective review of medical records, the exact proportion of patients who refused revisional surgery is not described in all studies. Several publications reported the number of patients who refused revisional surgery even though it was indicated due to advanced tumor stage[18,21,23,32,33]. Refusal of radical cholecystectomy is one of the more difficult issues encountered in clinical practice. As described before, because R0 resection is the most important factor determining prognosis, reoperation for revisional surgery should be strongly recommended.

In this systematic review, the pooled proportion of patients with unresectable disease when attempting revisional surgery was 23.0% (95%CI: 0.177-0.294). Even though GB cancer was not suspected before surgery, the disease was too advanced to perform radical surgery. Therefore, precise preoperative evaluation is necessary to assess the extent of disease before revisional surgery, especially in patients who undergo reoperation after a relatively long time interval from the first operation. For preoperative diagnosis of GB cancers, multidetector computed tomography (MDCT) is now widely available and has a reported accuracy of up to 84% for determining local extent or the T stage of primary gallbladder carcinoma[43] and 85% for predicting resectability[44]. Positron emission tomography (PET)-CT scanning might also be an option, and has been reported to have value for the detection of regional lymph node metastasis and distant metastases that are not diagnosed by MDCT[45,46]. Biliary magnetic resonance imaging is also useful for the detection of GB cancer[47]. However, considering the impact of postoperative change, it is not possible to draw conclusions about the efficacy of CT, PET, or MR to detect residual cancerous lesions or metastatic disease after cholecystectomy, and there was a lack of evidence on this issue in our review of the literature for incidentally found GB cancers. Further preoperative evaluation might be necessary taking into consideration the relatively significant proportion of patients who had unresectable disease when attempting revisional surgery.

The prognostic impact of incidentally diagnosed GB cancer on survival compared with preoperatively suspected GB cancer has not been widely studied. It is not clear whether incidental GB cancer has the same prognosis, or poorer prognosis, compared

with the same stage of non-incidental GB cancer. For incidental GB cancers, it is likely that the combined presence of cholecystitis complicates the diagnosis of GB cancer. Several studies have reported the negative impact of cholecystitis on survival[22,29,48] although the exact mechanism has not been investigated. Incomplete en bloc resection during cholecystectomy that causes spillage of cancer cells might affect the prognosis of GB cancer considering the relatively high pooled proportion of patients with residual cancerous lesions after revisional surgery in this study. However, the results of most of the studies warrant radical resection to improve survival[3,24,27,28]. In contrast, one study reported that the tumor characteristics differed between suspected and incidental GB cancer, and suggested that incidental GB cancer has a significant better median survival[49].

When comparing the survival impact of laparoscopic versus open procedures for the treatment of GB cancer, several studies reported no significant prognostic difference between the two procedures, suggesting that laparoscopic cholecystectomy does not decrease survival[9,13,50-52]. However, another study showed that laparoscopic cholecystectomy had an increased risk of disseminating tumor cells, suggesting that open surgery is warranted in cases of known or suspected GB cancer[53]. However, more recently, several authors have reported that early lesions of GB cancer can be managed successfully using laparoscopic cholecystectomy, achieving a satisfactory survival result and a low rate of port-site recurrence[54,55].

Whether port site resection should be performed is one of the major issues in revisional surgery after laparoscopic cholecystectomy. In our review, not all of the centers adopted port site resection as part of revisional surgery, and the pooled proportion in which cancer cells were detected in the port site was low. Maker *et al*[30] focused on the necessity for port site resection in the surgical management of incidental GB cancer. They concluded that port site metastases were associated with poorer survival. However, port site resection was not associated with improved survival and should not be considered mandatory during definite surgical treatment for incidental GB cancer. In the early laparoscopic era, many authors reported that laparoscopic surgery might promote peritoneal seeding during the surgical treatment of cancer patients[56,57]. However, there was no definite difference in the oncologic outcome between the two procedures in more recent studies[9,13].

In conclusion, incidental (unsuspected) GB cancers were not all early lesions; in fact, T2 and T3 lesions accounted for a large proportion of these cancers. Our data indicated that revisional surgery for radical cholecystectomy is warranted to gain a survival benefit in T2 and more advanced cancers, although surgical procedures were not homogeneous and were determined according to the extent of disease. Furthermore, even though these GB cancers were found incidentally, some incidental GB cancers were unresectable when attempting revisional surgery. Therefore, additional imaging studies to determine the extent of disease and resectability are necessary before performing revisional surgery.

COMMENTS

Background

Laparoscopic cholecystectomy (LC) is the gold standard for surgical treatment of benign GB diseases. Gallbladder (GB) cancer is diagnosed during or after cholecystectomy at a low incidence. The aim of this study was to perform a systematic review of incidental or unsuspected GB cancer diagnosed during or after cholecystectomy (laparoscopic or open). The incidence and clinical characteristics of the incidentally found GB cancers were investigated.

Research frontiers

R0 resection is the treatment of choice for GB cancers. Although the incidence of GB cancers diagnosed during or after cholecystectomy, is low, incidental GB cancers can cause difficult problems in clinical practice. In this study a systematic review of incidental GB cancer was performed, based on a relatively large number of patients with incidental GB cancers, focusing on the clinical characteristics and significance of incidental GB cancers; incidence, T stage, revisional surgery, and proportion of unresectable disease.

Innovations and breakthroughs

The prognosis of GB cancer is poor, and a high proportion of patients are diagnosed at an advanced stage. LC is the gold standard for surgical treatment of benign GB diseases. Although benign GB disease was diagnosed preoperatively, GB cancer can be diagnosed during or after cholecystectomy at a low incidence. If GB cancer is suspected during LC, conversion to open surgery to perform radical resection after confirmation of the cancer by intraoperative frozen biopsy is considered. When GB cancer is diagnosed after cholecystectomy, reoperation for radical resection according to the depth of invasion of the cancer (T stage) is inevitable. However, reoperation with radical surgery is not performed for all patients for several reasons including refusal to undergo radical surgery, poor medical condition, or cancer progression suggesting unresectability. This study is based on the systematic review of incidental GB cancers. Most published studies on incidentally diagnosed GB cancer are based on a single-center experience with a relatively small number of patients comparing the clinical significance of incidental GB cancer. Therefore, this study provides clinical information on incidental GB cancers diagnosed during or after cholecystectomy based on a relatively large number of patients.

Applications

The results of this study suggest that approximately three-quarters of incidental GB cancers were T2 and more advanced GB cancers. Therefore, a large proportion of patients with incidental GB cancers required revisional surgery. However, more than 20% of patients had unresectable disease when revisional surgery was attempted. Therefore, additional imaging studies are necessary in patients with GB cancers diagnosed following cholecystectomy.

Terminology

Revisional surgery is radical cholecystectomy including liver resection and/or extrahepatic bile duct resection and lymph node dissection. Although the extent of revisional surgery is different according to the stage of tumor, the aim of revisional surgery is to perform R0 resection.

Peer review

A large proportion of incidental GB cancers were T2 and T3 lesions. Revisional surgery for radical cholecystectomy is warranted to gain a survival benefit in T2 and more advanced cancers. Some incidental GB cancers were unresectable when attempting revisional surgery; therefore, additional imaging studies for revisional surgery are necessary to determine the extent of disease.

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CASE REPORT

Transanal endoscopic microsurgery: The first attempt in treatment of rectal amyloidoma

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Abstract

Localized amyloidosis is characterized by amyloid protein deposition restricted to one organ or tissue without systemic involvement. Gastrointestinal manifestations of localized amyloidoma are unusual, which makes amyloidoma restricted to the rectum a very rare diagnosis requiring a high index of suspicion. We present a rare account for rectal amyloidoma with an unusual presentation of obstructive symptoms and its treatment using a sophisticated surgical modality, transanal endoscopic microsurgery (TEM), which resulted in complete excision of the lesion without hospitalization and complications. The successful treatment for this rectal amyloidoma using TEM emphasizes the need to broaden its application in the treatment of various rectal lesions while preserving organ function and decreasing recurrence.

Key words: Transanal endoscopic microsurgery; Transanal endoscopic microsurgery; Amyloidoma; Localized amyloidosis; Rectal amyloidoma

Core tip: This case represents the first transanal endoscopic microsurgery (TEM) approach for full-thickness excision to treat organ restricted amyloidosis of the rectum, a very rare entity requiring high suspicion for diagnosis and treatment. Although TEM is the preferred modality to treat early rectal cancers and rectal adenomas, it should also be considered for other benign and non-advanced rectal lesions, such as localized amyloidoma. TEM is a less invasive procedure that provides lower morbidity and mortality by decreasing incidence of local recurrence and complications while preserving rectal continence and function.

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INTRODUCTION

Amyloidosis is a rare group of disorders with an annual incidence of eight patients per million and is characterized by pathological deposition of fibrillar protein named amyloid, which disrupts organ struc-

ture and function[1]. The diagnosis of amyloidosis requires a high index of suspicion since the symptoms are nonspecific and may involve a single or multiple organ systems. Additionally, slow progression of the disease often delays diagnosis causing limited or palliative treatments. The manifestation of amyloidosis can be classified as primary, secondary, localized, or familial with prognosis varying in regards to the specific type of disease process[2,3].

Localized amyloidosis, an extremely rare condition, is limited to a single organ. Furthermore, systemic features such as urinary and serum monoclonal proteins and/or clonal plasma cells in the bone marrow are absent making diagnosis difficult. Common sites of organ-restricted deposition include respiratory[4,5], genitourinary tract[6,7], in addition to, skin and soft tissue[8,9]. The mechanism underlying the formation of a localized amyloidoma remains poorly understood. *De novo* amyloid production[10], in addition to, local plasmacytosis in chronic inflammatory diseases[6,11] have been proposed for tissue restricted amyloidoma. Additionally, localized amyloid has been found to arise from certain fibrillary proteins produced by neoplasms such as calcitonin in medullary thyroid carcinoma[12,13], amylin in insulinoma[14] or prolactin in prolactinoma[15,16].

Localized amyloidoma of the gastrointestinal tract is extremely unusual. All reported cases affecting the large bowel presented clinically with lower gastrointestinal bleeding[17-20]. Rarely amyloid of the colon produces a mass lesion causing obstructive symptoms[21]. Only two cases have been described in literature for rectal amyloidoma[22,23]. This case study is a rare account of the presentation of rectal amyloidoma and its surgical resection using an older but sophisticated surgical device, transanal endoscopic microsurgery (TEM).

CASE REPORT

A 66-year-old Caucasian male with past medical history of diabetes mellitus type Ⅱ, hypertension, hyperlipidemia, and obstructive sleep apnea presented with unusual lower abdominal pain in September 2013. On digital rectal examination, the lesion was easily graspable and characterized as a hard, lobulated mass at the dentate line. Computed tomography scan was obtained, which showed a thickening of the left lower rectal wall with adjacent free gas due to the local perforation of the amyloidoma (Figure 1). A follow up colonoscopy found an irregular, poor defined, semi circumferential rectal mass occupying about 50% of the rectal lumen located 2 cm from the dentate line and biopsy of the mass was taken. Pathology evaluation demonstrated abnormal deposit, which was found to be positive for congo red stain and was confirmed to be a rectal amyloidoma. No personal or family history of amyloid diseases or chronic inflammatory diseases was reported.

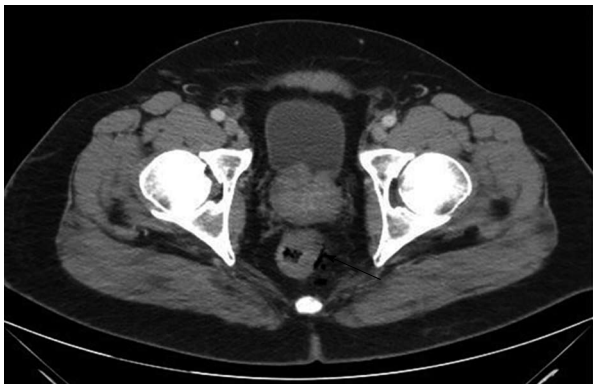

Figure 1 Computed tomography of the pelvis demonstrates a thickening of the left lower rectal wall with adjacent free gas. No pathologically enlarged lymphadenopathy was found. Arrow mark the extraluminal air or free air in the rectum.

Proper workup was performed to assess for systemic amyloidosis. Serum blood count, comprehensive metabolic panel, coagulation studies, and liver and kidney function tests were all within standard limits. Serum total protein, albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin were normal. Urine analysis revealed a low level of monoclonal peak is present on electrophoretogram but was too small to quantitate and immunofixation yielded no abnormal bands. Furthermore, serum free kappa and free lambda protein levels were within regular limits resulting in a normal free kappa: lambda ratio of 1.02 (0.26-1.65). Chest X-ray, electrocardiogram, and echocardiogram showed no findings. Conclusively, work up was negative for systemic amyloidosis.

Amyloidoma of the rectum is an extremely rare rectal tumor. Lesions this low and of this size are more commonly treated by radical surgical intervention (low anterior resection or abdominal perineal resection) to achieve negative margins and evaluate the lymphovascular system for invasion. Due to the benign behavior of localized amyloid tumors, we offered the patient a local excision with a TEM.

Surgical technique and post-operative course

TEM includes an adjustable, multi-port proctoscope combined with CO_2 mediated insufflation for optimal exposure of the rectum. This closed *in-vivo* system is then connected to a stereoscopic angulated optical system, which allows visualization and projection on a screen with higher resolution capabilities.

Briefly, for this case, the patient was placed in a lithotomy position. After examination of the rectum with a rigid, beveled, proctoscope, patient was positioned appropriately to localize the lesion inferiorly. The TEM device was connected to the anesthesia table. The TEM proctoscope was advanced to visualize the lesion in the left posterior aspect of the rectum at the inferior part of the TEM

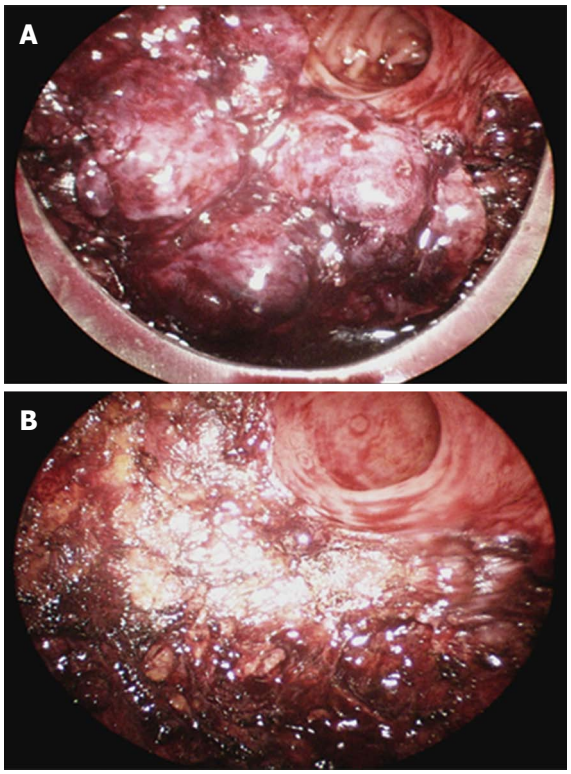

Figure 2 Rectal amyloidoma before transanal endoscopic microsurgery excision (A) and rectal wall after full thickness excision of the mass (B). Little bleeding was noted during and after the procedure.

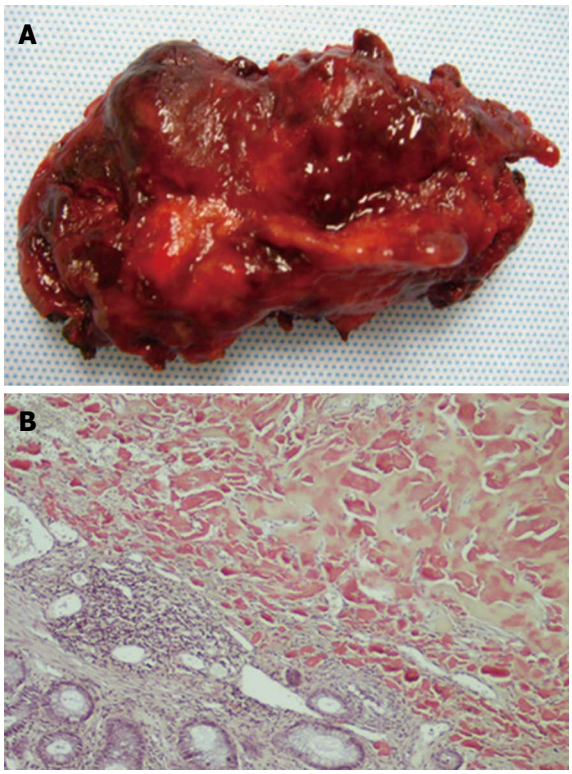

Figure 3 Five cm amyloidoma specimen excised from the rectum 2 cm from the dentate line (A) and photomicrograph (x 40) demonstrating amyloid deposition with Congo red staining in rectal amyloidoma (B). Amyloid protein is seen as acellular, homogeneous, and eosinphilic material deposited uniformly throughout the mass.

proctoscope (Figure 2A). Insufflation of CO_2 was started. The diameter of the patient's rectum was too large to keep the lesion in the center, which led to mobilization of the lesion 1 cm distally and using this edge as a handle in order to allow optimal exposure for full thickness excision of the rectal wall using the monopolar cautery. After establishing the posterior dissection, we advanced in the mesorectal fat where the mass was divided full-thickness circumferentially from left and right and lastly, proximal aspect (Figure 2B). Small bleeding was controlled with the cautery device. The mass was completely excised, margins were obtained, and specimen was sent for pathology (Figure 3A). The defect was left open due to the large size and the lack of bleeding after cautery, in addition to, decreasing the likelihood of complications such as abscess formation and increased pain that have occurred in previous TEM cases when closing the defect.

Post-operatively, the patient did not have any bleeding from the surgical site and was able to go home the same day of surgery after recovering from anesthesia. Patient was gas incontinent for 2 wk post-operatively but resumed normal bowel habits afterwards without any local recurrence thus far. Findings of the histopathological report revealed acellular, homogenous, eosinophilic material underlying benign colonic mucosa positive for congo red stain and identified as local rectal amyloidoma (Figure 3B). Small fibers of the internal sphincter were also noted within the specimen (Figure not shown).

DISCUSSION

Many classifications have emanated for Amyloidosis over the years due to the advancement in knowledge for the disease process. Amyloidosis can be classified into four major categories. Primary amyloidosis, also known as immunoglobulin light chain amyloidosis, is diagnosed in individuals without previous diseases or coexisting conditions except multiple myeloma. Secondary or reactive amyloidosis is associated with chronic inflammatory conditions resulting in accumulation of hazardous byproducts. Familial amyloidosis includes variety of heritable mutations of proteins, such as transthyretin (TTR - most common form), apolipoprotein A-I, fibrinogen, cystatin and gelsolin[2,3]. Furthermore, the central nervous system and its' exclusive environment is susceptible to amyloid deposition, which manifests as various forms of familial dementias[24]. The gastrointestinal system is a common site of amyloid deposition in patients with primary amyloidosis (70%) and secondary amyloidosis (50%) and the colon is frequently involved within the multi-systemic disease process[25]. However, single organ or localized form of amyloidosis, without systemic involvement, is rarely found in the colon[26-28].

Localized amyloidomas of the gastrointestinal

track are extremely rare, usually affecting the colon with bleeding as a chief complaint due to the lesion outgrowing the blood supply causing necrosis. In this case review, a low rectal amyloidoma, measuring 5 cm and occupying 50% circumference, presented with unusual obstructive symptoms and surgery was performed with the first TEM approach for full-thickness excision to treat this organ restricted amyloidosis. TEM is discovered in Germany, gained much popularity in Europe for resection of large rectal tumors since 1983. However, the US, housing merely 9% of TEM systems worldwide, has considered the technique appropriate only for the treatment of early rectal cancers and rectal adenomas[29,30]. Due to the characteristics of localized amyloidomas such as absence of systemic disease, slow growth of the lesion with clearly defined margins, and no malignant transformation, resection is the best treatment. TEM is proposed as the most optimal technique for resection of a low rectal lesion due to its allowance for superior visualization with adequate insufflation, so that the lesion can be clearly seen and removed with adequate margins while preserving all functionality of the gastrointestinal tract instead of a radical surgery that requires a colostomy. Furthermore, several studies have compared the use of TEM versus the conventional transanal resection for removal of localized rectal lesions[29,31-35]. These studies demonstrate TEM to have a markedly higher rate of negative margins and a significantly lower incidence rate of local recurrence. In addition, TEM allows for accurate pathological evaluation for staging of rectal lesions. Furthermore, TEM offers a less invasive option for full-thickness excision, which is much preferred over a partial wall, piecemeal endoscopic resection[29].

Diagnosis of localized amyloidosis is challenging owing to the non-specific clinical presentation, normal serum and urine protein screen, frequent lack of family history, and the rarity of organ-restricted amyloidosis. It is imperative to establish the correct diagnosis so that unnecessary procedures, incorrect therapy, and delay of diagnosis can be avoided to afford the patient the best chance for a cure, often by a surgical intervention. TEM is a safe, minimally invasive procedure that provides significantly lower morbidity and mortality as compared to traditional treatments for rectal lesions. Functional outcomes are better since sphincter complex is preserved while having minimal to no damage to the pelvic region/structures. This technique provides superior visibility due to the stereoscopic capability in combination with revolutionary instrumentation that allows manipulation of larger and difficult to reach lesions. Also, use of both hands of the surgeon gives better exposure and precise excision of the lesion. More importantly, TEM has minor complications and the patient in this case was able to receive the procedure on an outpatient basis with restoration of normal bowel function within 2 wk. The patient will have close follow up of every 6 mo for surveillance of local recurrence, an extremely rare and almost negligible occurrence. Conclusively, TEM could prove to be an excellent, non-invasive, and effective technique for removal of large rectal lesions such as an amyloidoma.

COMMENTS

Case characteristics

Patient presented with lower abdominal pain.

Clinical diagnosis

Digital rectal examination revealed a hard, lobulated, and easily graspable lesion at the dentate line.

Differential diagnosis

Due to the symptoms and physical exam findings, computed tomography (CT) was the appropriate next step which revealed thickening of left lower rectal wall with adjacent free air. Follow up colonoscopy visualized an irregular and large mass with biopsies, which revealed positive congo red stain leading to the diagnosis of rectal amyloidoma.

Laboratory diagnosis

CBC, CMP, and UA within normal limits with a normal free serum kappa:lambda ratio of 1.02 (0.26-1.65) leading to exclusion of systemic amyloidosis.

Imaging diagnosis

CT scan showed thickening of the left lower rectal wall with adjacent free gas due to the local perforation of the amyloidoma.

Pathological diagnosis

Acellular, homogenous, eosinophilic deposit underlying benign colonic mucosa positive for congo red stain and identified as local rectal amyloidoma.

Treatment

Full-thickness excision of the rectal amyloidoma using transanal endoscopic microsurgery (TEM) without hospitalization and complications.

Related reports

Rectal amyloidosis is a very rare entity that presents with non-specific symptoms requiring a high index of suspicion to establish the correct diagnosis so that the patient can receive timely surgical intervention, which often leads to cure.

Term explanation

Localized amyloidoma is a benign, pathological deposition of fibrillar protein named amyloid, which can disrupt organ structure and function.

Experiences and lessons

The successful treatment for this rectal amyloidoma using TEM emphasizes the need to broaden its application worldwide.

Peer review

The case report is an intresting case presentation of a rare rectal disorder. Gastrointestinal manifestations of localized amyloidoma are unusual, which makes amyloidoma restricted to the rectum a very rare diagnosis requiring a high index of suspicion. Using TEM as a surgical treatment for it is worth to try.

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CASE REPORT

Mixed adenoneuroendocrine carcinoma of gastrointestinal tract: Report of two cases

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Author contributions: Gurzu S wrote the manuscript and carried out the study design; Kadar Z carried out the oncological management and interpretation of oncological data; Bara T carried out the surgical interventions and interpretation of the data from literature; Bara TJ participated at surgical interventions and interpretation of clinical data; Tamasi A participated at histological and immunohistochemical interpretation of data; Azamfirei L carried out the draft of the manuscript and revision of technical data; Jung I carried out the histological examination and immunoassays coordinated the study design and the draft of the manuscript.
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Abstract

Mixed adenoneuroendocrine carcinoma (MANEC) is a rare tumor of the gastrointestinal tract that consists of a dual adenocarcinomatous and neuroendocrine differentiation, each component representing at least 30% of the tumor. To date, only seven cases have been reported in the cecum, and less than 40 in the stomach. Our first case was diagnosed in a 74-years-old female as a polypoid lesion of the cecum with direct invasion in the transverse colon, without lymph node metastases. The second case was diagnosed in the stomach of a 46-years-old male as a polypoid tumor of the antral region that invaded the pancreas and presented metastases in 22 regional lymph nodes. The metastatic tissue was represented by the glandular component. In both cases, the tumor consisted of a moderately-differentiated tubular adenocarcinoma (with mucinous component in Case 1) intermingled with neuroendocrine carcinoma. Ki67 index was lower than 20% in Case 1, respectively higher than 20% in Case 2. The neuroendocrine component was marked by synaptophysin and neuron specific enolase, being negative for Keratins 7/20. The neuroendocrine component represented 60% in Case 1, and 40% in Case 2, respectively. The glandular components were marked by carcinoembryonic antigen, maspin and keratin 20/7 (Case 1/2). Both cases were proved to be microsatellite stable. Independently by the localization and tumor stage, MANECs appear to be highly malignant tumors, with high risk for distant metastases. The aggressiveness seems to depend on the endocrine component, independent of its proportion. The neuroendocrine component could be a dedifferentiated adenocarcinoma with a neuroendocrine phenotype.

Key words: Mixed adenoneuroendocrine carcinoma; Composite tumor; Mixed tumor; Colorectal; Stomach; Cecum; Maspin; Carcinoembryonic antigen; Keratin 7; Keratin 20

Core tip: The aim of this paper was to report the clinicopathological data of two cases of mixed adenoneuroendocrine carcinomas (MANECs), one in the cecum and one in the stomach. MANEC is a rare tumor of the gastrointestinal tract that consists of a dual adenocarcinomatous and neuroendocrine differentiation. To date, only seven cases have been reported in the cecum, and less than 40 in the stomach. The characteristics of these cases, in correlation with data from literature, proved that MANEC is a highly malignant tumor, its aggressiveness being related to the endocrine component, independent of its proportion.

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INTRODUCTION

The term mixed adenoneuroendocrine carcinoma (MANEC) was introduced by the World Health Organization in 2010 referring to a neoplasm with dual adenocarcinomatous and neuroendocrine differentiation, each component representing at least 30% of the tumor[1]. Before 2010, this tumor was reported as a mixed or composite tumor[2]. It should be distinguished from the collision tumor, in which the two components are closely juxtaposed but not admixed, and the amphicrine tumor with dual endo- and exocrine differentiation within the same cell[1,2]. Diagnosis is mainly based on the tumor architecture, being completed by the immunostains with specific neuroendocrine markers such as chromogranin, synaptophysin, CD56, and neuron-specific enolase (NSE), combined with the markers on non-endocrine differentiation such as keratin 7 (for gastric tumors) and Keratin 20, CDX2, and carcinoembryonic antigen (CEA), respectively, for colorectal segments.

MANECs have been described in several organs. Beside gastrointestinal segments, it was also reported in the pancreas, gallbladder, and uterine cervix[2]. However, this is an extremely rare tumor, with majority being presented as a case report. To date, in the English literature, only seven cases were reported to have occured in the cecum and about 35 cases in the stomach. Due to its rarity, few aspects regarding the origin and best therapeutic options are known.

In this paper, we present two unusual MANECs of the cecum and the stomach and a pertinent review of the literature.

CASE REPORT

Case 1

A 74-years-old female was admitted to the hospital with intestinal obstruction symptoms, and an emergency right hemicolectomy with terminal ileum resection was performed. Gross examination of the surgical specimen revealed a 70 mm × 18 mm polypoid tumor that produced obstruction of the cecum and presented direct invasion in the serosa of the transverse colon, without invasion in the appendix. The proximal and distal resected margins were free from tumor involvement. Histopathological examination of the surgical specimen confirmed the tumor infiltration of the transverse colon, without metastases in the 40 regional lymph nodes. Angiolymphatic invasion, without perineural invasion, was also noted (pT4bN0 stage). The tumor architecture was predominantly (60% of tumor) solid, consisting of clusters of monomorphic tumor cells with abundant cytoplasm and large nuclei, marked by synaptophysin and NSE, which did not display positivity for keratin AE1/AE3, keratins 7/20, CEA, and chromogranin. A low mitotic activity was seen (< 20 mitoses/10 HPFs). Among these tumor clusters, moderately differentiated glandular structures with focal intraluminal Alcian blue-positive mucus were also seen. The glandular component represented about 40% of the tumor, being marked by keratin AE1/AE3, keratin 20, and CEA, without positivity for chromogranin, synaptophysin, NSE, and keratin 7. Ki67 proliferative index was < 20% (G2), without differences among the two components. Maspin expression was cytoplasmic in the glandular component and slightly positive in the neuroendocrine areas (Figure 1). In the ascending colon, an adenomatous polyp without dysplasia was associated.

The molecular examinations, performed with Roche's LightCycler PCR- and melting-point analysis, revealed a stable microsatellite status. MLH-1 and MSH-2 were positive in both tumor components.

Based on the tumor stage, a classical FOLFOX therapy was recommended. The postoperative course was uneventful, and to date, the patient has survived 10 mo without any evidence of recurrence or metastases.

Case 2

A 46-years-old male was admitted to the hospital with symptoms suggesting gastric cancer: weight loss, hematemesis and melena, without signs of carcinoid syndrome. On upper gastrointestinal endoscopy, a type I-Borrmann's tumor was described in the antral region, which was surgically removed. Intraoperatively, direct invasion in the

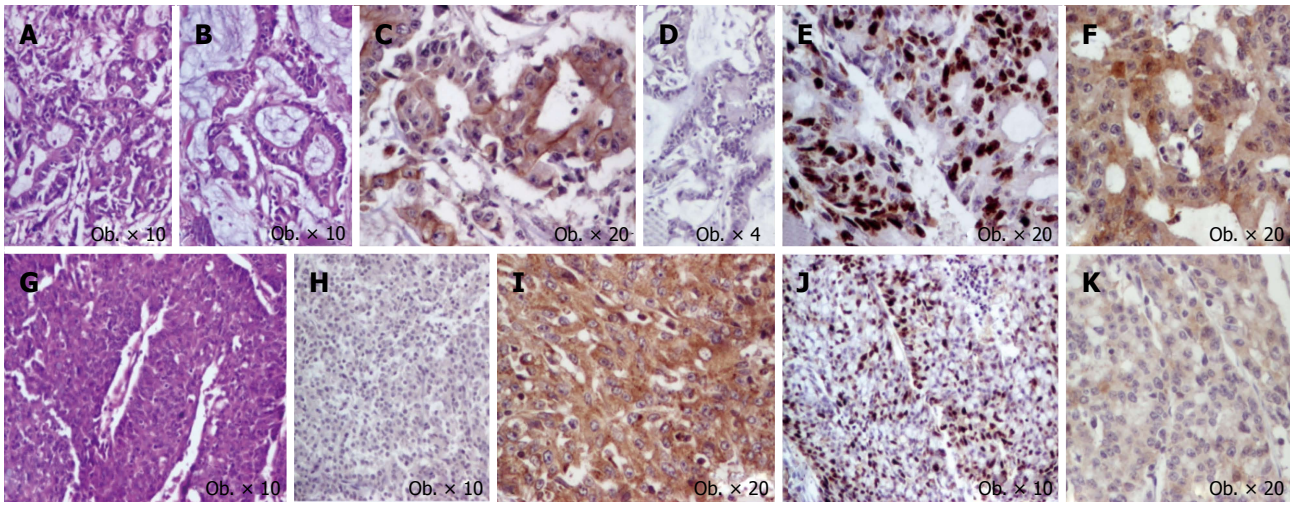

Figure 1 Cecum mixed adenoneuroendocrine carcinoma consists of proliferation of two components. First component is represented by glandular structures with mucinous foci (A-B) and displays positivity for keratin 20 (C) without immunoreactivity for synaptophysin (D), with a high Ki67 index (E) and cytoplasmic expression of maspin (F); The second component is represented by solid tumor islands (G) that are negative for keratin 20 (H) and are immunoreactive for synaptophysin (I), with a lower Ki67 index (J) and slightly cytoplasmic expression of maspin (K).

pancreas was identified. Total gastrectomy with D2 lymphadenectomy was performed. Gross examination of the surgical specimen revealed a 70 mm × 80 mm polypoid antral tumor that produced direct invasion in the pancreas. The tumor invaded the distal resection margin. Histopathological examination of the surgical specimen confirmed the pancreatic invasion, and the presence of metastases in 22 of the 32 lymph nodes, with peri lymphonodular invasion; celiac and hepatic hilum lymph nodes presented metastases. Angiolymphatic and perineural invasions were also noted (pT4N3b stage). The tumor architecture was predominantly (60% of tumor) glandular, with moderately differentiated glandular structures, without mucinous component, marked by keratin AE1/AE3, keratin 7, and CEA, without positivity for chromogranin, synaptophysin, NSE, HER-2, keratin 20, and vimentin. Among the glandular structures, solid sheets, cords, and islands of monomorphic tumor cells with scant cytoplasm and large pleomorphic nuclei were seen; they were immunoreactive for synaptophysin and NSE, and did not display positivity for keratin AE1/AE3, keratins 7/20, chromogranin, HER-2, CEA, and vimentin. Mitotic activity was high (> 20 mitoses/10 HPFs). Ki67 proliferation index was 80% (G3), without differences among the two components. Maspin expression was nuclear in the glandular component and negative in the neuroendocrine areas (Figure 2). In the metastatic lymph nodes, the glandular component was predominant.

The molecular examinations, performed with Roche's LightCycler PCR- and melting-point analysis, revealed a stable microsatellite status. MLH-1 and MSH-2 were positive in both components. The patient refused chemotherapy and died 5 mo after surgery.

DISCUSSION

The cecum MANEC was first described by Cardier in 1924, and another six cases have been reported later[3]. In the colorectal segments, independently by localization, a polypoid lesion has been reported arising in patients aged around 60 years, with the male/female ratio being about 1.5:1. All seven cases of cecum MANEC were reported in women. In majority of the cases, MANEC presents with an aggressive behavior and a high risk for liver metastases[3,4]. Independent from the proportion of the neuroendocrine component, the associated-carcinoid syndrome was not reported yet in the literature; the serum level of tumor markers such as CEA, CA125, and CA19-9 are also normal[1]. The clinical behavior seems to be influenced by the neuroendocrine component[1]. However, identification of the neuroendocrine component in tubular adenocarcinomas is not easily performed because the neuroendocrine cells are not always immunoreactive for specific markers, with the reported rate of positivity being 60%-70% for chromogranin, 75%-90% for synaptophysin, and 50% for CD56[1,5,6]. In our case, the neuroendocrine component was diffusely positive for synaptophysin and negative for chromogranin. The cytoplasmic expression of maspin in the glandular structures indicates an indolent behavior of this component[7], it's negativity in the neuroendocrine areas confirming that the clinical outcome depends on the characteristics of the neuroendocrine carcinoma. Angiolymphatic invasion was noted in our cases without any lymph node metastases.

The reported clinicopathologic characteristics of gastric MANEC are similar with those of colorectal segments, the non-endocrine component being

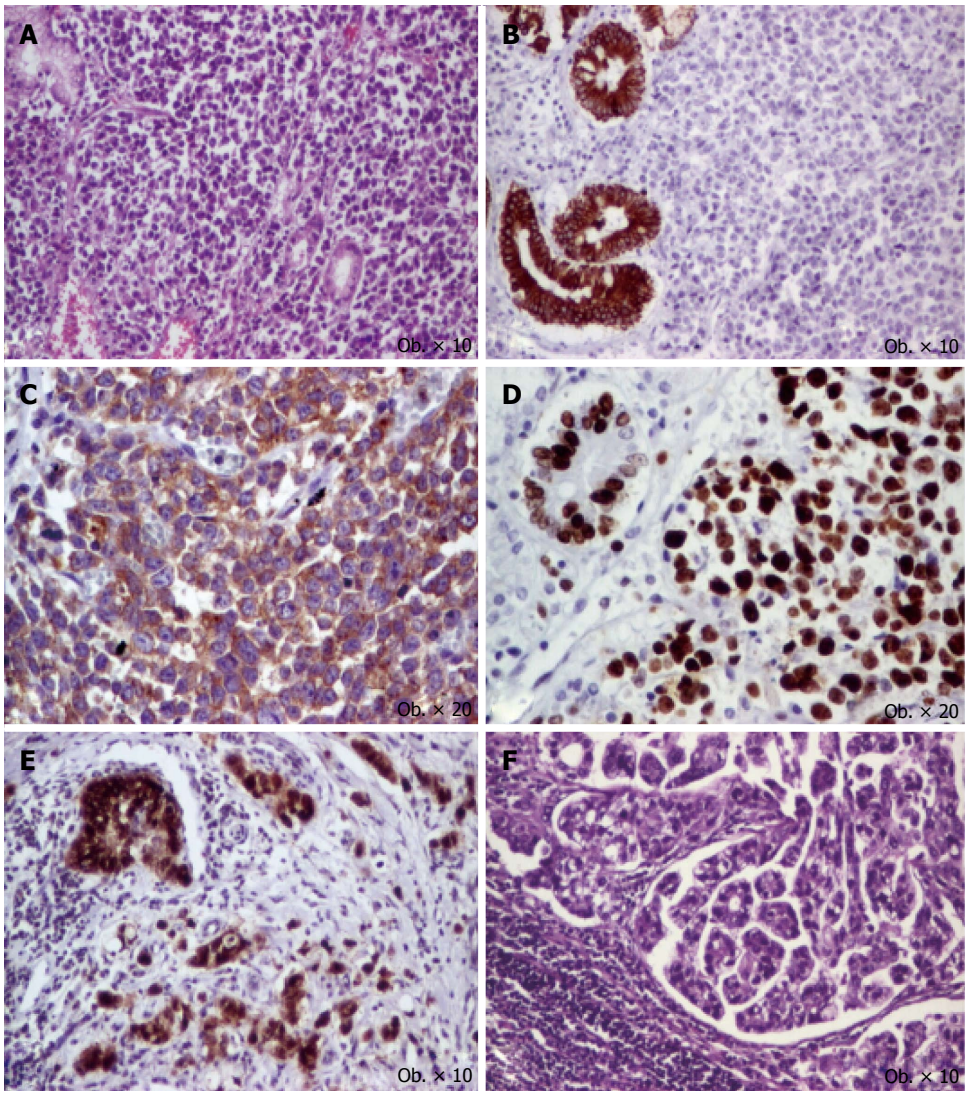

Figure 2 Gastric mixed adenoneuroendocrine carcinoma consists of mixed proliferation of glandular with specific immunoexpression. Solid tumor areas (A); The glandular structures are reactive for keratin AE1/AE3 (B) and solid structures express synaptophysin (C); A high Ki67 index can be seen in both tumor components (D); The nuclear expression of maspin in the glandular component (E) reflects the tumor aggressiveness that is confirmed by predominance of this component in lymph node metastases (F).

rather an intestinal type rather than a diffuse-type gastric carcinoma. The mean age is 58 years old (range: 30 to 80 years old), and the polypoid lesions are usually located in upper, middle, or lower third of the stomach[6]. Although some authors sustain that clinical behavior depends on the grade of the neuroendocrine component, some of them reveal that the characteristics of adenocarcinomatous part influence the outcome in well-differentiated neuroendocrine components[6,8]. In our case, the glandular component was predominant in lymph node metastases and nuclear expression of maspin in glandular structures compared with its negativity in the neuroendocrine part confirmed the higher aggressiveness of the glandular part, compared to the poorly differentiated neuroendocrine one[7].

Due to the rarity of this tumor, few aspects are known about its histogenesis, with most of the authors admitting its origin in a multipotent stem cell with bidirectional differentiation, opposite to collision tumor, in which a separate origin of the two components is supposed[1-3,6]. Because the poorly differentiated areas can also display positivity for synaptophysin in colorectal carcinomas[9], we tend to believe that it is not about a real multidirectional differentiation of a single neoplasm but rather a neuroendocrine phenotype of dedifferentiated areas of tubular adenocarcinoma. To sustain our hypothesis, it is necessary to also take into account the expression of CD133, the marker of cancer stem cells. Recently, it was reported that CD133 was expressed in 30% of well-differentiated neuroendocrine tumors, in 26% of poorly differentiated neuroendocrine carcinomas, and in 64% of MANEC of the digestive tract[10]. On the other hand, the proportion of CD133 positivity in colorectal adenocarcinomas was 47%, without correlation with the tumor grade but with a strong correlation

with the tumor aggressiveness[11]. In gastric adenocarcinoma, the CD133 positivity was 42%, being also correlated with the clinical outcome[12]. Comparing the proportion of CD133 positivity, we can also support that the neuroendocrine component of MANEC, independent of its location, is rather a dedifferentiated area of a classic adenocarcinoma.

Clarifying this hypothesis could help identify of the proper therapeutic management of these rare, highly malignant tumors, similar to that of ordinary adenocarcinomas or for neuroendocrine tumors.

COMMENTS

Case characteristics

A 74-years-old female with a locally advanced tumor of the cecum (Case 1) and an 46-years-old male with a node positive gastric cancer (Case 2).

Clinical diagnosis

Intestinal obstruction in Case 1, and weight loss, hematemesis, and melena, in Case 2.

Differential diagnosis

Intestinal infarction in Case 1, chronic peptic ulcer in Case 2.

Laboratory diagnosis

Non-specific - slight anemia in both of the cases (low hemoglobin and hematocrit); liver function tests were within normal limits.

Imaging diagnosis

Case 1-an emergency hemicolectomy was performed without supplementary examinations. Case 2-CT and MRI examinations were not performed. The upper gastrointestinal endoscopy revealed a type I - Borrmann's tumor in the antral region.

Pathological diagnosis

Examination of surgical specimens from cecum (case 1) and stomach (case 2) revealed a mixed adenoneuroendocrine carcinoma (MANEC) in both of the cases, that was immunohistochemically confirmed.

Treatment

Case 1 - the patient was treated with FOLFOX regimen; Case 2 - the patient refused the chemotherapy.

Experiences and lessons

This case report shows two microsatellite-sable MANECs of the gastrointestinal tract, tumors that are not only rare but also seems to have an aggressive behavior that depends on the characteristics of the neuroendocrine component, independend of its proportion. Because this component seems to be a deddiferentiated adenocarcinoma with acquired neuroendocrine properties, a very attentive evaluation should be microscopically performed in adenocarcinomas with dedifferentiated areas. Identification of the neuroendocrine components could have therapeutic relevance.

Peer review

This article applies a large panel of molecular markers, including the prognostic marker maspin, to explore the origins and behavior of gastrointestinal MANEC in two cases with distinct characteristics.

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CASE REPORT

Isolated intrapancreatic IgG4-related sclerosing cholangitis

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Author contributions: Nakazawa T analyzed data and wrote the paper; Ikeda Y collected clinical data; Kawaguchi Y collected clinical data; Kitagawa H collected clinical data; Takada H collected clinical data; Takeda Y collected clinical data; Makino I collected clinical data; Makino N collected clinical data; Naitoh I analyzed data and wrote the paper; and Tanaka A analyzed data.
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Abstract

Immunoglobulin G4-related sclerosing cholangitis (IgG4-SC) is frequently associated with type 1 autoimmune pancreatitis (AIP). Association with AIP can be utilized in the diagnosis of IgG4-SC. However, some cases of IgG4-SC are isolated from AIP, which complicates the diagnosis. Most of the reported cases of isolated IgG4-SC displayed hilar biliary strictures, whereas isolated IgG4-SC with intrapancreatic biliary stricture is very rare. Recently, we have encountered 5 isolated intrapancreatic IgG4-SC cases that were not associated with AIP, three of which were pathologically investigated after surgical operation. They all were males with a mean age of 74.2 years. The pancreas was not enlarged in any of these cases. No irregular narrowing of the main pancreatic duct was found. Bile duct wall thickening in lesions without luminal stenosis was detected by abdominal computed tomography in all five cases, by endoscopic ultrasonography in two out of four cases and by intraductal ultrasonography in all three cases. In three cases, serum IgG4 levels were within the normal limits. The mean serum IgG4 level measured before surgery was 202.1 mg/dL (4 cases). Isolated intrapancreatic IgG4-SC is difficult to diagnose, especially if the IgG4 level remains normal. Thus, this type of IgG4-SC should be suspected in addition to cholangiocarcinoma and pancreatic cancer if stenosis of intrapancreatic bile duct is present.

Key words: Immunoglobulin G4-related sclerosing cholangitis; Isolated immunoglobulin G4-related sclerosing cholangitis; Autoimmune pancreatitis

Core tip: If stenosis of intrapancreatic bile duct is present and no abnormal findings of pancreas are detected, cholangiocarcinoma is suspected. Recently, we have encountered 5 isolated intrapancreatic immunoglobulin G4-related sclerosing cholangitis (IgG4-SC) cases that were not associated with autoimmune pancreatitis, three of which were pathologically investigated after surgical operation. Isolated intrapancreatic IgG4-SC is difficult to diagnose, especially if the IgG4 level remains normal. Thus, this type of IgG4-SC should be suspected in addition to cholangiocarcinoma and pancreatic cancer if stenosis of intrapancreatic bile duct is present.

Nakazawa T, Ikeda Y, Kawaguchi Y, Kitagawa H, Takada H, Takeda Y, Makino I, Makino N, Naitoh I, Tanaka A. Isolated intrapancreatic IgG4-related sclerosing cholangitis. *World J Gastroenterol* 2015; 21(4): 1334-1343 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1334.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1334

INTRODUCTION

Immunoglobulin G4-related sclerosing cholangitis (IgG4-SC) is frequently associated with type 1 autoimmune pancreatitis (AIP). Association with AIP can be utilized in the diagnosis of IgG4-SC[1]. However, some cases of IgG4-SC are isolated from AIP, which complicates the diagnosis[2,3].

IgG4-SC displays various cholangiographic features similar to those of pancreatic cancer, primary sclerosing cholangitis (PSC), and cholangiocarcinoma (CC). The characteristic features of IgG4-SC can be classified into 4 types based on the stricture regions revealed by cholangiography and differential diagnosis (Figure 1)[4].

Most of the reported cases of isolated IgG4-SC displayed hilar biliary strictures, whereas isolated IgG4-SC with intrapancreatic biliary stricture is very rare[2,3]. Recently, we have encountered 5 isolated intrapancreatic IgG4-SC cases that were not associated with AIP, three of which were pathologically investigated after surgical operation. In the present study, we examined this series to clarify the clinical profiles of isolated intrapancreatic IgG4-SC.

CASE REPORT

We report 5 cases of isolated type1 IgG4-SC. One case was retrieved from 87 IgG4-SC case records in Nagoya City University. Two cases were presented during the 49th annual meeting of Japan Biliary Association. The other two cases were retrieved from the nationwide survey for PSC and IgG4-SC conducted in Japan. Informed consents were obtained from all the patients.

Below, we will first describe the details of these five cases. We will then characterize them in terms of serum IgG4 levels, pancreatic features, bile duct features, diagnosis, and treatment.

Case 1

An 82-year-old man was diagnosed with intrahepatic bile duct dilation during a follow-up after gastrectomy for gastric cancer. For further evaluation, he was referred to Kansai Rosai Hospital. On admission, serum hepatobiliary enzymes were elevated, but serum IgG4 was within the normal range. Endoscopic retrograde cholangiopancreatography (ERCP) revealed stenosis of the intrapancreatic bile duct but did not show irregular narrowing of the main pancreatic duct (MPD) (Figure 2A-C). Computed tomography (CT) showed thickening of the extrahepatic bile duct wall without enlargement of the pancreas (Figure 2D and E). Positron emission tomography-CT (PET-CT) imaging with 18F-fluorodeoxyglucose (FDG) detected intense 18F-FDG uptake in the intrapancreatic duct. Bile duct cytology and endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) revealed no malignant features. With a tentative diagnosis of bile duct cancer at the intrapancreatic duct, the patient underwent pancreaticoduodenectomy. Histological examination showed no malignant cells, but infiltration of lymphocytes and IgG4-positive plasma cells, storiform fibrosis, and obstructive phlebitis in the bile duct wall were present (Figure 2F-H). Examination of the adjacent pancreatic tissue did not reveal any signs of AIP (Figure 2I and J). On the basis of these findings, the disease was diagnosed as isolated intrapancreatic IgG4-SC.

Case 2

A 60-year-old man was initially admitted to an affiliate hospital for the evaluation of a gall bladder tumor. Under the suspicion of bile duct cancer he was then referred to Kanazawa University Hospital. The serum IgG4 level was within the normal range. Abdominal CT detected long segmental wall thickening in the middle and lower extrahepatic bile duct and cystic duct, but the pancreas was of normal size (Figure 3A and B). Wall thickening of the fundus of the gallbladder was also detected (Figure 3B). ERCP and magnetic resonance cholangiopancreatography (MRCP) revealed long segmental stenosis in the middle and lower extrahepatic bile duct and normal MPD with an exception of a cyst (Figure 3C and D). Transpapillary bile duct biopsy did not provide enough tissue for histopathological evaluation. Cytology of the bile duct showed Class Ⅳ, whereas PET-CT detected intense 18F-FDG uptake in the middle and lower extrahepatic bile duct. With a tentative diagnosis of cholangiocarcinoma in the middle and lower extrahepatic bile duct and adenomyomatosis of

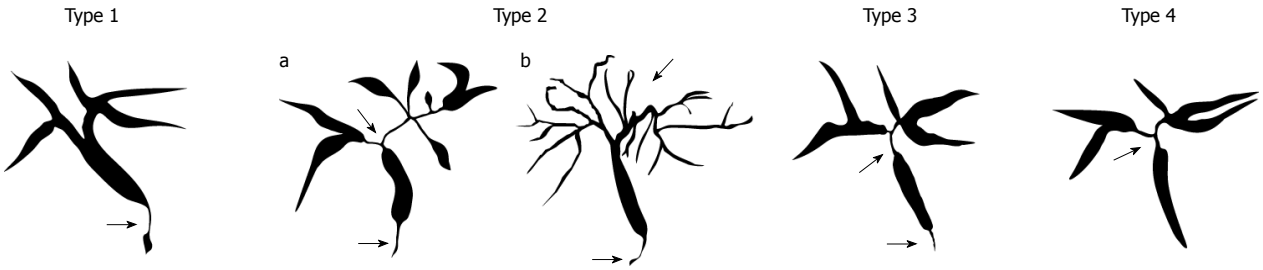

Figure 1 Cholangiographic classification of IgG4-related sclerosing cholangitis. Stenosis is located only in the lower part of the common bile duct in Type 1; stenosis is diffusely distributed in the intra-and extra-hepatic bile ducts in Type 2. Type 2 is further subdivided into 2 types. Extended narrowing of the intrahepatic bile ducts with prestenotic dilation is widely distributed in Type 2a. Narrowing of the intrahepatic bile ducts without prestenotic dilation and reduced bile duct branches are widely distributed in Type 2b; stenosis is detected in both the hilar hepatic lesions and the lower part of the common bile ducts in Type 3; strictures of the bile duct are detected only in the hilar hepatic lesions in Type 4.

gallbladder, the patient underwent pylorus-preserving pancreaticoduodenectomy.

Histological examination revealed characteristic findings of IgG4-SC (Figure 3E-G) and IgG4-related cholecystitis (Figure 3H). The adjacent pancreatic tissue was found to be normal (Figure 3E and I). On the basis of these findings, the disease was diagnosed as isolated intrapancreatic IgG4-SC and IgG4-related cholecystitis.

Case 3

An 81-year-old man was admitted to an affiliate hospital for the treatment of common bile duct stones. ERCP revealed a stricture in the intrapancreatic duct. The patient was then referred to Kasugai Municipal Hospital for further evaluation. ERCP and MRCP showed a short stenosis in the intrapancreatic bile duct and normal MPD (Figure 4A and B). Abdominal CT detected wall thickening in the middle and lower extrahepatic bile duct and the pancreas of normal size (Figure 4C and D). The patient underwent pylorus-preserving pancreaticoduodenectomy based on the diagnosis of cholangiocarcinoma.

Histological examination revealed characteristic findings of IgG4-SC (Figure 4E and F). Examination of the adjacent pancreatic tissue indicated normal pancreas (Figure 4G). Based on these findings, the diagnosis of isolated intrapancreatic IgG4-SC was made. The level of serum IgG4 measured after the surgery was within the normal range.

Case 4

A 61-year-old man was admitted with jaundice. ERCP showed a stenosis of intrapancreatic duct. He was then referred to Tokai University Hospital for further evaluation. As in previous cases, abdominal CT detected wall thickening in the intrapancreatic bile duct, but the pancreas was of normal size (Figure 5A and B). ERCP revealed a stenosis of intrapancreatic duct and normal MPD (Figure 5C and D), whereas intraductal ultrasonography (IDUS) showed symmetric and smooth thickening of the inner hypoechoic layer of the bile ducts spreading from the intrapancreatic duct to the intrahepatic ducts (Figure 5E-G). Transpapillary bile duct biopsy showed infiltration of lymphocytes and 23 IgG4-positive plasma cells/high power field. The ratio of IgG4/IgG-positive plasma cells was 43.5%. The serum IgG4 level was 509 mg/dL. In accordance with these findings, the disease was diagnosed as isolated intrapancreatic IgG4-SC. Steroid therapy was administered with the initial dose of 30 mg. ERCP showed an improvement of the stenosis 3 mo later. He underwent maintenance steroid therapy and IgG4-SC has not recurred until now.

Case 5

An 87-year-old man was admitted with jaundice. ERCP and MRCP detected a slight dilation of the MPD (Figure 6A and B). A stenosis in the intrapancreatic common bile duct was revealed with cholangiography (Figure 6A and B). Abdominal CT showed a normal-sized pancreas (Figure 6C). IDUS showed thickening of the inner hypoechoic layer of the bile ducts spreading from the intrapancreatic common bile duct to the middle common bile duct (Figure 6D-F). Transpapillary biopsy of the stenotic area of the bile duct did not reveal malignant cells. The level of serum IgG4 was 262 mg/dL. The patient was associated with IgG4-related sialadenitis and retroperitoneal fibrosis. He was diagnosed with isolated intrapancreatic IgG4-SC. Endoscopic biliary drainage was performed and followed up.

The clinical characteristics of the 5 patients are summarized in Table 1. They all were males with a mean age of 74.2 years. The pancreas was not enlarged in any of these cases. No irregular narrowing of the MPD was found. Bile duct wall thickening in lesions without luminal stenosis, which is typical of IgG4-SC, was detected by abdominal CT in all five cases, by endoscopic ultrasonography (EUS) in two out of four cases and by IDUS in all three cases. In three cases, serum IgG4 levels were within the normal limits. The mean serum IgG4 level measured before surgery was 202.1 mg/dL (4

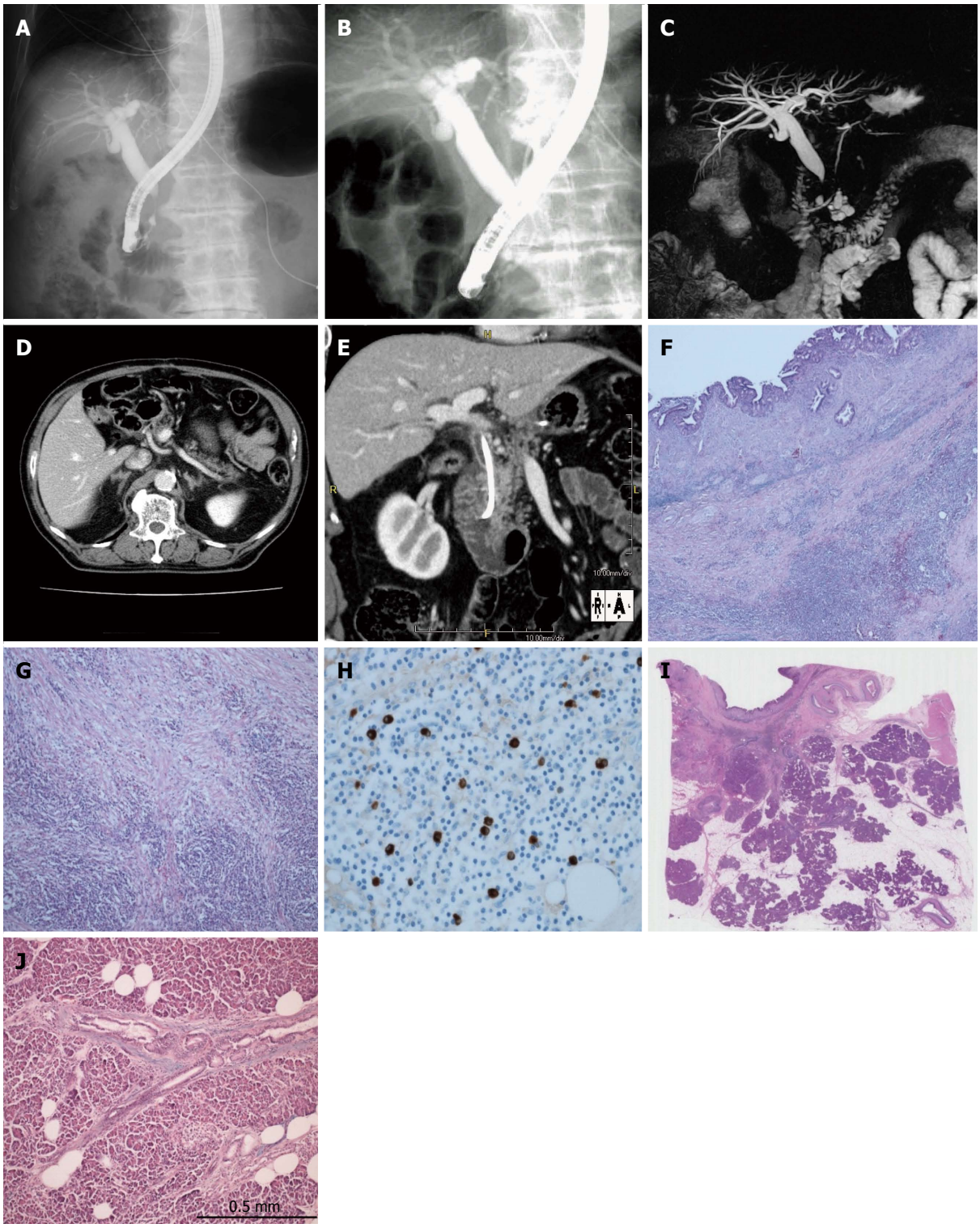

Figure 2 Imaging and pathological findings of Case 1. A: Stenosis of the intrapancreatic bile duct on endoscopic retrograde cholangiopancreatography (ERCP); B, C: No irregular narrowing of the main pancreatic duct on ERCP and magnetic resonance cholangiopancreatography; D: No enlargement of the pancreas on computed tomography (CT); E: Thickening of the extrahepatic bile duct wall on CT (arrowhead); F: Bile duct wall thicking in surgical specimen of bile duct wall (HE × 40); G: Abundant infiltration of lymphocytes and plasma cells in the bile duct wall (HE × 200); H: Abundant infiltration of IgG4-positive plasma cells in the bile duct wall (IgG4 staining × 800); I, J: No findings mimicking AIP in surgical specimen of adjacent pancreatic tissue (I: HE × 2), (J: HE × 400).

cases).

Three out of 5 cases (1, 2, and 3) were not diagnosed with IgG4-SC until the surgery. In the remaining two cases, however, the diagnosis was established without operation. Among those two, one (case 4) received steroid therapy, whereas the other (case 5) was treated with endoscopic biliary drainage only. Two out of three cases that were surgically treated (case 1 and 2) showed low serum IgG4 levels (22.8 mg/dL and 14.6 mg/dL, respectively)

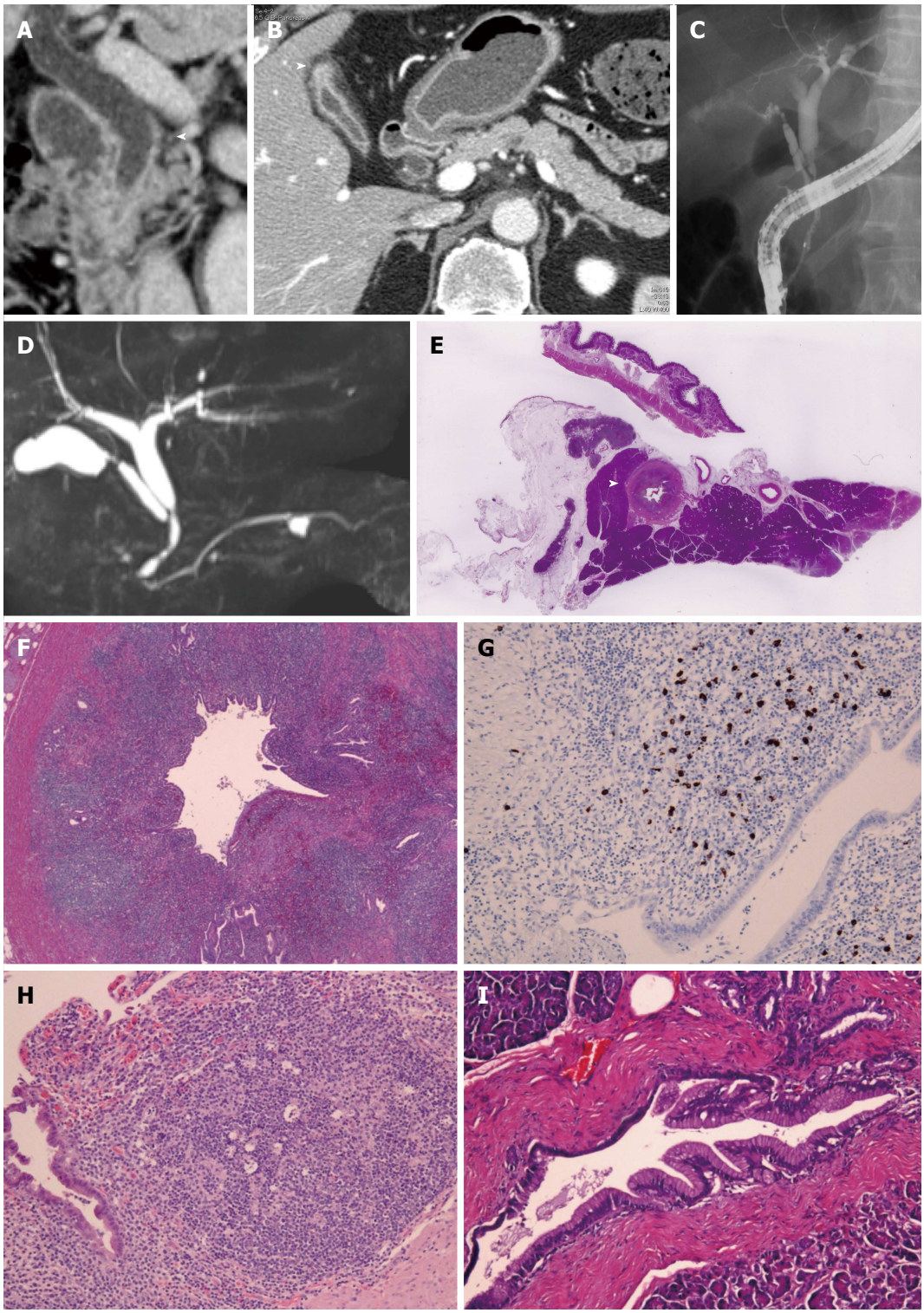

Figure 3 Imaging and pathological findings of Case 2. A: Long segmental wall thickness in the middle and lower extrahepatic bile duct on abdominal computed tomography (arrowhead); B: Wall thickness of the fundus of gall bladder (arrowhead) and normal size of the pancreas; C: Long segmental stenosis in the middel and lower extrahepatic bile duct on endoscopic retrograde cholangiography; D: Normal main pancreatic duct except a pancreatic cyst on magnetic resonance cholangiopancreatography; E: Bile duct wall thicking (arrow head) and no inflammation of pancreas tissue in surgical specimen (HE × 1); F: Abundant infiltration of lymphocytes and plasma cells in the bile duct wall (HE × 200); G: Abundant infiltration of IgG4-positive plasma cells in the bile duct wall (IgG4 staining × 400); H: Numerous lymphocytes and plasma cells in the wall of gall bladder (HE × 400); I: Normal pancreatic tissue in adjacent pancreas (HE × 400).

before the surgery, whereas in the remaining case (case 3) this parameter was not measured at that time. Only one case with high serum IgG4 (case 5) was associated with other organ involvements. The reasons for surgical treatment were as follows. Adenocarcinoma was suspected based on the results of brush cytology in two cases (case 2 and 3). The possibility of cholangiocarcinoma could not be ruled

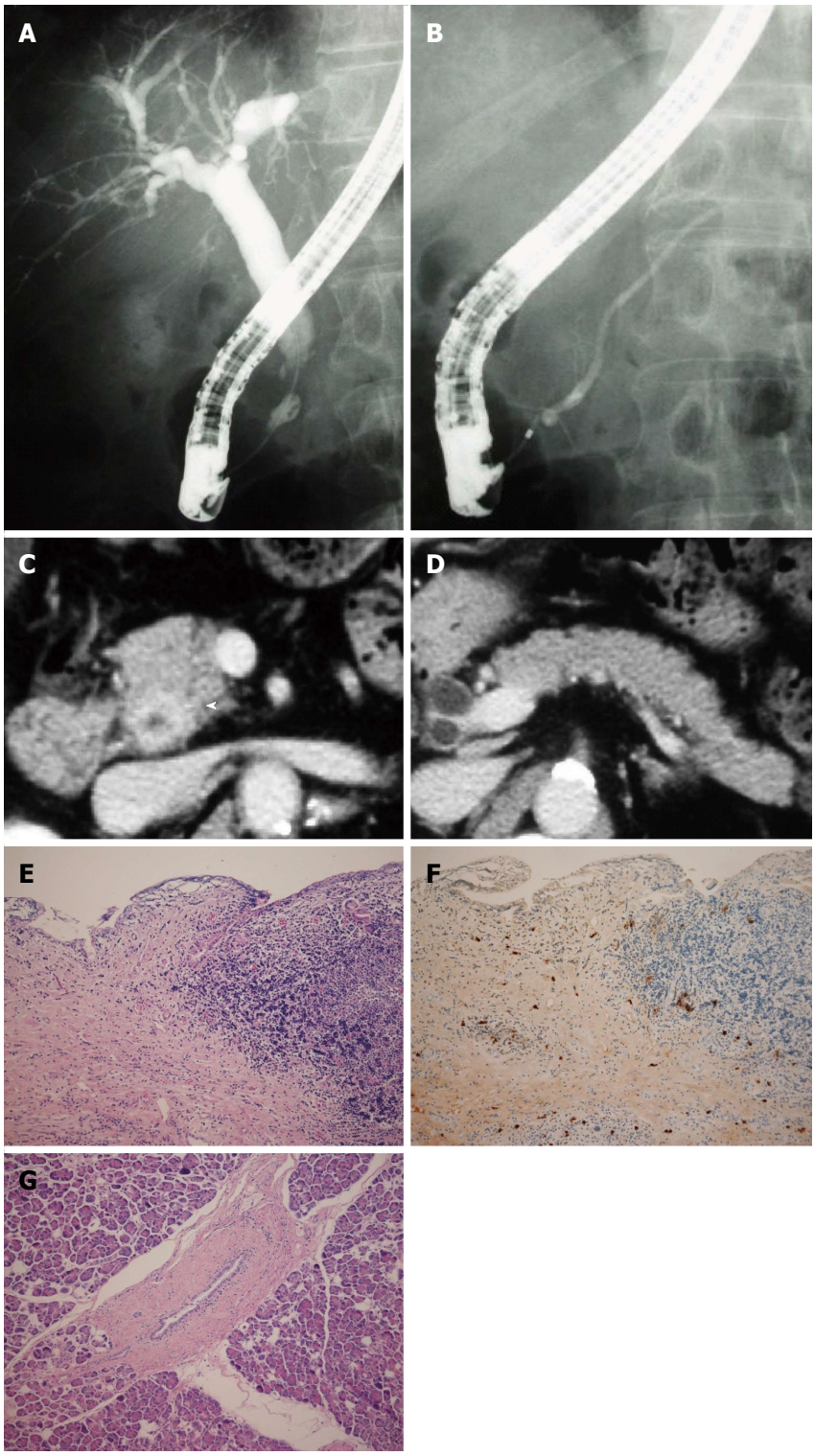

Figure 4 Imaging and pathological findings of Case 3. A: Stricture in the intrapancreatic duct on endoscopic retrograde cholangiography; B: Normal main pancreatic duct on endoscopic retrograde pancreatography; C: Wall thickening in the middle and lower extrahepatic bile duct on abdominal computed tomography (arrowhead); D: Pancreas of normal size; E: Bile duct wall thicking (HE × 40); F: Abundant IgG4-positive plasma cells in bile duct wall (IgG4 staining × 200); G: No inflammation of pancreas tissue (HE × 400).

out in spite of the negative results of brush cytology and EUS-FNA in the remaining case (case1).

In three cases (case 1, 2, and 3), pathological findings in the surgical specimens of the bile duct showed severe infiltration of lymphocytes and IgG4-positive plasmacytes as well as prominent fibrosis in the bile duct. These findings were compatible with the diagnosis of IgG4-SC. However, no inflammatory changes compatible with AIP in the adjacent pancreas tissue were found in any of these three cases.

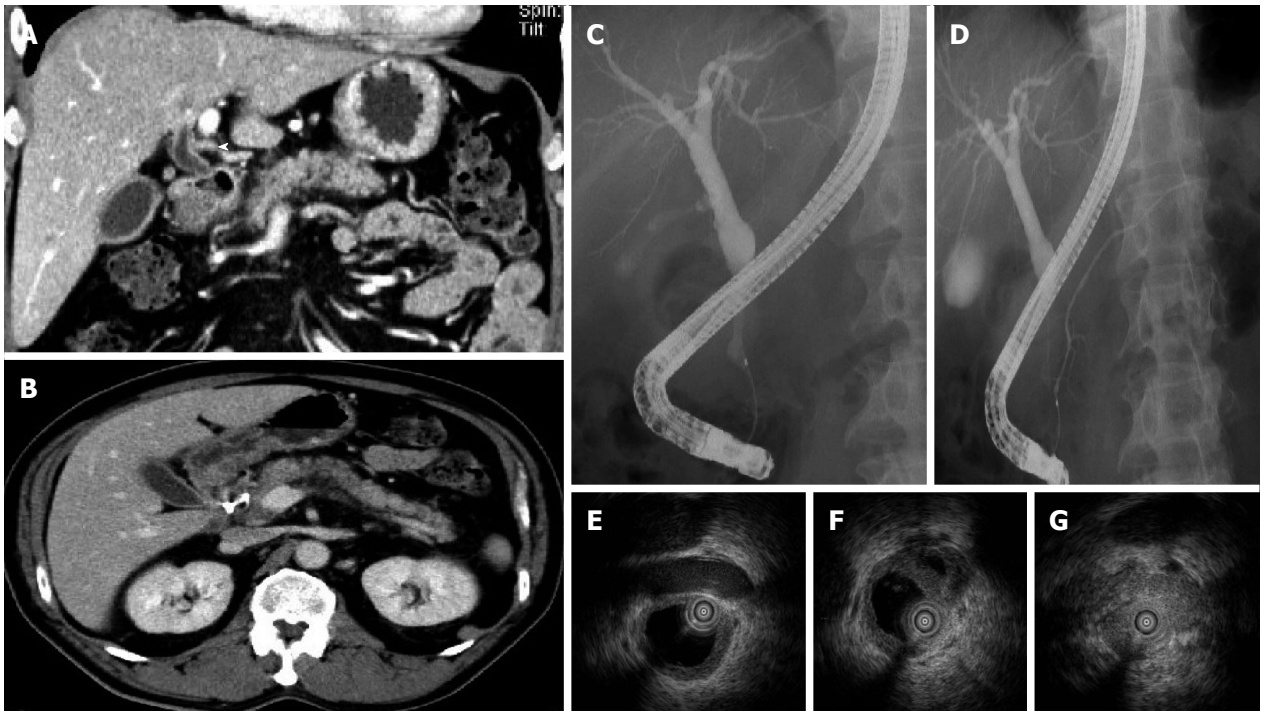

Figure 5 Imaging findings of Case 4. A: Wall thickness of extrahepatic bile duct on abdominal computed tomography (CT); B: Normal size of the pancreas on abdominal CT; C: Stenosis in the lower extrahepatic bile duct on endoscopic retrograde cholangiography; D: Normal main pancreatic duct on endoscopic retrograde pancreatography; E (at the hilar hepatic lesion); F (at the bifurcation of cystic duct); G (at the intrapancreatic lesion): Bile duct wall thickening with smooth inner and outer margin in areas with stenosis (G) and without (E, F) on intraductal ultrasonography.

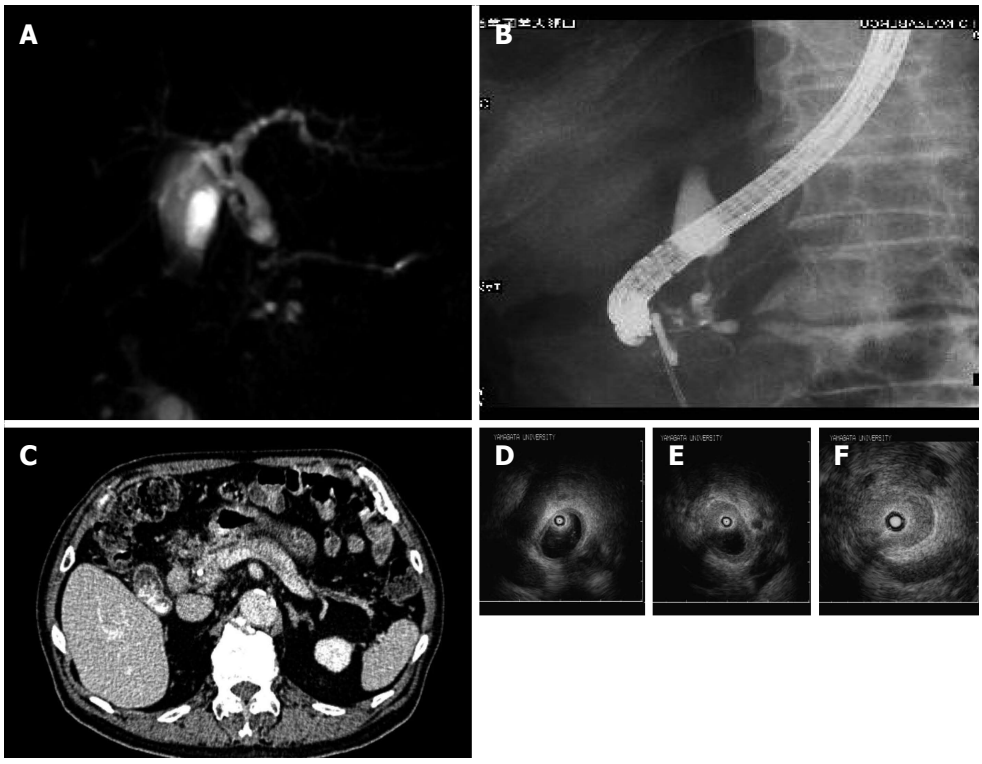

Figure 6 Imaging findings of Case 5. A, B: Stenosis in the lower extrahepatic bile duct and a slight dilation of the main pancreatic duct on endoscopic retrograde cholangiopancreatography (A) and magnetic resonance cholangiopancreatography (B); C: Normal size of the pancreas on abdominal computed tomography; D (at the hilar hepatic lesion); E (at the bifurcation of cystic duct); F (at the intrapancreatic lesion): Bile duct wall thickening with smooth inner and outer margin in areas with stenosis (F) and without (D, E) on intraductal ultrasonography.

Table 1 Clinical profile of isolated type1 immunoglobulin G4-related sclerosing cholangitis without autoimmune pancreatitis

Case No.	Age	Sex	Year	Serum IgG4	Pancreatic enlargement	Narrowing of MPD	Bile duct biopsy/ cytology	IDUS/EUS	Wall thickening In lesions without luminal stenosis	Other modalities	First diagnosis	Treatment
(1) Kansai Rosai Hospital	82	M	2012	22.8	Atrophic	Normal (MRCP)	Negative (cytology)	Symmetric, smooth wall thickening (EUS)	Yes (CT)	Intense uptake (PET-CT)	Cholangio carcinoma	PD
(2) Kanazawa University	60	M	2012	14.6	Normal	Normal	Inadequate Sample (biopsy) Suspicious of adenocarcinoma (cytology)	Symmetric, smooth wall thickening (EUS)	Yes (CT)	Intense uptake (PET-CT)	Cholangio carcinoma Cholangiocarcinoma Adenomyomatosis of gall bladder	PPPD
(3) Kasugai municipal Hospital	81	M	2009	76 (after operation)	Normal	Normal	Suspicious of adenocarcinoma (biopsy)	Symmetric, smooth wall thickening (IDUS)	Yes (CT, IDUS)		Cholangio carcinoma	PPPD
(4) Tokai University	61	M	2010	509	Normal	Normal	IgG4-SC (biopsy)	Symmetric, smooth wall thickening (IDUS, EUS)	Yes (CT, IDUS, EUS)		IgG4-SC	Predoniso lone
(5) Yamagata University	87	M	2009	262	Normal	Dilation	No malignancy (biopsy)	Symmetric, smooth wall thickening (IDUS, EUS)	Yes (CT,IDUS, EUS)		IgG4-SC	Endoscopic biliary drainage

M: Male; F: Female; IDUS: Intraductal ultrasonography; EUS: Endoscopic ultrasonography; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving PD; MPD: Main pancreatic duct; CT: Computed tomography; PET-CT: Positron emission tomography-CT; IgG4-SC: Immunoglobulin G4-related sclerosing cholangitis; MRCP: Magnetic resonance cholangiopancreatography.

Based on the Japanese clinical diagnostic criteria of 2012[5], all five cases were diagnosed as definite IgG4-SC.

DISCUSSION

IgG4-SC is often associated with AIP, and the frequency of isolated IgG4-SC is low. Most of the reported cases of isolated IgG4-SC showed type 3 or type 4 cholangiogram (Figure 1), and a series of isolated type 1 (intrapancreatic) IgG4-SC cases has not been reported until now[2,3]. In the present study we have described 5 such cases and evaluated their clinical features. Recently, two Japanese studies have evaluated the frequency of IgG4-SC in large population samples. First, a multi-institutional study has utilized our cholangiographic classification system to reveal that out of the total of 349 IgG4-SC cases, 334 (95.7%) were associated with AIP[6]. Specifically, 244/246 (99.2%), 51/56 (91.1%), 28/29 (96.5%), and 11/18 (61.0%) cases of types 1, 2, 3, and 4 IgG4-SC, respectively, were found to be associated with AIP. Of note, type 4 IgG4-SC cases showed a lower frequency of association. Second, a nationwide survey for primary and IgG4-related sclerosing cholangitis conducted by the Japanese Biliary Association has revealed that type 1, 2, 3, and 4 IgG4-SC was not associated with AIP in 7%, 7%, 9%, and 51% of the cases, respectively, whereas this percentage was 26 for the unclassified type[7]. These results indicate that only type 4 IgG4-SC is frequently found in the isolated form. Thus, the cases of isolated type 1 IgG4-SC are very rare.

The feasibility of including type 1 IgG4-SC into the IgG4-SC category has been disputed. Some researchers suggest that the stricture of the lower common bile duct, which is observed in type 1 IgG4-SC, is caused by compression due to AIP. This claim is based on the fact that the frequency of type 1 IgG4-SC was

Table 2 Characteristic features of isolated intrapancreatic IgG4-related sclerosing cholangitis

Isolated intrapancreatic IgG4-SC is rare among isolated IgG4-SC
Isolated intrapancreatic IgG4-SC is misdiagnosed as cholangiocarcinoma of intrapancreatic duct
Frequency of cases with higher serum IgG4 level is low in isolated intrapancreatic IgG4-SC cases
Bile duct wall thickening in lesions without luminal stenosis detected by abdominal CT, EUS and IDUS is useful finding in the diagnosis of isolated intrapancreatic IgG4-SC

IgG4-SC: Immunoglobulin G4-related sclerosing cholangitis; CT: Computed tomography; IDUS: Intraductal ultrasonography; EUS: Endoscopic ultrasonography.

low (16%) in AIP without pancreatic head lesion[8]. However, we believe that type 1 IgG4-SC should be classified as one of the IgG4-SC types because of the following reasons. First, pathological examination of the bile duct wall obtained from surgically resected samples showed abundant IgG4-positive plasma cell infiltration, storiform fibrosis, and obstructive phlebitis, which are characteristics of IgG4-SC-associated inflammation[9]. Second, the results of an IDUS study showed continuous thickening of the bile duct wall from the intrapancreatic to the extrapancreatic bile duct[10]. In addition, this paper revealed that some cases showed inflammation of only the bile duct wall and not of the pancreas. In fact, it is difficult to identify which factor is the main contributor to the thickening of the bile duct wall, inflammation of the bile duct or compression due to AIP. However, we believe that type 1 IgG4-SC should be included into our cholangiographic classification system as an independent type because the purpose of this system is to facilitate clinical awareness of these conditions in order to avoid unnecessary surgical resection under the diagnosis of cholangiocarcinoma or liver transplantation under the diagnosis of PSC.

Some cases of IgG4-SC isolated from AIP are difficult to diagnose[2,3]. There seem to be several reasons for this. Bile duct biopsy may not be able to provide a large enough sample that would allow identification of characteristic pathological features of IgG4-SC[10], whereas the results of cytology, which has been performed in two cases presented in the current study, suggested adenocarcinoma. PET-CT showed intense 18F-FDG uptake in two cases. In addition, two studies reported that the serum IgG4 level is often normal in IgG4-SC[2,3]. In agreement with this observation, serum IgG4 levels were found to be within the normal limits in 2 out of 4 cases presented here for which the analysis was done prior to surgery.

We summarized the key findings of isolated intrapancreatic IgG4-SC in Table 2.

Frequency of cases with higher serum IgG4 level is low in isolated intrapancreatic IgG4-SC cases. However, bile duct wall thickening in lesions without luminal stenosis detected by abdominal CT, EUS and IDUS is useful finding in the diagnosis of isolated intrapancreatic IgG4-SC.

In conclusion, isolated intrapancreatic IgG4-SC is difficult to diagnose, especially if the IgG4 level remains normal. Thus, this type of IgG4-SC should be suspected in addition to cholangiocarcinoma and pancreatic cancer if stenosis of intrapancreatic bile duct is present.

COMMENTS

Case characteristics

Five male patients with isolated intrapancreatic IgG4-related sclerosing cholangitis.

Clinical diagnosis

Three patients were misdiagnosed as cholangiocarcinoma and two patents were correctly diagnosed as isolated intrapancreatic IgG4-related sclerosing cholangitis.

Differential diagnosis

Intrapancreatic cholangiocarcinoma.

Laboratory diagnosis

In three cases, serum IgG4 levels were within the normal limits.

Imaging diagnosis

Stenosis and wall thickness of intrapancreatic bile duct. Bile duct wall thickening in lesions without luminal stenosis detected by abdominal computed tomography, endoscopic ultrasonography and intraductal ultrasonography is useful finding in the diagnosis.

Pathological diagnosis

Three surgical specimen and one bile duct biopsy showed infiltration of abundant IgG4-positive plasma cells

Treatment

Three patients were surgically treated. Another underwent steroid therapy and the other endoscopic biliary drainage.

Related reports

There are no case reports with isolated intrapancreatic IgG4-related sclerosing cholangitis.

Term explanation

Isolated intrapancreatic IgG4-related sclerosing cholangitis is type 1 IgG4-related sclerosing cholangitis without autoimmune pancreatitis.

Experiences and lessons

Isolated intrapancreatic immunoglobulin G4-related sclerosing cholangitis (IgG4-SC) is difficult to diagnose, especially if the IgG4 level remains normal. Thus, this type of IgG4-SC should be suspected in addition to cholangiocarcinoma and pancreatic cancer if stenosis of intrapancreatic bile duct is present.

Peer review

In the manuscript, the authors described the clinical findings of 5 cases of isolated IgG4-SC, the manuscript is well written, and the cases are detailed introduced. The current manuscript enriches the knowledge of isolated IgG4-SC.

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CASE REPORT

Pulmonary embolism after arterial chemoembolization for hepatocellular carcinoma: An autopsy case report

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Abstract

We report an extremely rare case of pulmonary lipiodol embolism with acute respiratory distress syndrome (ARDS) after transcatheter arterial chemoembolization (TACE) for hepatocellular carcinoma (HCC). A 77-year-old man who was diagnosed with a huge HCC was admitted for TACE. Immediately after the procedure, this patient experienced severe dyspnea. We suspected that his symptoms were associated with a pulmonary lipiodol embolism after TACE, and we began intensive treatment. However, his condition did not improve, and he died on the following day. A subsequent autopsy revealed that the cause of death was ARDS due to pulmonary lipiodol embolism. No cases have been previously reported for which an autopsy was performed to explain the most probable mechanism of pulmonary lipiodol embolism; thus, ours is the first report for such a rare case.

Key words: Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Pulmonary lipiodol embolism; Acute respiratory distress syndrome; Autopsy

Core tip: Transcatheter arterial chemoembolization (TACE) has become the first treatment choice for patients with non-surgical hepatocellular carcinoma (HCC). Common complications associated with TACE have been reported, which include acute hepatic failure, liver abscess, intrahepatic biloma, hepatic infarction, hepatic artery occlusion, gallbladder infarction, acute renal failure, and/or gastrointestinal mucosal ulceration. However, fatal complications are rare. Although a few cases with pulmonary lipiodol embolism were previously reported, to our knowledge there have been no pathological autopsy reports. Here we present a pathological autopsy report for a patient with a huge HCC who died due to pulmonary lipiodol embolism after TACE.

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INTRODUCTION

Transcatheter arterial chemoembolization (TACE) has become the first treatment choice for patients with non-surgical hepatocellular carcinoma (HCC). Common complications associated with TACE have been reported, which include acute hepatic failure, liver abscess, intrahepatic biloma, hepatic infarction, hepatic artery occlusion, gallbladder infarction, acute renal failure, and/or gastrointestinal mucosal ulceration. However, fatal complications are rare.

Although a few cases with pulmonary lipiodol embolism were previously reported, to our knowledge there have been no pathological autopsy reports. Here we present a pathological autopsy report for a patient with a huge HCC who died due to pulmonary lipiodol embolism after TACE.

CASE REPORT

A 77-year-old man with a huge HCC was admitted to our hospital in April 2013 for the purpose of undergoing TACE. Since May 2011, he had been treated with chemotherapy for advanced lung cancer, although this was not sufficiently effective. Thus, after his admission, the treatment plan for his lung cancer was palliative therapy. Although contrast enhanced computed tomography (CECT) had shown a liver mass in November 2011, we thought this to be a metastatic tumor from his lung cancer.

One month previously, hepatic CECT with arterial phase and portal phase revealed a huge HCC (16 cm × 16 cm) in the right liver lobe (Figure 1). His hepatic function was classified as "A" according to the Child-Pugh classification. On physical examination, a non-tender smooth-surfaced mass was palpated at the right upper abdomen. Laboratory results on admission showed that hemoglobin (9.0 g/dL) and albumin (2.9 g/dL) levels were low. His coagulation time was normal, although liver function studies were slightly abnormal. Serological studies for hepatitis viral markers were negative (Hepatitis B surface antigen and Hepatitis C virus antibody). Alpha-fetoprotein and protein-induced vitamin K absence Ⅱ were 1717 ng/mL and 337000 mAU/mL, respectively. Because of his clinical signs and advanced lung cancer, a surgical resection could not be performed. Thus, TACE was offered to this patient.

Angiography demonstrated that the huge HCC was supplied from the right hepatic artery (RHA) and right inferior phrenic artery (RIP), concomitant with arteriovenous shunting (Figure 2). TACE was performed through RHA and RIP using an emulsion of miriplatin (70 mg) and lipiodol (40 mL). Gelfoam fine particles were injected into the feeding artery. During this procedure, the patient had no complaints, although his peripheral oxygen saturation decreased to 90%. However, after this operation, the patient immediately experienced dyspnea and he required a non-rebreathing mask. The clinical picture suggested acute respiratory distress syndrome (ARDS).

A chest CT scan revealed diffuse increased attenuation and interstitial thickening. There was also an accumulation of multiple iodized oil-like high-density materials, particularly in the right lung lobe (Figure 3). This patient underwent bilevel positive airway pressure, and methylprednisolone (1000 mg/d) was administered. Despite vigorous resuscitation and immediate artificial ventilation, this patient's condition did not improve, and he died the following day. After his death, written consent was obtained from his relatives, and we performed an autopsy to investigate the underlying pathological condition.

Histopathological micrographs revealed pathological changes in the lung. Hematoxylin and eosin (H and E) staining showed alveolar hemorrhagic edema with fat droplet deposition and fibrin thrombi. Fat staining showed multiple fatty droplets in the lung. Fat specific staining with Sudan Ⅲ indicated fat droplets in the pulmonary arteriolar lumen (Figure 4). His huge liver tumor was a moderately differentiated hepatocellular carcinoma with hemorrhage and necrosis, although the background of the liver was normal.

DISCUSSION

TACE is associated with several severe complications. ARDS associated with a pulmonary lipiodol embolism that develops after TACE is one of the most severe of these complications. The incidence of symptomatic pulmonary lipiodol embolism after TACE is in the range of 0.05%-1.8%[1,2], and ARDS that develops due to pulmonary lipiodol embolism is extremely rare. The respiratory symptoms that have been reported were non-specific including dyspnea, cough, tachypnea, and hemoptysis. A previous report showed that the onset of respiratory distress symptoms occurred within 2-5 d[1]. For our case, respiratory symptoms were apparent within two hours after TACE.

The mechanisms underlying symptomatic pulmonary injury associated with TACE are not well understood. The most likely mechanism is chemical injury caused by free fatty acid components. This develops because a high concentration of unbound free fatty acids released from the breakdown of oil microemboli may lead to pulmonary capillary leakage[1]. Most hypotheses for the pathogenesis of lung injury due to fat embolisms are related to the toxicity of free fatty acids. Silvestri *et al*[3] and Clouse *et al*[4] reported on inflammatory reactions to iodized

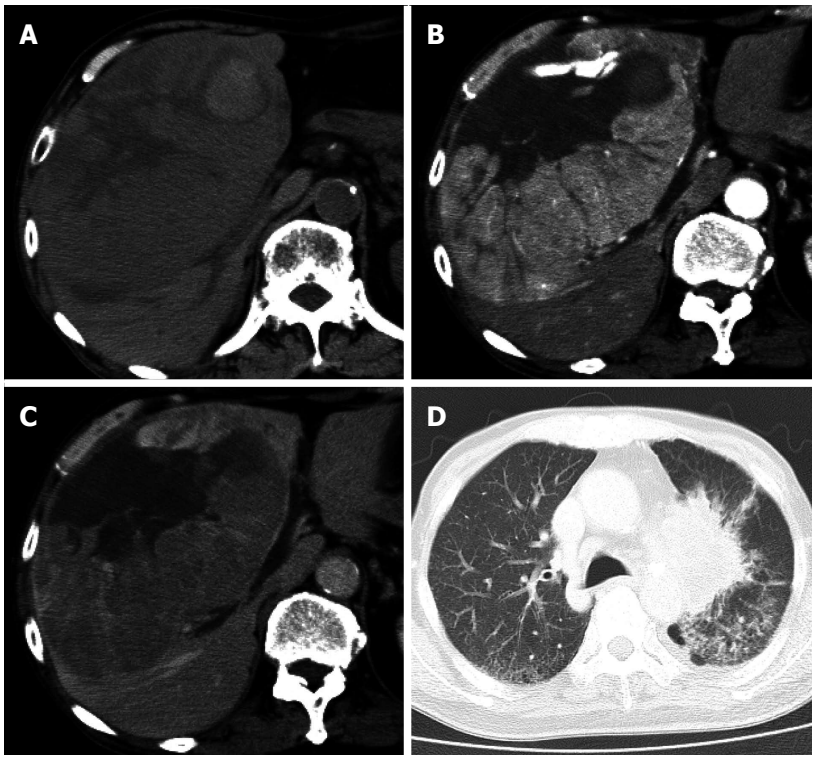

Figure 1 Contrast enhanced computed tomography image of the abdomen shows a huge tumor that occupies the right hepatic lobe, with enhancement in a viable lesion. A: Non-contrast phase; B: Hepatic arterial phase; C: Portal vein phase; D: Computed tomography image of the chest shows a primary lung cancer in the hilar side of the left lung.

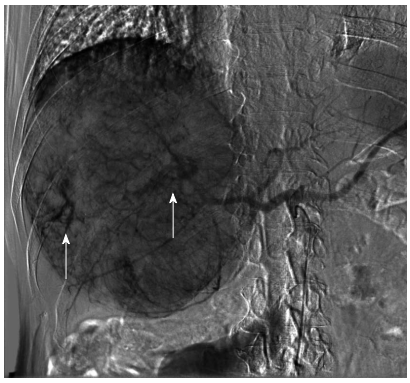

Figure 2 Hepatic arteriography shows arteriovenous shunting from hepatic arteries to hepatic veins (arrows).

oil. Kao *et al*[5,6] reported on patients who suffered from traumatic injuries and developed fat embolism syndrome with fulminant ARDS within 2 h. They suggested that nitric oxide (NO), phospholipase A2, free radicals, and pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-1β, interleukin-10) played a role in the pathogenesis of fat embolism syndrome-induced ARDS. They also proposed that alveolar macrophages were probably the major source of inducible nitric oxide synthase for producing NO in the lung. For our case, histopathological micrographs of the lung showed that alveolar

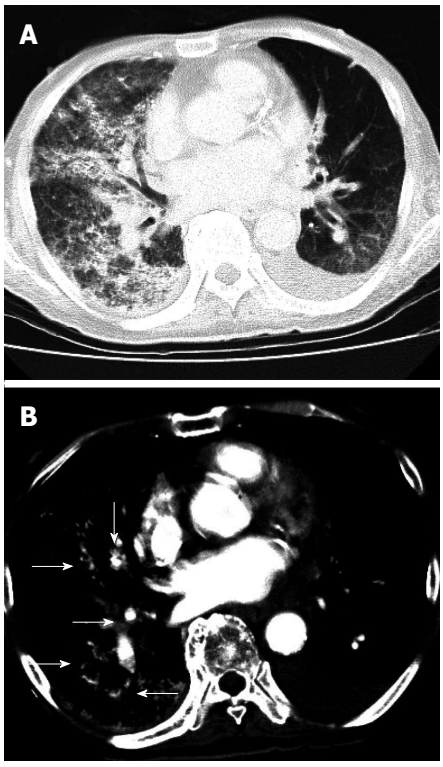

Figure 3 Computed tomography image. A: The chest 2 h after transarterial chemoembolization demonstrates diffuse increased attenuation and interstitial thickening; B: There is also an accumulation of lipiodol (arrows), particularly in the right lung lobe.

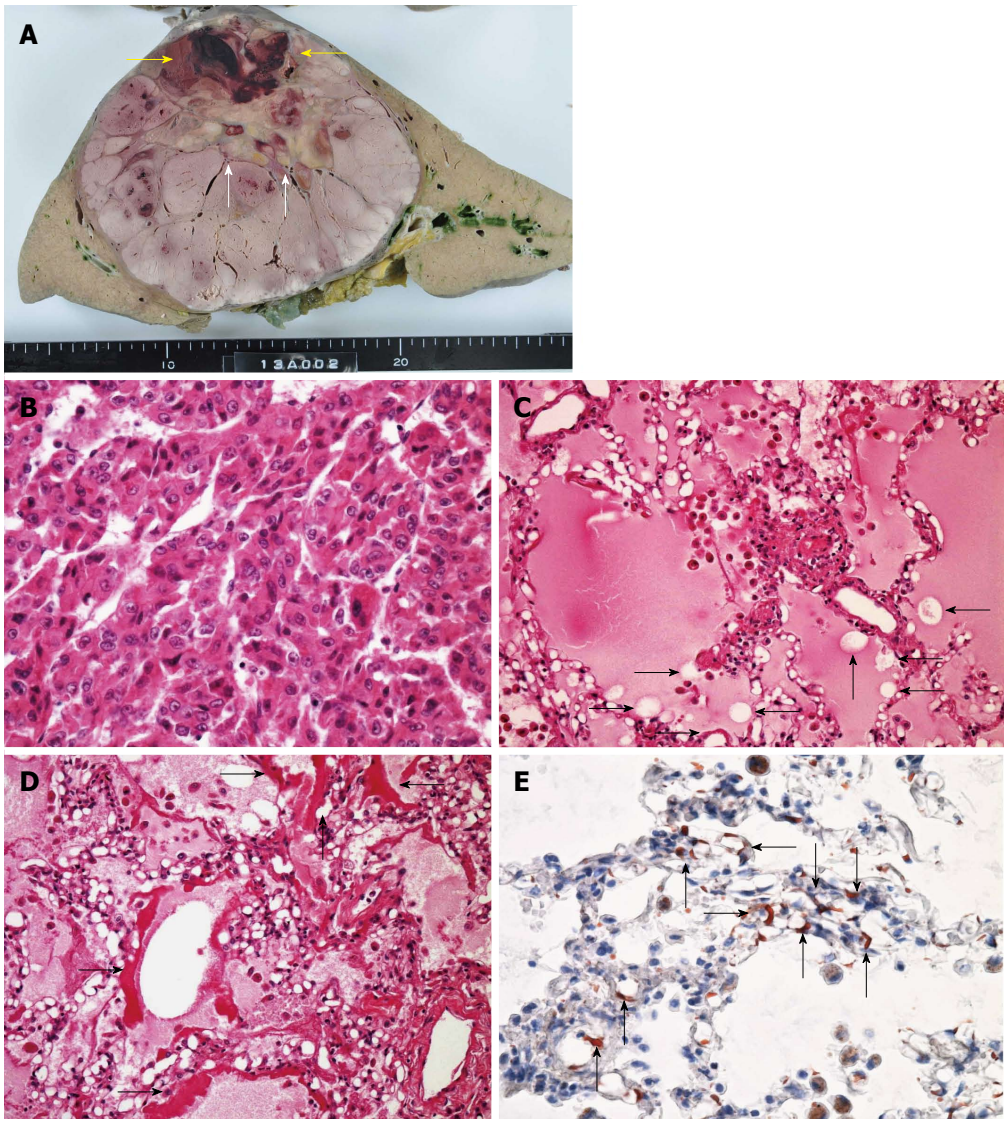

Figure 4 Histopathological findings. A: Macrograph of the liver shows a huge 16 cm × 16 cm tumor with necrotic changes (white arrows) and hemorrhage (yellow arrows); B: Micrograph of the liver tumor [hematoxylin and eosin (H and E) staining] shows moderately differentiated hepatocellular carcinoma with thin and thick mixed trabeculae. The background of the liver was normal (not shown); C: Micrograph of the lung (H and E staining) reveals alveolar edema and hemorrhage with fat droplet depositions (arrows); D: Fibrin thrombi (arrows); E: Fat specific staining with Sudan III indicates the presence of fat droplets in the pulmonary arteriolar lumen (arrows).

capillaries were focally distended by iodized oil. We speculate that the etiology of lung injury after TACE is similar to the pathological conditions associated with fat embolism syndrome.

The risk factors for pulmonary lipiodol embolism after TACE include the amount of lipiodol that is injected, arteriovenous shunting in a tumor, and communication between the inferior phrenic artery and the pulmonary artery[7-9]. Chung *et al*[1] proposed that the amount of injected lipiodol was the most important among these risk factors. They recommended that the amount of lipiodol used should be less than 20 mL (0.25 mL/kg). They also demonstrated that using more than 0.3 mL/kg of lipiodol was associated with the development of pulmonary lipiodol embolism in 43% of their patients. For our case, the amount of lipiodol injected was 40 mL, which was greater than the amount recommended by Chung *et al*[1].

Another risk factor for pulmonary lipiodol embolism is arteriovenous shunting in a tumor. It is considered that the communication between a tumor feeding artery and the hepatic vein leads to pulmonary lipiodol embolism after TACE. In addition, the communication between the inferior phrenic artery and the pulmonary artery is similar. Vascular abnormalities can be found in patients with advanced liver disease or a huge HCC[10]. Ho *et al*[11] demonstrated that lung shunting was influenced by the type, size, and vascularity of HCC. With HCC, mean lung shunting increases with increasing tumor size, up to 15 cm, although mean lung shunting remains nearly unchanged up to a tumor size of > 20 cm. Mean lung shunting also increases with increased vascularity grades.

To reduce the effect of arteriovenous shunting,

Lee *et al*[12] reported that temporary balloon occlusion of the hepatic vein with arteriovenous shunting prevented pulmonary complications during TACE. For our case, we speculate that lipiodol had passed through the hepatic arteriovenous shunt, and subsequently entered the systemic circulation. Thus, our patient suffered from a pulmonary embolism. Therefore, we should pay more attention to those patients who receive injections with large amounts of lipiodol, particularly if they have intrahepatic arteriovenous shunting, large tumors, and abundant tumor vascularity.

Unfortunately, there is no definitive, effective therapy for pulmonary lipiodol embolism. Yamaura *et al*[7] used heparin, nitroglycerine, furosemide, high-dose methylprednisolone, and mechanical ventilation with positive end-expiratory pressure and pressure support. Shiah *et al*[13] used oxygenation and high-dose methylprednisolone. Although these treatments may facilitate the recovery from a fulminant pulmonary lipiodol embolism with ARDS, they have not been shown to reduce the morbidity or mortality associated with this condition.

In conclusion, pulmonary lipiodol embolism after TACE is rare, although it can be a fatal complication. To prevent this complication, it is important to consider the therapeutic strategy with regard to the following points: limiting the amount of lipiodol injected and evaluating for the presence of arteriovenous shunting.

COMMENTS

Case characteristics

A 77-year-old man who was diagnosed with a huge hepatocellular carcinoma (HCC).

Clinical diagnosis

Hepatocellular carcinoma.

Differential diagnosis

Metastatic liver cancer.

Laboratory diagnosis

Alpha-fetoprotein and protein-induced vitamin K absence Ⅱ were 1717 ng/mL and 337000 mAU/mL, respectively.

Imaging diagnosis

Hepatic contrast-enhanced computed tomography (CT) with arterial phase and portal phase revealed a huge HCC (16 cm × 16 cm) in the right liver lobe. After the transcatheter arterial chemoembolization, chest CT scan revealed diffuse increased attenuation and interstitial thickening.

Pathological diagnosis

Hematoxylin and eosin staining showed alveolar hemorrhagic edema with fat droplet deposition and fibrin thrombi. Fat staining showed multiple fatty droplets in the lung. Fat specific staining with Sudan Ⅲ indicated fat droplets in the pulmonary arteriolar lumen.

Treatment

This patient underwent bilevel positive airway pressure, and methylprednisolone (1000 mg/d) was administered.

Experiences and lessons

To prevent pulmonary lipiodol embolism after transcatheter arterial chemoembolization, it is important to consider the therapeutic strategy.

Peer review

This paper reports on a rare case of pulmonary lipiodol embolism with acute respiratory distress syndrome after transcatheter arterial chemoembolization for HCC. It is a rare case and autopsy was performed to explain the probable mechanism of pulmonary lipiodol embolism.

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CASE REPORT

Coincidence between malignant perivascular epithelioid cell tumor arising in the gastric serosa and lung adenocarcinoma

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Abstract

A 4-mo history of both epigastralgia and back pain was presented in a 39-year-old male. Computed tomography showed right lung nodule and abdominal mass attached to the gastric wall, measuring approximately 30 mm and 70 mm in diameter. Since biopsy samples from the lung and abdomen revealed poorly differentiated adenocarcinoma and malignant tumor, clinicians first interpreted the abdominal mass as metastatic carcinoma, and a right lower lobectomy with following resection of the mass was performed. Gross examination of both lesions displayed gray-whitish to yellow-whitish cut surfaces with hemorrhagic and necrotic foci, and the mass attached to the serosa of the lesser curvature on the gastric body. On microscopic examination, the lung tumor was composed of a proliferation of highly atypical epithelial cells having abundant eosinophilic cytoplasm, predominantly arranged in an acinar or solid growth pattern with vessel permeation, while the abdominal tumor consisted of sheets or nests with markedly atypical epithelioid cells having pleomorphic nuclei and abundant eosinophilic to clear cytoplasm focally in a radial perivascular or infiltrative growth pattern. Immunohistochemically, the latter cells were positive for HMB45 or α-smooth muscle actin, but the former ones not. Therefore, we finally made a diagnosis of malignant perivascular epithelioid cell tumor (PEComa) arising in the gastric serosa, combined with primary lung adenocarcinoma. Furthermore, small papillary car-

cinoma of the thyroid gland was identified. The current case describes the coincidence of malignant PEComa with other carcinomas, posing a challenge in distinction from metastatic tumor disease.

Key words: Perivascular epithelioid cell tumor; Malignant; Gastric serosa; Lung adenocarcinoma; Metastatic carcinoma

Core tip: We reported the first single-case of malignant perivascular epithelioid cell tumor (PEComa) arising in the gastric serosa, combined with primary lung adenocarcinoma of poorly differentiated type. It is likely that the present malignant PEComa might pose a challenge in distinction from metastatic lung carcinoma on the examination of the small inadequate biopsy specimen. Pathologists should be aware that its characteristic features could lead to a misdiagnosis especially in this case. Furthermore, we suggest that a large panel of antibodies including various melanocytic, muscle or epithelial markers in immunohistochemistry should be useful and critical aids for reaching the correct diagnosis of malignant PEComa.

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INTRODUCTION

Perivascular epithelioid cell (PEC) was first introduced by Pea *et al*[1] and Bonetti *et al*[2] in the early 1990s, in order to present the concept of a family of tumor, *i.e.*, perivascular epithelioid cell tumor (PEComa), characterized by a proliferation of peculiar muscle cells having a specific expression of melanoma-associated antigens, such as HMB45[1,2]. In 1996, Zamboni *et al*[3] subsequently described the term PEComa to introduce this rare family of mesenchymal tumors containing characteristic epithelioid cells with a close association with blood vessels. PEComa family tumors include angiomyolipoma of the kidney and liver, pulmonary lymphangioleiomyomatosis, clear cell "sugar" tumor (CCST) of the lung, extrapulmonary CCST, clear cell myo melanocytic tumor of the falciform ligament/ligamentum teres, and abdominopelvic sarcoma of PECs[1-4]. In fact, the World Health Organization have already accepted the designation of PEComa as a distinct mesenchymal neoplasm predominantly composed of histopathologically unique PECs since 2002[5]. PEComas have been reported in various organs, such as the uterus and adnexa, pancreas, small and large intestine, mesentery, breast, skull base, soft tissue and so on[3-15]. Until now, the case number reported as PEComas of the digestive tract in the English literatures is small, less than 50, within our thorough investigation, as previously described in stomach, jejunum, ileum, cecum, descending colon, and rectum[5,9-11,16,17]. The most common site of involvement with gastrointestinal PEComas is the colon, followed by the small intestine, as more recently reported[17]. Although PEComas show a wide spectrum of biological behavior, classified into "benign", of uncertain "malignant potential", and "malignant" categories[4,5], the histopathological criteria for the diagnosis of malignant PEComa have not been clearly established to date, due to its rarity in part. Indeed, there have been 6 histopathological features suggestive of high risk factors of malignancy: (1) tumor size > 5 cm or 8 cm; (2) infiltrative growth pattern; (3) high nuclear grade and hypercellularity; (4) a high rate of mitosis, more than 1 per 50 high-power fields; (5) coagulative necrosis; and (6) vascular invasion[4,5,11,12], even though "true" malignant PEComas are extremely rare and its histogenesis and cytogenesis remain to be elucidated. Large PEComas (> 5 cm) without any above features have uncertain malignant potential, whereas any PEComas with the 2 or more high-risk features might be considered as malignant[4,5,11,12]. In contrast, "benign" PEComas lacking all these features only rarely metastasize[5]. Nevertheless, those above criteria have not yet been validated in larger series. However, it would be critical to establish an accurate initial diagnosis, including "benign", "of uncertain malignant potential", or "malignant" PEComas, even by small biopsy specimens.

We report an extremely rare case of malignant PEComa arising in the gastric serosa combined with primary lung adenocarcinoma of poorly differentiated type and thyroid papillary carcinoma, likely confused with metastatic carcinoma in the gastric wall, based on an inadequate volume of biopsy sample.

CASE REPORT

The patient was a 39-year-old middle-aged Japanese male. The surgical tumor specimens after fixation in 10% neutral buffered formalin were embedded in paraffin for histological or immunohistochemical examinations. All immunohistochemical stainings were carried out using Dako Envision kit (Dako, Glostrup, Denmark) according to the manufacturer's instructions, and using commercially available pre-diluted monoclonal or polyclonal antibodies against the following antigens: cytokeratins (Cam5.2; Becton Dickinson Immunocytometry Systems, San Jose, CA, diluted 1:1, and AE1/AE3; Dako, diluted 1:5000), epithelial membrane antigen (EMA; Dako, diluted

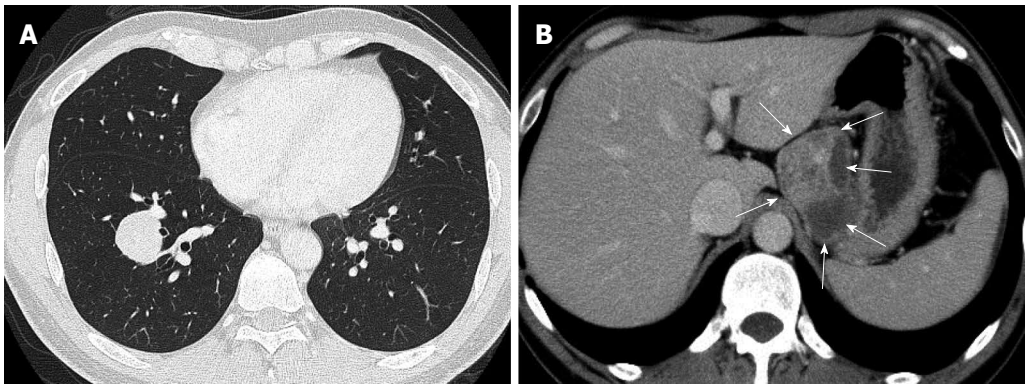

Figure 1 Findings of chest and abdominal computed tomography scan at surgery. A: A chest computed tomography (CT) scan showed a relatively well-demarcated nodule, measuring approximately 30 mm × 30 mm, in the right lower lobe, S9; B: An abdominal CT scan showed a relatively well-defined huge mass with heterogeneously enhancement (arrows), measuring approximately 70 mm × 60 mm, attached to the gastric wall and separated from the left kidney and adrenal gland.

1:100), thyroid transcription factor 1 (TTF-1; Dako, diluted 1:100), Napsin A (Nichirei Bioscience, Tokyo, Japan, diluted 1:1), CD10 (NOVOCASTRA laboratories Ltd., Newcastle, United Kingdom, diluted 1:20), CD34 (Immuno Tech. Co., Ltd., Osaka, Japan, diluted 1:150), CD45 (Dako, diluted 1:400), CD56 (NICHIREI, Tokyo, Japan, diluted 1:1), CD68 (KP-1; Dako, diluted 1:100), CD117 (c-Kit; IBL, Gunma, Japan, diluted 1:15), synaptophysin (Dako, diluted 1:20), chromogranin A (Dako, diluted 1:200), S-100 protein (Dako, diluted 1:900), HMB45 (Enzo Life Sciences Ltd., New York, diluted 1:100), Melan A (NOVOCASTRA, 1:50), microphthalmia transcription factor (MiTF; NOVOCASTRA, diluted 1:10), TFE3 (Santa Cruz Biotechnology, Santa Cruz, CA, United States, 1:600), α-smooth muscle actin (α-SMA; Dako, diluted 1:150), pan-muscle actin (HHF-35; Enzo, New York, United States, diluted 1:20), desmin (Dako, diluted 1:300), h-caldesmon (Dako, diluted 1:50), and Ki-67 (MIB-1; Dako, diluted 1:50). However, no chromosome studies have been performed.

Clinical summary

The patient was admitted to hospital due to a 4-mo history of both epigastralgia and back pain. The patient had neither signs of tuberous sclerosis complex nor any family history of it. He was a non-smoker. There was no history of malignancy, immunosuppressive disorders, use of immunosuppressive medications, or unusual infections.

Laboratory data, including blood cell count, chemistry and tumor markers, were almost within normal limits, except for slightly high levels of carcinoembryonic antigen (3.7 ng/mL). A chest computed tomography (CT) scan revealed a relatively well-demarcated nodule, measuring approximately 30 mm × 30 mm, in the right lower lobe, S9 (Figure 1A). Moreover, an abdominal CT scan showed a relatively well-defined huge mass with heterogeneously enhancement, measuring approximately 70 mm × 60 mm, attached to the gastric wall and separated from the left kidney and adrenal gland (Figure 1B). Besides, a view of neck ultrasound revealed a well-demarcated nodule, measuring approximately 9 mm, in the right lobe of the thyroid gland. CT scans of the head, chest and abdomen disclosed no definite evidence of neoplastic foci or other metastases in the lymph nodes or other organs, including the bilateral kidney or adrenal gland. The patient had neither recurrence nor metastases of malignant PEComa, lung carcinoma, and thyroid carcinoma, respectively, and was alive and well at 6 mo after the operation.

Pathological findings

The first bronchial brushing and washing cytology specimens were predominantly consisted of clusters of cohesive and three-dimensional tumor cells having large hyperchromatic nuclei and prominent nucleoli with necrotic backgrounds. Based on that, we first interpreted it as poorly differentiated adenocarcinoma, confirmed by following transbronchial lung biopsy from the pulmonary nodule. On the other hand, the percutaneous biopsy specimen from the abdominal mass showed extensively necrotic and hemorrhagic tissue, admixed with quite tiny fragments of tumor lesion, composed of a solid proliferation of highly atypical cells having hyperchromatic pleomorphic nuclei and abundant eosinophilic to clear cytoplasm (data not shown). Clinicians first interpreted the gastric serosal mass as metastatic carcinoma of the lung carcinoma, but we pathologists tentatively made a diagnosis of malignant tumor. Therefore, the surgeons performed an ordinary right lower lobectomy with following laparoscopic combined resection of the gastric serosal mass and one part of the gastric wall. Finally, the fine needle aspiration cytology from the thyroid small (less than 1 cm) tumor revealed papillary carcinoma, however, careful follow-up but not thyroidectomy was done.

On gross examination, the cut surface of the lung tumor showed a solid firm and lobulated mass, measuring 32 mm × 30 mm × 29 mm, which

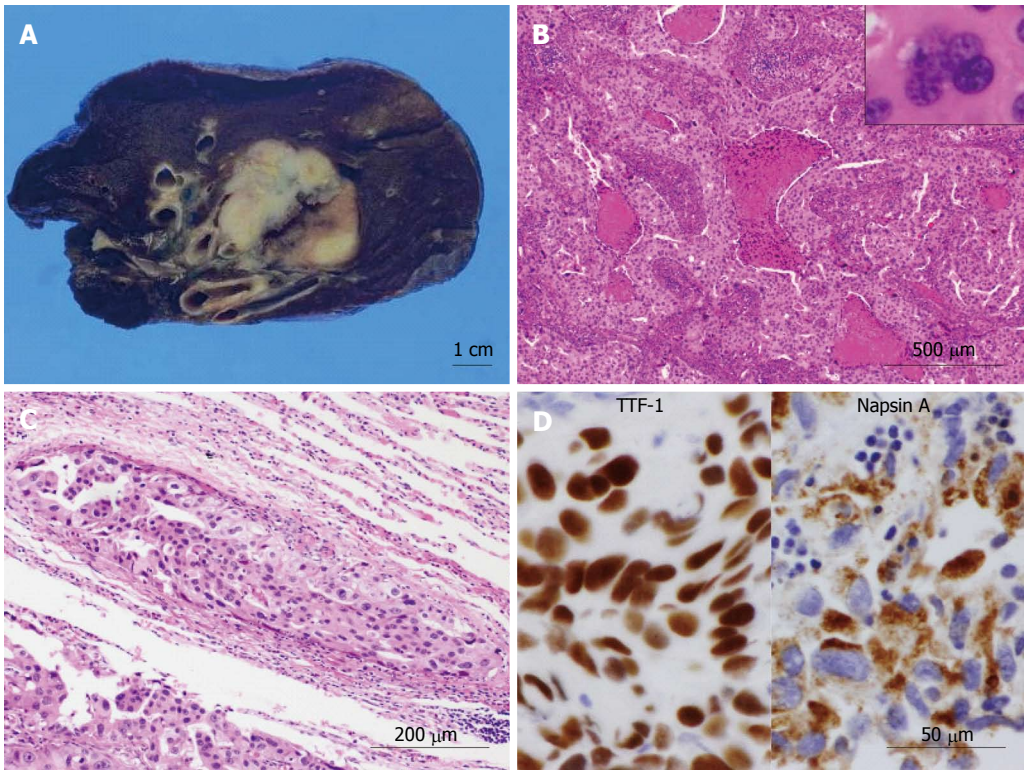

Figure 2 Gross, histological and immunohistochemical findings of poorly differentiated adenocarcinoma of the lung. A: On gross examination, the cut surface showed a solid firm and lobulated mass, measuring 32 mm × 30 mm × 29 mm, which looked from gray-whitish to yellow-whitish in color, accompanied with focal necrosis and hemorrhage. The background had no remarkable change. Bar = 1 cm; B: Low to medium power view exhibited a proliferation of medium-sized to large atypical epithelial cells having hyperchromatic pleomorphic nuclei and abundant eosinophilic cytoplasm, predominantly arranged in an acinar or solid fashion with frequent necrotic foci (HE stains). Multi-nucleated giant tumor cells were readily encountered (inset). Bar = 500 μm; C: The tumor nests peripherally involved the vascular vessel (HE stains). Bar = 200 μm; D: In immunohistochemistry, these adenocarcinoma cells were specifically positive for thyroid transcription factor 1 (TTF-1) (left) and Napsin A (right), in nuclear and intracytoplasmic pattern, respectively. Bar = 50 μm.

looked from gray-whitish to yellow-whitish in color, accompanied with focal necrosis and hemorrhage (Figure 2A). The background of the lung had no remarkable change, *e.g.*, not emphysematous (Figure 2A). Microscopic findings revealed a proliferation of medium-sized to large atypical epithelial cells having hyperchromatic pleomorphic nuclei and abundant eosinophilic cytoplasm, predominantly arranged in an acinar or solid fashion with frequent necrotic foci (Figure 3B), involving the adjacent bronchial wall with vessel permeation (Figure 2C). On high-power view, mitotic counts were high (more than 10 per 50 high-power fields) and multi-nucleated giant tumor cells were readily encountered (Figure 2B). Immunohistochemically, these adenocarcinoma cells were positive for TTF-1 and Napsin A (Figure 2D). Based on all these features, we indicated that these carcinoma cells were characteristic of glandular differentiation, and finally made a diagnosis of moderately to poorly differentiated adenocarcinoma of the lung, further classified as invasive adenocarcinoma, acinar predominant, based on the histological classification system from the International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society/European Respiratory Society International Multidisciplinary Classification of Lung Adenocarcinoma[18]. Final pathological stage was determined as $pT_{2a}N_0M_0$, stage IB, according to the IASLC classification[19].

Next, gross examination of the surgical specimen from the abdominal mass showed that the huge tumor, measuring 73 mm × 65 mm × 61 mm, had gray-whitish to yellow-whitish cut surfaces with hemorrhagic and yellowish necrotic foci, attached to the serosa of the lesser curvature on the gastric body (Figure 3A) and separated from the left kidney and adrenal gland. Microscopically, the abdominal tumor consisted of sheets or nests of markedly atypical epithelioid cells having hyperchromatic pleomorphic nuclei and abundant eosinophilic to clear cytoplasm, admixed with a large number of multi-nucleated giant cells, supported by delicate fibrovascular septa (Figure 3B). Spindle cell-predominant components were very few. These tumor nests predominantly showed an alveolar or trabecular growth pattern with coagulative necrotic foci, occasionally and characteristically displaying a radial perivascular fashion (Figure 3C). On high-power view, the large tumor cells sometimes showed atypical mitosis with relatively high mitotic rates (more than 2 per 10 high-power fields) (Figure 3C). On the other hand, vascular permeation of the infiltrative tumor nests

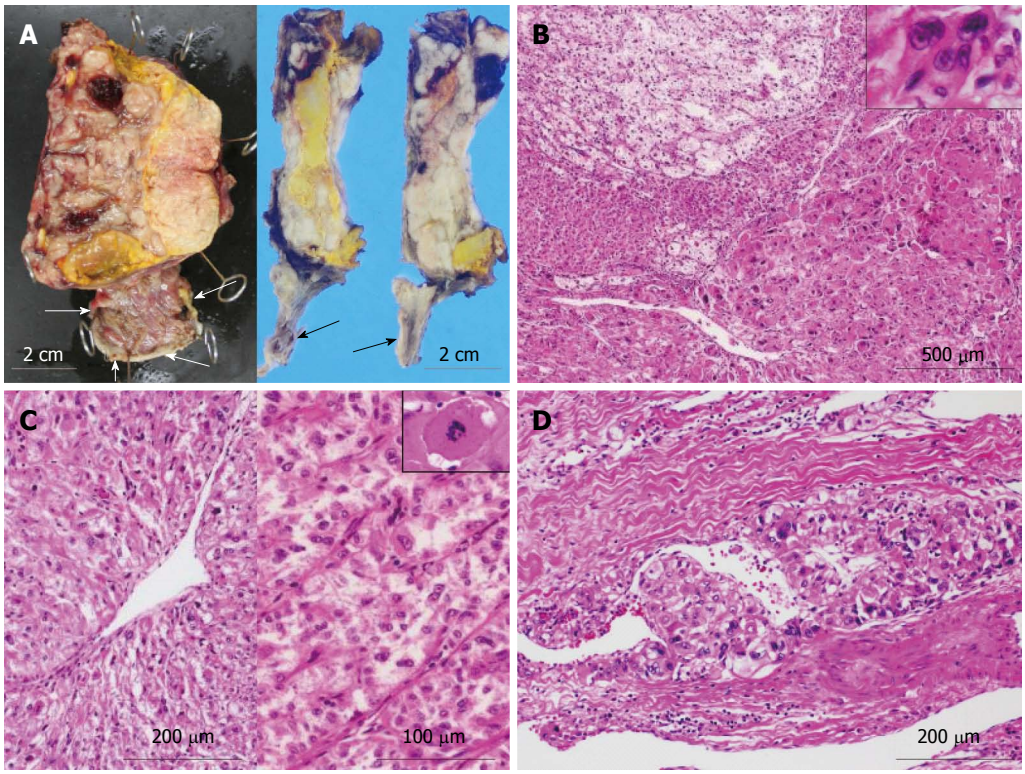

Figure 3 Gross and microscopic examination of the resected specimen of malignant perivascular epithelioid cell tumor arising in the stomach. A: Gross examination at surgery (left) and after fixation (right) showed that the huge tumor, measuring 73 mm × 65 mm × 61 mm, had gray-whitish to yellow-whitish cut surfaces with some hemorrhagic and yellowish necrotic foci, attached to the serosa of the lesser curvature on the gastric body (arrows) and separated from the left kidney and adrenal gland. Bars = 2 cm; B: Microscopically (low to medium power view), the abdominal tumor predominantly consisted of sheets or nests of markedly atypical epithelioid cells having hyperchromatic pleomorphic nuclei and abundant eosinophilic to clear cytoplasm, admixed with a large number of multi-nucleated giant cells (inset), supported by delicate fibrovascular septa (HE stains). Bar = 500 μm; C: On high-power view, these nests displayed an alveolar or trabecular growth pattern (right), characteristically displaying a radial perivascular fashion (left) (HE stains). The large tumor cells sometimes showed atypical mitosis with relatively high mitotic rates (more than 2 per 10 high-power fields) (inset). Bars = 200 μm or 100 μm; D: Moreover, vascular permeation of the infiltrative tumor nests was partly noted in the peripheral areas (HE stains). Bar = 200 μm.

was partly noted in the peripheral areas (Figure 3D). In immunohistochemistry, these epithelioid cells were specifically positive for melanocytic markers, such as HMB45 (Figure 4A), Melan A (Figure 4B), and MiTF (Figure 4C), and muscle markers, such as α-SMA (Figure 4D), desmin (Figure 4D), HHF-35, and h-caldesmon, and focally positive for CD10, whereas negative for TTF-1, Napsin A, epithelial markers, including EMA, Cam 5.2, and AE1/AE3, neuroendocrine markers, such as CD56, chromogranin A, and synaptophysin, S-100 protein, TFE3, CD34, c-Kit, CD45, and CD68. Moreover, relatively higher MIB-1 labeling index, 3% to 5%, was found within the gastric serosal tumor cells. Based on all the clinicopathological features, we made a final diagnosis of malignant PEComa arising in the gastric serosa. All immunohistochemical profiles of malignant PEComa arising in the gastric serosa and primary lung adenocarcinoma are summarized in Table 1.

DISCUSSION

The most important clinical differential diagnosis in the present malignant PEComa case is with metastatic lung adenocarcinoma of poorly differentiated type. Immunohistochemical analyses can resolve the distinction from metastatic carcinoma very easily, since the PEComa cells were specifically positive for melanoma-associated antigens, representing as HMB45, and muscle markers, such as α-SMA and desmin, whereas completely negative for lung adenocarcinoma markers, TTF-1 and Napsin A, but in striking contrast, the lung carcinoma cells were not. However, the adenocarcinoma cells in our case microscopically shares with malignant PEComa not only a solid sheet-like growth pattern but markedly cellular atypia displaying hyperchromatic pleomorphic nuclei, abundant eosinophilic cytoplasm, and occasionally multi-nucleated giant cells, admixed with a number of mitotic figures. Thus, we pathologists should be aware that its features possibly make us misinterpret as a metastatic focus only on small or inadequate biopsy specimens. On the other hand, among malignant tumors, histopathologically differential diagnoses include epithelioid extra-gastrointestinal stromal tumor (extra-GIST), malignant melanoma, epithelioid leiomyosarcoma or metastatic clear cell renal cell carcinoma (RCC)[4-17]. Although malignant PEComa

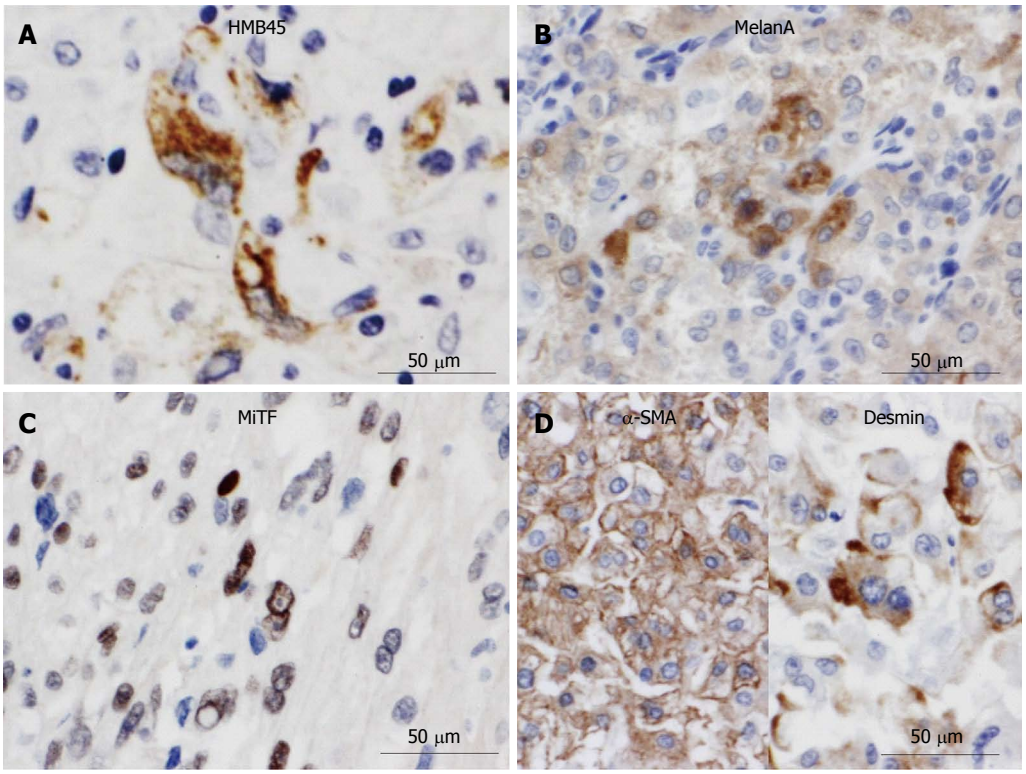

Figure 4 Immunohistochemical examination of malignant perivascular epithelioid cell tumor of the stomach. The highly atypical epithelioid cells were specifically positive for melanocytic markers, such as HMB45 (A), Melan A (B), and microphthalmia transcription factor (MiTF) (C), and muscle markers, such as α-smooth muscle actin (SMA) markers (D) or desmin (D). Bars = 50 μm.

and above each neoplasm share variable histological features, immunohistochemical profiles (Table 1) of malignant PEComa also can readily distinguish from epithelioid extra-GIST, epithelioid leiomyosarcoma, and metastatic RCC mainly by positive staining for melanocytic markers and negative staining for c-Kit, CD34, and epithelial markers including cytokeratin, and differentiate from melanoma chiefly by negative staining for S-100 protein and positive staining for muscle markers, respectively[1-17].

It is very likely that the present case is clinicopathologically remarkable for two reasons at least: first, to the best of our knowledge, this is the first single-case report of malignant PEComa arising in the gastric serosa, and the fourth occurrence of gastric PEComa[4,5,9-11,16,17]. Actually, to date, the number of "true" cases reported as PEComa of the digestive tract in the English literatures is not large, and the most recent reference of single-case paper (in fact, gastric "benign" PEComa) is from 2010[16]. According to those previous papers, the criteria of "benign", "of uncertain malignant potential" to "malignant" for PEComa have not been clearly established[4,5,11], and intriguingly, there has been no known normal counterpart of PEComa. Fu *et al*[12] have recently proposed that infiltrating appearance (*e.g.*, vascular invasion) and extensive coagulative necrosis should be much more pivotal factors to be used for the evaluation of "malignant" PEComa, corresponding to our case, rather than hypercellularity or numerous mitotic figures. By contrast, more recently, the relatively larger series of PEComa especially arising in the gastrointestinal tract have revealed that, similar to us, the presence of marked nuclear atypia, diffuse pleomorphism, and more than 2 mitoses per 10 high-power fields have a significantly close relationship with the development of metastatic disease, manifesting as malignant PEComa[17]. Malignant PEComas are still extremely rare, and thus, it is interesting and critical to study this topic with regard to histopathological criteria of "malignancy" after further collecting and investigating a substantial number of surgical cases of PEComas in the future.

Second, this middle-aged (*i.e.*, relatively young) male patient suffered from multifocal malignancy: (1) malignant PEComa arising in the gastric serosa; (2) primary lung adenocarcinoma of poorly differentiated type in the right lower lobe; and (3) thyroid papillary carcinoma of the right lobe. Within our thorough investigation, the present case is the first report of PEComa combined with multiple malignancy, as well. We might provide the possible evidence for the first time that one part of malignant PEComas have a predilection for multifocal growth fashion, including other malignant neoplasms, rather than metastasis, even though it is known that the most common metastatic sites of PEComas include the

Table 1 Immunohistochemical profile of highly atypical epithelioid cells in our case of malignant perivascular epithelioid cell tumor arising in the stomach

Antibodies	Malignant PEComa	Lung adenocarcinoma
EMA	-	+
AE1/AE3	-	+
Cam5.2	-	+
TTF-1	-	+
Napsin A	-	+
Synaptophysin	-	-
Chromogranin A	-	-
CD56	-	-
NSE	-	-
S-100 protein	-	-
CD34	-	-
c-Kit	-	-
TFE3	-	-
α-SMA	+	-
h-caldesmon	+	-
HHF-35	+	-
Desmin	+	-
MelanA	+	-
HMB-45	+	-
MiTF	+	-
CD10	+	-

MiTF: Microphthalmia transcription factor; α-SMA: α-smooth muscle actin; EMA: Epithelial membrane antigen; TTF-1: Thyroid transcription factor-1; NSE: Neuron-specific enolase; TFE3: Transcription factor E3; HMB-45: Human melanoma black; PEComa: Perivascular epithelioid cell tumor.

lungs, liver, lymph nodes, peritoneum, bone, brain, and adrenal gland[5,17]. According to the first series of gastrointestinal PEComas[17], of 13 patients who developed metastatic foci, 3 patients (23%) showed lung metastases. Nevertheless, future convincing data will be further required to determine whether our speculation is significant or not.

In summary, we reported an extremely rare case of malignant PEComa arising in the gastric serosa, combined with primary lung adenocarcinoma of poorly differentiated type and papillary carcinoma of the thyroid gland. It is likely that the current malignant PEComa might pose a challenge in distinction from metastatic lung carcinoma on the examination of the small biopsy specimen, since its section contained tiny foci of viable tumor epithelioid cells in the background of extensively necrosis and hemorrhage. All pathologists should be aware that its characteristic features could lead to a misdiagnosis especially in case of inadequate specimens. Furthermore, we suggest that a large panel of antibodies including various melanocytic, muscle or epithelial markers in immunohistochemistry should be useful and critical aids for reaching the correct diagnosis of malignant PEComa. PEComa arising in digestive tract may be more common than generally considered.

COMMENTS

Case characteristics

A 39-year-old male with a 4-mo history of both epigastralgia and back pain.

Clinical diagnosis

The patient had neither signs of tuberous sclerosis complex nor any family history of it. There was no history of malignancy, immunosuppressive disorders, use of immunosuppressive medications, or unusual infections.

Differential diagnosis

Metastatic carcinoma of the gastric serosa from primary lung cancer.

Laboratory diagnosis

Laboratory data, including blood cell count, chemistry and tumor markers, were almost within normal limits, except for slightly high levels of carcinoembryonic antigen (3.7 ng/mL).

Imaging diagnosis

A chest computed tomography (CT) scan revealed a relatively well-demarcated nodule, measuring approximately 30 mm × 30 mm, in the right lower lobe, S9. Moreover, an abdominal CT scan showed a relatively well-defined huge mass with heterogeneously enhancement, measuring approximately 70 mm × 60 mm, attached to the gastric wall and separated from the left kidney and adrenal gland.

Pathological diagnosis

The authors made a final diagnosis of malignant perivascular epithelioid cell tumor (PEComa) arising in the gastric serosa, combined with primary lung adenocarcinoma of poorly differentiated type.

Treatment

The surgeons performed an ordinary right lower lobectomy with following laparoscopic combined resection of the gastric serosal mass and one part of the gastric wall.

Related reports

Until now, the case number reported as PEComas of the digestive tract in the English literatures is small, less than 50, within our thorough investigation, as previously described in stomach, jejunum, ileum, cecum, descending colon, and rectum. The most common site of involvement with gastrointestinal PEComas is the colon, followed by the small intestine.

Term explanation

Perivascular epithelioid cell was first introduced by Pea *et al* in the early 1990s, in order to present the concept of a family of tumor, *i.e.*, PEComa, characterized by a proliferation of peculiar muscle cells having a specific expression of melanoma-associated antigens, such as HMB45.

Experiences and lessons

To the best of our knowledge, this is the first single-case report of malignant PEComa arising in the gastric serosa, and the fourth occurrence of gastric PEComa.

Peer review

This article reports an extremely rare case of malignant PEComa arising in the gastric serosa combined with primary lung adenocarcinoma of poorly differentiated type and thyroid papillary carcinoma, likely confused with metastatic carcinoma in the gastric wall.

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CASE REPORT

Pancreatic carcinosarcoma: First literature report on computed tomography imaging

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Abstract

Carcinosarcoma of the pancreas is an extremely rare tumor and has a dismal prognosis. To the best of our knowledge, the histopathological features of the lesion have been illustrated in the literature but to date no reported cases have been documented on imaging characteristics. We report a female case of pancreatic carcinosarcoma presenting as a mucinous cystadenoma on computed tomography (CT). We also summarize the CT characteristics according to the appended CT images in the reported cases. This is the first report of CT features of pancreatic carcinosarcoma in the English literature.

Key words: Computed tomography; Carcinosarcoma; Pancreas; Neoplasm

Core tip: Pancreatic carcinosarcoma is an extremely rare tumor with a poor prognosis. We report a case of pancreatic carcinosarcoma in a 74-year-old woman and describe its appearance on computed tomography (CT). Detailed analysis and conclusion of the CT characteristics were performed according to the appended CT images in reported cases. This is the first report of radiological findings of carcinosarcoma originating from the pancreas.

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INTRODUCTION

Pancreatic carcinosarcoma is an uncommon entity comprising a fairly small subset of all pancreatic neoplasms. It is histologically characterized by a mixture of carcinomatous and sarcomatous elements. The prognosis of pancreatic carcinosarcoma is very poor. From the data of reported cases, the majority of patients survived an average of only 6 mo after surgery. Because of the rarity and difficulty of diagnosis, radiological findings of the lesion have not been well illustrated in the literature. Here, we

report the clinical and contrast-enhanced computed tomography (CT) findings of carcinosarcoma originating from the pancreas in a 74-year-old woman.

CASE REPORT

A 74-year-old woman was referred to our hospital with acute calculous cholecystitis. Contrast material-enhanced dual-phase multidetector row CT of the abdomen was performed and revealed a 2.2 cm × 2.0 cm well-circumscribed cystic lesion that was located at the pancreatic tail and did not invade the adjacent viscera. After intravenous injection of contrast media, the lesion had a peripheral enhancing thick wall surrounding the non-enhancing low-attenuation area which is consistent with cystic fluid (Figure 1). Mural nodules and intratumoral septa were not seen on the CT imaging. There was no main pancreatic duct dilatation or any abnormalities in the rest of the pancreatic tissue. We diagnosed it as mucinous cystadenoma. The patient underwent a cholecystectomy and was then discharged.

Follow-up CT imaging was performed 13 mo later. The lesion in the pancreatic tail had grown up to 2.9 cm in diameter with a more thickened wall and a solid component appeared in the cystic lesion. The arterial phase revealed heterogeneous enhancement in the wall and solid part of the mass (Figure 2A). In the portal phase, the enhancement became more pronounced. The cystic part revealed non-enhancing low-attenuation (Figure 2B).

After nearly six months, the patient was referred to our hospital again for intermittent abdominal pain and distention lasting for more than 2 wk. She reported no association with food. The abdomen was soft and tender at physical examination. Laboratory analysis revealed elevated CA19-9 (148.40 U/mL), CA72-4 (19.19 U/mL), CA24-2 (51.7 U/mL) and CEA (10.05 ng/mL) levels. Liver function tests and complete blood counts were all within the normal range. CT showed that the original lesion in the pancreatic tail manifested as a heterogeneous complex mass that contained cystic and mixed solid areas and measured 4.0 cm in diameter. The solid components increased and septa in the lesion were aroused. The lesion showed progressive and heterogeneous enhancement (Figure 3). No evidence of metastasis was identified. The CT findings coupled with tumor markers raised the possibility of pancreatic mucinous cystadenocarcinoma. The patient underwent a distal pancreatectomy with splenectomy, followed by uneventful postoperative course.

On gross examination, the resected specimen consisted of a 9 cm × 6 cm × 3 cm segment of the pancreas with a mass in the tail and the spleen. Sectioning through the mass revealed a well-circumscribed tumor consisting of a 5 cm × 4 cm × 2 cm cystic lesion with a thick wall that measured 0.1-0.3 cm. The cystic lesion contained dark-red substances.

Histologically, the tumor of pancreas showed two components separated from each other. The first component was composed of columnar mucin-producing epithelial cells with marked cellular pleomorphism and prominent mitoses, consistent with carcinoma. The second component revealed a sarcomatous growth pattern composed predominantly of highly cellular areas with spindle cells (Figure 4A). Immunohistochemically, the carcinoma component was strongly reactive for antibodies to cytokeratin 7, cytokeratin 19 and cytokeratin AE1/AE3 (Figure 4B). The sarcomatous component was strongly reactive for vimentin (Figure 4C). According to the morphology and the immunohistochemical staining pattern, pancreatic carcinosarcoma was diagnosed.

DISCUSSION

The concept of carcinosarcoma is that a malignant neoplasm is composed of an intimate admixture of carcinomatous and sarcomatous elements, without areas of transition between both components, and with each of these elements showing distinct immunohistochemical or ultrastructural features pertaining to their different lines of differentiation. Cases fulfilling these criteria have been reported in many organs but rarely in the pancreas. The origin of mixed carcinosarcoma is unknown. Although controversy remains, several studies using diverse immunohistochemical and molecular analyses have suggested that pancreatic carcinosarcoma could be of monoclonal origin and that the sarcomatous component might have arisen from metaplastic transformation of the carcinomatous component[1,2].

The carcinomatous components are varied. Pancreatic ductal adenocarcinoma is the most commonly reported, followed by mucinous cystadenocarcinoma[3-6]. Okamura *et al*[7] reported intraductal papillary mucinous carcinoma (IPMC) in pancreatic carcinosarcoma for the first time, which is extremely rare. There are also different sarcomatous elements, including spindle cell sarcoma, leiomyosarcoma, malignant fibrous histiocytoma and osteosarcoma.

From the summary of the reported literature, we found that pancreatic carcinosarcoma is common in middle aged and elderly people and few patients were identified incidentally. For patients with symptoms, the most common were abdominal pain, anorexia, nausea and vomiting. When tumors are located in the pancreatic head, they cause early jaundice, commonly seen in other malignant neoplasms. Serum CA19-9 can be elevated in some patients[7-9].

Due to the rarity of this tumor, the prognosis has not been well defined. According to the reported

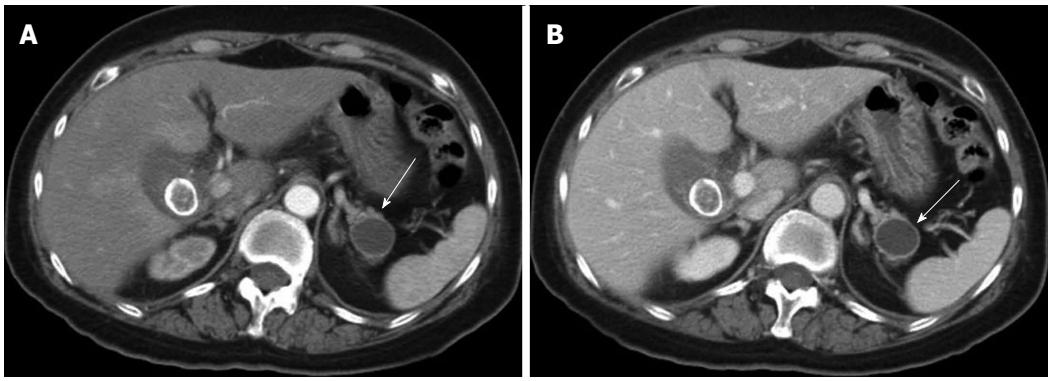

Figure 1 Computed tomography imaging of the abdomen the first time. The arterial phase (A) and portal venous phase (B) revealed a 2.2 cm × 2.0 cm well-circumscribed cystic lesion that was located at the pancreatic tail with a peripheral enhancing thick cystic wall that surrounded a non-enhancing low-attenuation area consistent with cystic fluid. We diagnosed it as mucinous cystadenoma.

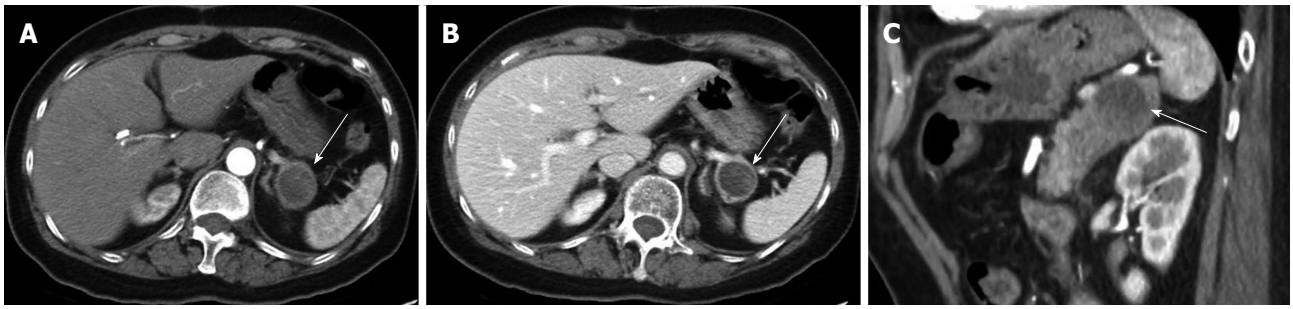

Figure 2 Follow-up computed tomography imaging 13 mo later. The arterial phase (A), portal venous phase (B) and oblique sagittal contrast-enhanced computed tomography image (C) showed the lesion grew up to 2.9 cm in diameter with heterogeneous enhancement in the more thickened wall and solid component.

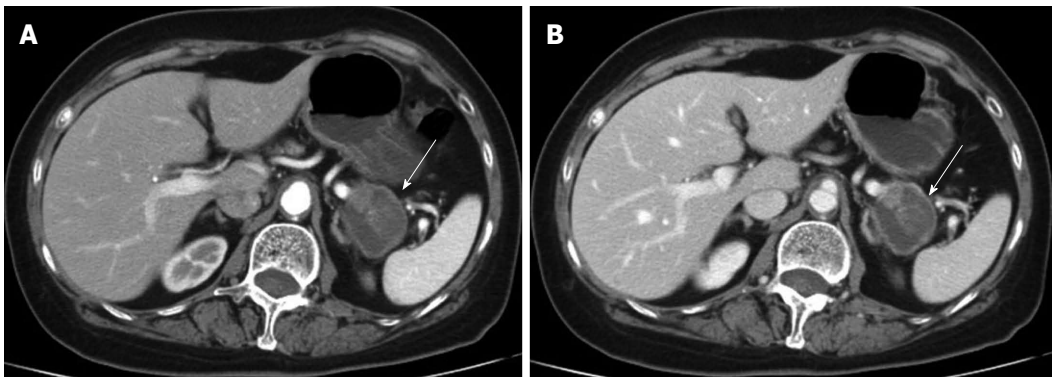

Figure 3 Follow-up computed tomography imaging after nearly six months. Computed tomography (A: Arterial phase; B: Portal venous phase) showed that the original lesion in the pancreatic tail manifested as a heterogeneous complex mass that contained cystic and mixed solid areas that measured 4.0 cm in diameter. The solid components increased. The lesion showed progressive and heterogeneous enhancement.

literature, the majority of patients survived an average of only 6 mo after surgery. Zhu *et al*[9] reported that a 53-year-old woman had remained free of recurrence for 20 mo, the longest recurrence-free survival time recorded for this tumor. Pancreatic carcinosarcoma can also disseminate and recur[10,11]. However, a strategy to improve the prognosis is still not available because of the limited experience of it.

To our best knowledge, the imaging features of pancreatic carcinosarcoma have not been reported. By summarizing the reported cases in the literature, combined with our case, we found that the preferential location for pancreatic carcinosarcoma is the pancreatic head and tail and the size is variable (ranging from 2.5 cm to 20 cm). It is worth noting that pancreatic carcinosarcoma can grow quickly, as observed in our case. During follow-up, the lesion in the pancreatic tail grew from 2.2 cm to 4.0 cm in diameter and a more solid component was also seen 18 mo later. On CT images, the lesion in the pancreatic head more frequently appears as a solid mass with cystic regions and necrosis, while the one in the pancreatic tail is characterized by a cystic neoplasm with mural nodules and a solid component,

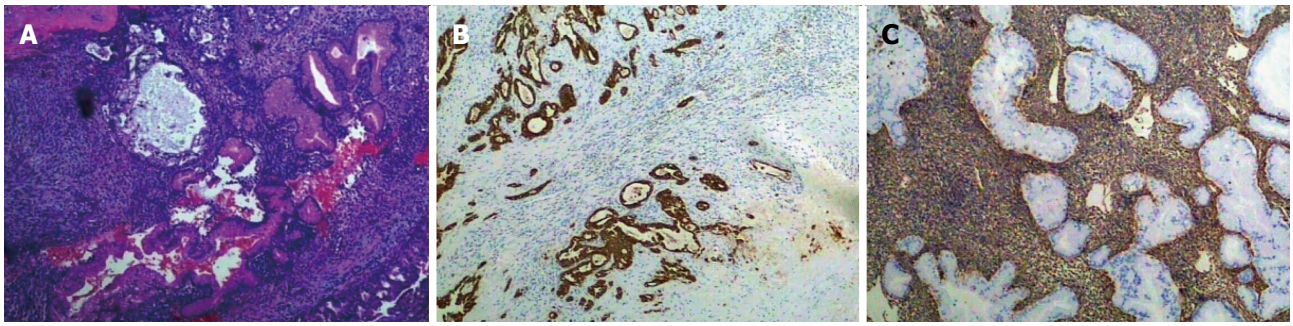

Figure 4 Histological examination of the lesion. A: Microscopy of the tumor indicated a predominance of dual disparate sarcomatous and carcinomatous components. Spindle-shaped tumor cells and well-differentiated adenocarcinoma cells coexisted and intermingled (HE staining, × 200); B: Cytokeratin 7 immunostaining showed strong and diffuse expression in the ductal adenocarcinoma cells (× 200); C: Sarcomatous cells were immunopositive for vimentin (× 200).

without accompanying ductal dilatation, although with some exceptions[1,7,11,12]. Tumors located in the pancreatic head can cause pancreatic main duct and intra- and extra-hepatic bile duct dilatation. After intravenous injection of a gadolinium chelate, the solid component, mural nodules and cystic wall show moderate enhancement. Unlike pancreatic ductal adenocarcinoma, carcinosarcoma of the pancreas often has a well-circumscribed border and seldom invades the adjacent organs, extrapancreatic nerve and vascular system. However, they easily metastasize to the liver and peritoneum which is the main cause of death[1,10].

The predominant differential diagnosis with pancreatic carcinosarcoma is pancreatic ductal adenocarcinoma. Compared with ductal adenocarcinoma that is poorly vascularized, pancreatic carcinosarcoma has more vascularity. In addition, extrapancreatic perineural and vascular invasion, atrophy of the pancreatic parenchyma and duct dilatation are less common in carcinosarcoma. When pancreatic carcinosarcoma manifests as a cystic tumor, it is difficult to distinguish it from mucinous cystadenoma or cystadenocarcinoma. They share many features in common, such as focal thickening of the wall, heterogeneous content, mural nodules and so on. However, the preponderance of calcification in the septa and cystic wall enable one to readily distinguish this entity from pancreatic carcinosarcoma because intratumoral calcification in pancreatic carcinosarcoma has not been described so far.

Given the tumor's rarity, it is difficult to establish more classical imaging findings. Nevertheless, a few characteristic imaging features have been described in this paper. We believe that the characterization of imaging features of pancreatic carcinosarcoma can increase the awareness of this entity among radiologists.

COMMENTS

Case characteristics

A 74-year-old woman was referred to our hospital for acute calculous cholecystitis and a lesion in the pancreatic tail was discovered. The patient was referred again to our hospital for intermittent abdominal pain and distention lasting for more than 2 wk.

Clinical diagnosis

Physical examination showed tenderness on the upper abdominal region.

Differential diagnosis

Pancreatic mucinous cystadenoma or cystadenocarcinoma.

Laboratory diagnosis

Laboratory analysis revealed elevated CA19-9 (148.40 U/mL), CA72-4 (19.19 U/mL), CA24-2 (51.7 U/mL) and CEA (10.05 ng/mL) levels.

Imaging diagnosis

Computed tomography showed that a mass in the pancreatic tail manifested as a heterogeneous complex mass that contained cystic and mixed solid areas and measured 4.0 cm in diameter.

Pathological diagnosis

Histological examination demonstrated characteristic histological findings of an intimate admixture of carcinomatous and sarcomatous elements.

Treatment

The patient underwent a distal pancreatectomy with splenectomy.

Related reports

The histopathological features of pancreatic carcinosarcoma have been illustrated in the literature but to date no reported cases have been documented on imaging characteristics.

Experiences and lessons

The predominant differential diagnosis of pancreatic carcinosarcoma is pancreatic ductal adenocarcinoma, mucinous cystadenoma or cystadenocarcinoma.

Peer review

This study determines the histopathological features of carcinosarcoma lesions of the pancreas as this is an extremely rare tumor with a poor prognosis. The authors report the computed tomography (CT) appearance of pancreatic carcinosarcoma which presents as a mucinous cystadenoma in one female patient. Detailed analysis and conclusion of the CT characteristics were performed according to the appended CT images in other reported cases. The authors claim that this is the first report of CT features of pancreatic carcinosarcoma in the English literature.

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CASE REPORT

Unusual case of digestive hemorrhage: Celiac axis-portal vein arteriovenous fistula

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Author contributions: Liu YR and Huang B are joint first authors and performed the majority of experiments; Zhao JC designed the study and wrote the manuscript; Yuan D was involved in editing the manuscript; and Wu ZP collected the human samples.

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Abstract

A case of intractable upper gastrointestinal-hemorrhage was reported in a patient with portal hypertension caused by an arterioportal fistula (APF), namely, celiac axis-portal vein arteriovenous fistula. Portal hypertension caused by extrahepatic-APFs is extremely rare. Trauma, malignancy, and hereditary causes are the common etiology of APFs; but were absent in our patient. Our patient represents an unusual case of unexplained APF who presented with portal hypertension and was successfully managed through endovascular aortic repair.

Key words: Arterioportal fistula; Portal hypertension; Endovascular aortic repair

Core tip: We present a rare case of intractable upper gastrointestinal hemorrhage due to celiac axis-portal vein arteriovenous fistula. Through this case report, we hope to highlight diagnosis of this unusual condition, and discuss our subsequent management. This case represents an unusual cases of unexplained presenting with portal hypertension that was successfully managed through endovascular aortic repair.

Liu YR, Huang B, Yuan D, Wu ZP, Zhao JC. Unusual case of digestive hemorrhage: Celiac axis-portal vein arteriovenous fistula. *World J Gastroenterol* 2015; 21(4): 1362-1364 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1362.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1362

INTRODUCTION

Portal hypertension is defined as the increase in porto-systemic resistance and/or flow[1]. The etiology of portal hypertension can be divided into pre-, intra-, and post-hepatic causes. The primary cause of portal hypertension is liver cirrhosis, which accounts for 90% of the cases in the United States[2]. The second - most common - etiology of portal hypertension is extrahepatic portal vein thrombosis, which accounts for 7% of cases. The remaining 3% of cases, including our case, are the result of a variety of rare etiologies.

CASE REPORT

A-17-years old child with no history of liver disease or gastric ulcers was admitted to West China Hospital, Sichuan University for a five-day history of hematemesis and tarry stools. The physical examination revealed a blood pressure of 122/63 mmHg, a heart rate of 73 beats per minute, and a platelet count reduced to 45 × 10^9/L. Pertinent laboratory values of liver function, renal function, and electrolytes were normal; moreover all marks for hepatitis, and autoimmune disease were negative. Gastroscope revealed esophageal varices (Figure 1). Based upon this finding, it was speculated that the overflowing of the portal vein due to the shunt between the artery and the portal vein was causing perisinusoidal portal hypertension. To further confirm this hypothesis, digital subtraction angiography was performed and revealed a mass of 52 mm × 48 mm. The portal vein was highlighted at the arterial phase, strongly suggesting that the mass was an arterioportal fistula involving an arteriole from celiac axis feeding directly into the portal vein (Figure 2). Taken together, the arterioportal fistula was concluded to be the cause of this patient's non-cirrhotic portal hypertension. Surgery was performed under local anesthesia with full hemodynamic monitoring. The right femoral artery was isolated, and 5-F catheter sheaths were placed into the right femoral artery *via* guidewire. A covered stent (10 mm × 40 mm. Fluency Plus-) was deployed slowly and accurately in the celiac axis. An aortogram revealed no endoleak and no blood flow entering into the portal vein, but the proper hepatic artery and left gastric artery were not observed (Figure 3). The post-operation recovery of the patient has been uneventful, and no additional episodes of upper gastrointestinal bleeding have been reported by the patient on three subsequent clinic visits during the last six months (Figure 4).

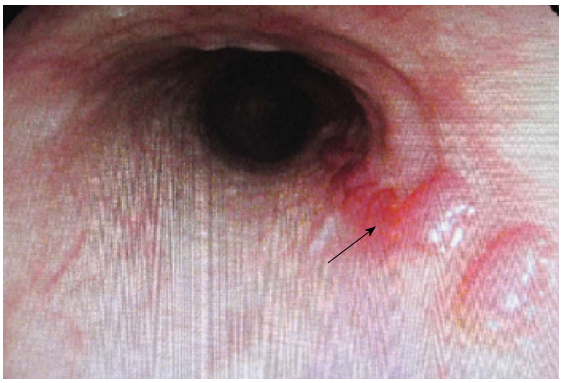

Figure 1 Gastroscope revealed esophageal varices (black arrow).

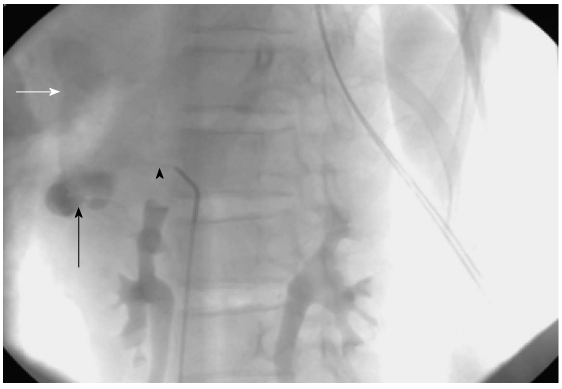

Figure 2 DSA showed a mass of 52 mm × 48 mm (black arrow), the portal vein(white arrow) was highlighted at the arteriaphase(black arrowhead), strongly suggesting the existence of a hepatic arterioportal communication.

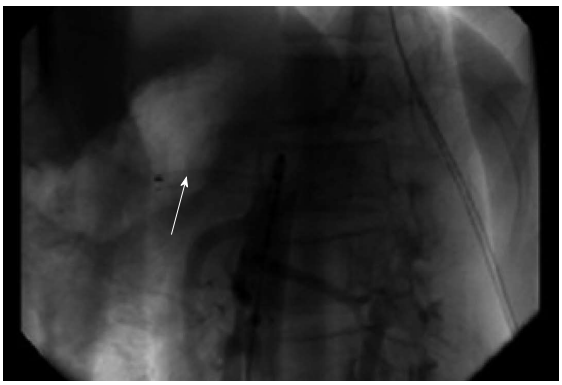

Figure 3 Aortogram revealed no endoleak and no blood flow entering into Portal Vein anymore (arrow).

DISCUSSION

Most reports on the etiology of arterioportal fistula (APF) in the literature is focuses on trauma, hepatocellular carcinoma (HCC), and congenital causes[3]. The clinical manifestations of APFs include congestive heart failure (40%-60%), portal hypertension (20%-40%), and diarrhea with abdominal pain (20%)[4]. Hepatic trauma is a common cause of APFs. HCC can cause APFs, and studies have demonstrated that APFs may occur in up to 60% of patients with HCC[5,6]. Congenital APFs are associated with portal hypertension and gastrointestinal hemorrhage[3,7]. The portal hypertension in our patient manifested as splenomegaly on computed tomography, as esophageal varices on gastroscope and, as blood platelet counts reduced to 45 × 10^9/L. Our patient did not have the any history of congenital APF, abdominal trauma, or malignancy. Thus, we believe that the present report is a case of unexplained AFP. Based on literature search, this is one of the few cases of unexplained APF presenting with portal hypertension.

The treatment of APF is either surgical or minimally invasive though interventional radiology (IR) techniques. Surgical ligation of the supplying artery can performed, but the surgical wound of patient

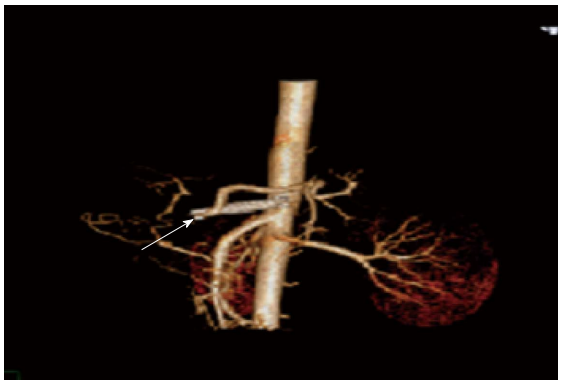

Figure 4 Post-operation recovery of the patient has been uneventful, and no additional episodes of upper gastrointestinal bleeding have been reported by the patient on three subsequent clinic visits during the last six months (arrow).

is not inconsequential. IR offers many advantages over surgical treatment. These advantages include decreased morbidity and mortality, reduced risk of subsequent complications, and a significant reduction in the time required for recovery. With the advancement of interventional radiology techniques, the trend has shifted to IR techniques[8]. The AFP adhesion in our patient was serious; therefore, we selected IR technique.

IR is accomplished through artery embolization. The therapy in our case was accomplished through celiac axis endovascular aortic repair; Because we were concerned about the risk of celiac axis embolism of the coils of the IR intervention. Endovascular aortic repair complications include non-target repair, hepatic infarction, and ischemic cholangitis. The proper hepatic artery in our case was covered with a stent because of stent migration, however, the proper hepatic artery in our case exhibited a decreased diameter, and no complications were observed.

In summary, we described a case of portal hypertension due to unexplained APF. The patient was successfully treated through endovascular aortic repair and is being monitored regularly in our outpatient clinic without any further episodes of bleeding.

COMMENTS

Case characteristics

The child with no history of liver disease or gastric ulcers was admitted to hospital for a five-day history of hematemesis and tarry stools.

Clinical diagnosis

Digestive hemorrhage: celiac axis-portal vein arteriovenous fistula.

Differential diagnosis

Portal hypertension was caused by liver diseases and gastric ulcers, but the patient with no history of liver diseases and gastric ulcers.

Laboratory diagnosis

Platelet count reduced to 45 × 10^9/L. Pertinent laboratory values of liver function, renal function, and electrolytes were normal; moreover all marks for hepatitis, and autoimmune disease were negative.

Imaging diagnosis

Computed tomography (CT) revealed a mass of 52 mm × 48 mm behind the head of the pancreas. The portal vein was highlighted at the arterial phase, strongly suggesting the existence of a hepatic arterioportal communication.

Treatment

Patient was successfully managed through endovascular aortic repair.

Related reports

Intractable upper gastrointestinal hemorrhage for portal hypertension caused by an arterioportal fistula (APF) is rare, however, though CT or digital subtraction angiography we can find the cause.

Experiences and lessons

Patients with intractable upper gastrointestinal hemorrhage, with no history of liver disease or gastric ulcers, should be diagnosed as portal hypertension caused by an APF.

Peer review

This manuscript illustrates an unusual cause of gastrointestinal hemorrhage. This article encourages the clinician to consider a unique source of portal hypertension caused by APF.

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CASE REPORT

Partial embolization as re-treatment of hypersplenism after unsuccessful splenic artery ligation

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Author contributions: Xu ZJ conceived and coordinated the study, and participated in data collection and manuscript writing; Pan XN participated in the study design, data collection, and manuscript writing; Zheng LQ participated in data collection and manuscript writing.

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Abstract

Ligation of splenic artery (LSA) is used for the treatment of liver cirrhosis with hypersplenism. However, hypersplenism is not significantly improved following LSA treatment in some cases, and there are few reports of retreatment of hypersplenism after LSA. We report the case of a 47-year-old man with liver cirrhosis and hypersplenism who underwent LSA treatment, but did not significantly improve. Laboratory tests revealed severe leukocytopenia and thrombocytopenia. Celiac computed tomography arteriogram and digital subtraction angiography revealed two compensatory arteries connected to the hilar splenic artery from the left gastro-epiploic artery and from the dorsal pancreatic artery. Partial splenic embolization (PSE) was performed through the compensatory arteries. As a result, the patient achieved partial splenic ischemic infarction, and white blood cell and platelet counts rose and remained in the normal range. PSE is an effective therapeutic modality for the retreatment of hypersplenism when other modalities have failed.

Key words: Hypersplenism; Partial splenic embolization; Splenic artery ligation; Liver cirrhosis; Leukocytopenia; Thrombocytopenia

Core tip: Ligation of splenic artery (LSA) is used for the treatment of hypersplenism due to liver cirrhosis. However, hypersplenism is not always improved following LSA. We report a case of cirrhosis and hypersplenism which underwent LSA treatment, but failed to respond as manifested by persistent severe leukocytopenia and thrombocytopenia. Celiac computed tomography arteriogram and digital subtraction angiography revealed two compensatory arteries supplying the spleen. Partial splenic embolization (PSE) was performed through those arteries resulting in increased leukocyte and thrombocyte counts that remained within the normal range. PSE is an effective therapeutic modality for the retreatment of hypersplenism when other modalities have failed.

Xu ZJ, Zheng LQ, Pan XN. Partial embolization as re-treatment of hypersplenism after unsuccessful splenic artery ligation. *World J Gastroenterol* 2015; 21(4): 1365-1370 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i4/1365.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i4.1365

INTRODUCTION

Hypersplenism is a common complication of liver cirrhosis. Peripheral cytopenia is frequent manifestation in patients with hypersplenism secondary to liver cirrhosis. The traditional treatment method for hypersplenism secondary to cirrhosis is splenectomy. However, due to the potential disadvantages of severe postoperative complications, and the high rates of perioperative mortality after splenectomy[1-3], the clinical application of this traditional mode of treatment has been limited. In recent years, partial splenic embolization (PSE) has been shown to be an effective way to relieve hypersplenism[4-7]. Ligation of the splenic artery (LSA) is also used in the treatment of hypersplenism due to other causes[8-11]. However, some cases of hypersplenism are not significantly improved following LSA, and there are few cases reporting the retreatment of hypersplenism after LSA. We report a case of PSE performed through the compensatory arteries for the treatment of hypersplenism after unsuccessful LSA. The aim of the current report was to highlight the use of PSE in the treatment of hypersplenism due to cirrhosis, and emphasize its value when other modalities such as LSA have failed.

CASE REPORT

A 47-year-old Chinese man with severe leukocytopenia and thrombocytopenia due to hypersplenism secondary to liver cirrhosis was admitted to our hospital (180th Hospital of the People's Liberation Army, Quanzhou, China). Physical examination revealed palmar erythema, mild anemia, and moderate splenomegaly.

He had a history of severe upper gastrointestinal bleeding once three years ago. The patient was diagnosed with hepatitis B-related decompensated liver cirrhosis, portal hypertension, hemorrhage from ruptured esophageal varices, and splenomegaly with hypersplenism. The vasopressin analog, terlipressin, was administered to reduce the portal venous pressure, and the bleeding stopped. Abdominal computed tomography (CT) scan showed liver cirrhosis with portal hypertension and splenomegaly. Gastroscopy revealed the esophageal and gastric varices. Routine blood test (RBT) results were: white blood cells (WBC), 1.75 × 10^9 cells/L; granulocytes (G), 1.18 × 10^9 cells/L; red blood cells (RBC), 2.67 × 10^{12} cells/L; hemoglobin (HGB), 73 g/L; platelets (PLT), 32 × 10^9/L. Bone marrow biopsy results showed hyperplasia of bone marrow with nucleated cells, and a slight maturation disorder in granulocytes. The reticulocyte count (RC) was 3.5%. His model for end-stage liver disease (MELD) assessment score was 19.52. As there was no evidence of a toxic cause, or of an underlying hematologic disease, the most likely diagnosis was hypersplenism secondary to cirrhosis. Because the MELD assessment score was nearly 20, LSA was selected instead of transjugular intrahepatic portosystemic shunt (TIPS) treatment to avoid possible hepatic decompensation. Prior to the LSA treatment, celiac computed tomography angiogram (CTA) was performed to confirm patency of the collateral arteries. The patient underwent laparoscopic LSA and ligation of the gastric coronary vein at the end of December, 2010. Follow-up over three years showed that the WBC and PLT counts did not increase indicating that the hypersplenism had not significantly improved.

Over the following 3 years the patient remained in stable clinical condition but required a few admissions to the hospital, mainly for respiratory infections. However, RBT revealed recurrent severe leukocytopenia and thrombocytopenia. He was admitted to our hospital for further treatment. On admission, the RBT results: WBC, 1.65 × 10^9 cells/L; G, 1.10 × 10^9 cells/L; RBC, 3.67 × 10^{12} cells/L; HGB, 109 g/L; PLT, 30 × 10^9/L, PLT size was normal. The RC was 3.3%. Bone marrow biopsy results again showed hyperplasia, a slight maturation disorder in granulocytes with a slightly increased proportion of phagocytes. Serum test results were: albumin, 32.1 g/L; total bilirubin, 33.8 μmol/L; alanine aminotransferase, 43 IU/L; aspartate aminotransferase, 56 IU/L. Clotting test results were: international normalized ratio, 1.23; prothrombin time, 16.5 s; prothrombin activity, 50.3%. Abdominal ultrasound examination revealed an enlarged spleen (175 mm × 63 mm) and liver cirrhosis. The liver was shrunken, and the portal diameter was 14.5 mm with hepatic inflow at a velocity of 181 mm/s. An abdominal CT scan showed liver cirrhosis with portal hypertension and splenomegaly. Ischemic necrosis was not seen, but the bio-clamp (Figure 1A) was seen in the splenic artery area. Celiac CTA (Figure 1B) showed that the proximal splenic artery was not enlarged, but the gastroduodenal artery and the right gastro-epiploic artery were enlarged, and the right gastro-epiploic artery and the left gastro-epiploic artery were anastomosed. There were two compensatory arteries supplying the spleen. The collateral arteries were confirmed as connected to the hilar splenic artery from the left gastro-epiploic artery and from the dorsal pancreatic artery on a celiac arteriogram. The Child-Pugh score was 8 (B-class), and the MELD assessment score was 18.53. He was diagnosed with liver cirrhosis, portal hypertension, and splenomegaly with hypersplenism.

Because of the high risk of complications with splenectomy, PSE was performed through the compensatory arteries. Prior to embolization, selective angiography of the celiac trunk, the splenic artery and the superior mesenteric artery were performed in the patient through the right

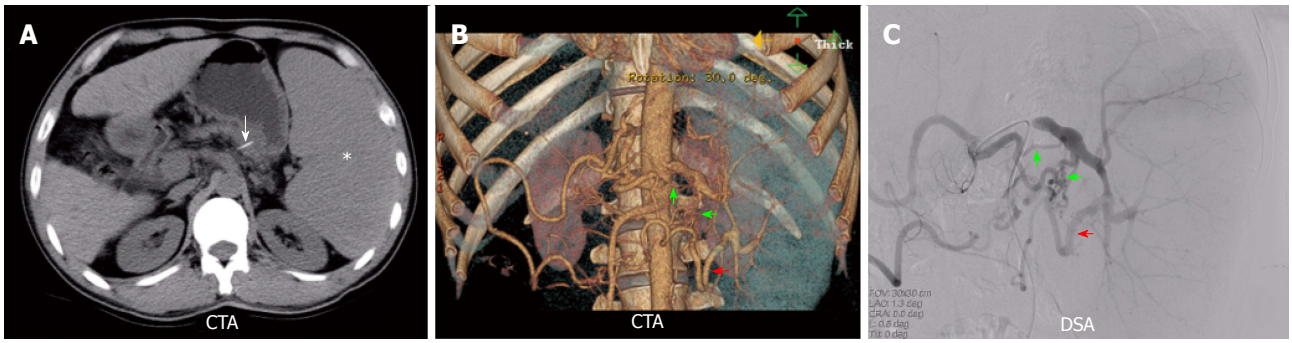

Figure 1 Imaging studies. A: After ligation of splenic artery treatment, abdominal computed tomography showed that the size of the spleen was enlarged (white star). Ischemic necrosis was not seen in the spleen. However, the bio-clamp (white arrow) was seen in the splenic artery area; B and C: Splenic compensatory arteries: the dorsal pancreatic artery (green arrow), and the left gastro-epiploic artery (red arrow). CTA: Computed tomography angiogram; DSA: Digital subtraction angiography.

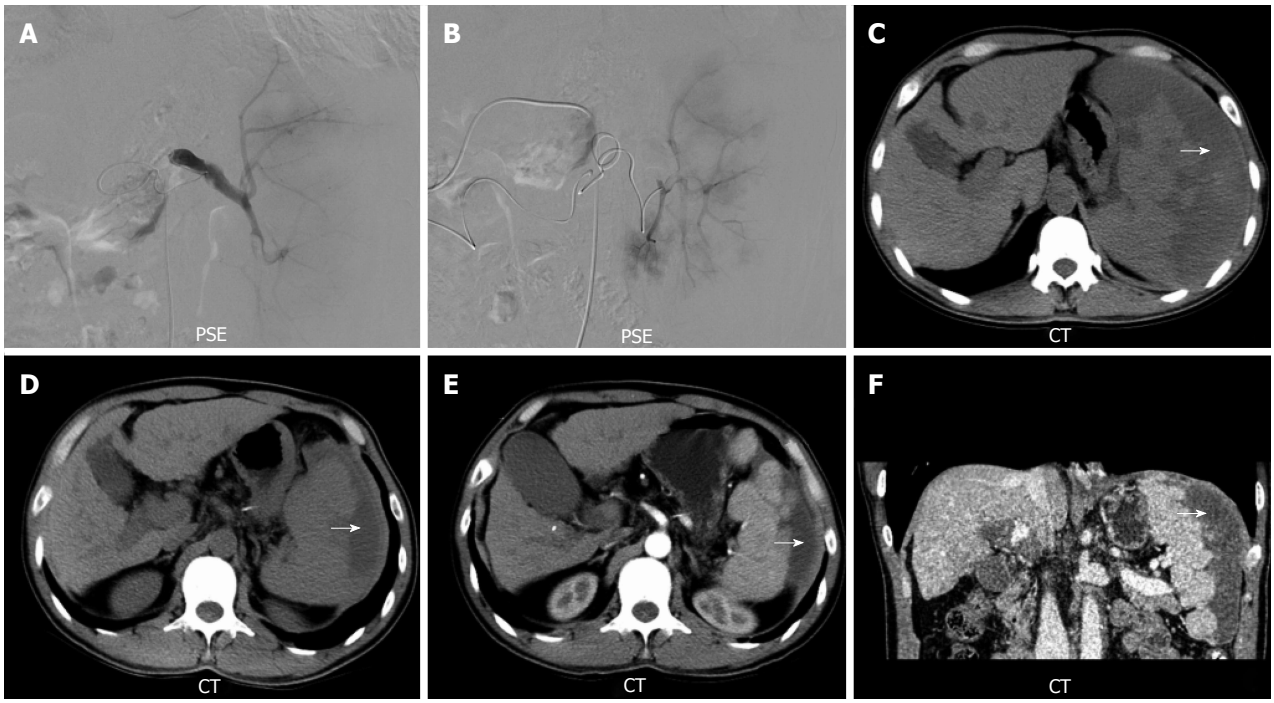

Figure 2 Partial splenic embolization therapy. A: Partial splenic embolization (PSE) therapy through the collateral artery of dorsal pancreatic artery; B: PSE therapy through the left gastro-epiploic artery; C: Abdominal computed tomography (CT) revealed that ischemic infarction (black arrow) was seen in the spleen 2 wk after PSE; D-F: Follow-up abdominal CT was performed after 4 mo (D) and 9 mo (E and F), showing that the size of the spleen and infarction (black arrows) progressively decreased.

femoral artery using a 5 Fr diagnostic catheter. Digital subtraction angiography (Figure 1C) showed similar findings in the splenic artery and collateral circulation arteries as seen previously on CTA. PSE was performed by cannulation of the hilar splenic artery through the compensatory arteries with a Radifocus SP microcatheter (Terumo Corporation; Tokyo, Japan) (Figure 2A and B). Sixty-five percent of the spleen was embolized with fine particles of gelatin sponge. Preoperative antibiotic prophylaxis was administered 1 d prior to the procedure. Following embolization, he was monitored clinically, and antibiotics were administered after the procedure for 7 d. The patient complained of post-embolization syndrome including fever, left upper abdominal pain, nausea, vomiting, ascites, and left pleural effusion. These were effectively relieved by anti-inflammatory, antipyretic, analgesic and diuretic medications. After 2 wk, the symptoms resolved.

At 2 wk post-operation, an abdominal CT (Figure 2C) scan showed a shadow pattern of low density in the spleen, suggesting splenic ischemic infarction. The blood results were: WBC, 7.54 × 10^9 cells/L; G, 6.05 × 10^9 cells/L; RBC, 3.31 × 10^{12} cells/L; HGB, 100 g/L; and PLT, 231 × 10^9/L. Follow-up abdominal CT scan was performed 4 mo (Figure 2D) and 9 mo (Figure 2E and F) after the PSE, and showed that the size of both the spleen and the infarction progressively decreased. Both WBC and PLT counts increased significantly, peaked at 2 wk,

then gradually fell during one year follow-up period, and remained within the normal range. There were no significant changes in RBC counts either after PSE or during the follow-up period. Follow-up at one year by abdominal ultrasound examination revealed that the portal diameter was 13.5 mm with a hepatic inflow velocity of 203 mm/s. The patient and the size of spleen remained stable.

DISCUSSION

Liver cirrhosis with hypersplenism can result in severe thrombocytopenia and/or leukocytopenia. The management of hypersplenism includes several possible approaches such as splenectomy, PSE, total splenic artery embolization (TSAE), LSA, *etc*. Splenectomy can cause a sustained and long-term increase in WBC and PLT counts. However, severe complications after splenectomy have been reported and range from 9.6%-26.6%[2,3,12]. In addition, splenectomy is often associated with an increased long-term risk of septic events[3,12]. PSE was first performed in the treatment of hypersplenism by Spigos *et al*[13] in 1979, and has gained increasing popularity as an alternative to splenectomy[7,14]. The net result of PSE is a partial splenectomy which allows for retention of splenic immune function[15]. PSE is accomplished by segmental and subsegmental arterial embolization of the spleen, and can be controlled to result in partial to complete splenic ischemic infarction[16]. The curative effect of PSE is long-term, and there are few complications, and low mortality. In PSE, the splenic infarction rate is a critical factor for the improvement of thrombocytopenia. To ensure a sustained and long-term increase in PLT and WBC counts, the extent of splenic infarction needs to be greater than 50%[6]. However, severe postoperative complications occurred more frequently in these patients[17]. In addition, quantitative control of the splenic infarction is difficult in this procedure, and is dependent on the experience of the operators. The reduction in portal hypertension reduces the risk of hemorrhage of the upper digestive tract. In addition, PSE has been suggested to be an effective method for the treatment of bleeding from gastric varices and portal hypertension[18]. TSAE has been shown to be a safe and effective method for the treatment of splenic artery aneurysms[19,20], and hypersplenism[21]. LSA is also used to treat hypersplenism, and leads to increased PLT and WBC counts in the short term[22], but there have been problems after long-term follow up. The effect of total splenic artery ligation or embolization is limited once the splenic collateral circulation is established[23], resulting in a recurrence of hypersplenism. Because the spleen has an abundant collateral circulation network, collateral circulation is frequently established within a short period of time after ligation. The main splenic collateral arteries are the left gastric artery, short gastric artery, dorsal pancreatic artery and the left gastro-epiploic artery[24,25]. Therefore, TSAE and LSA do not usually cause complete ischemic necrosis of the spleen.

PSE, LSA and TSAE are reasonably safe and effective procedure for controlling hypersplenism in patients with cirrhosis. However, hypersplenism is not significantly improved following the treatment in some cases, and there are very limited reports of retreatment required to control hypersplenism after LSA. The retreatment of hypersplenism includes several possible approaches, including splenectomy, ablation[24-26], and TIPS[27,28]. Despite of technological advances, splenectomy is still the most commonly used treatment modality. In this case, splenectomy might be an alternative treatment modality when LSA was failed. However, splenectomy is associated with high rates of morbidity and mortality. In the current case, the patient was not able to receive such treatment due to severe thrombocytopenia and leukocytopenia. These conditions presented increased risks of life-threatening hemorrhage and infection for the patient. Celiac CTA revealed two compensatory arteries supplying the spleen. Therefore, PSE was selected for our patient to treat the severe thrombocytopenia and leukocytopenia. The complications observed in the immediate post-operative period, post-embolization syndrome, included fever, left upper abdominal pain, nausea, vomiting, malaise, gastrointestinal symptoms, ascites, and left pleural effusion[29,30]. This is a self-limiting and benign adverse event. The most frequent side effect of post-embolization syndrome consists of associated fever and/or abdominal pain which have been reported to occur in 55%-100% of patients[4,31] and can last up to 40 d[4]. Serious complications of PSE such as splenic abscess and septicemia, are very rare[4,31]. Our patient developed moderate fever and left upper abdominal pain which lasted for 15 d. Potentially lethal postoperative complications of venous thrombosis, septicemia and splenic abscess were not observed in the current case. As a result, the patient achieved successful treatment of hypersplenism. Moreover, the portal venous diameter was decreased, and blood flow velocity was increased after PSE, which is also consistent with previous studies that reported a reduction of portal venous pressure and portal venous blood flow after PSE[18,32]. Because this is only a case report with a short follow-up period, we plan to increase the number of cases, and the follow-up period in future studies.

In conclusion, PSE can be an effective alternative for the management of hypersplenism in patients with cirrhosis, and is an effective therapeutic modality for the retreatment of hypersplenism when

other modalities have failed.

COMMENTS

Case characteristics

A 47-year-old man with severe leukocytopenia and thrombocytopenia due to hypersplenism secondary to liver cirrhosis.

Clinical diagnosis

Palmar erythema, mild anemic and moderate splenomegaly.

Differential diagnosis

Aplastic anemia, myelodysplastic syndrome, low proliferative leukemia, thrombocytopenia.

Laboratory diagnosis

White blood cells: 1.65 × 10^9 cells/L; granulocytes: 1.10 × 10^9 cells/L; red blood cells: 3.67 × 10^{12} cells/L; hemoglobin: 109 g/L; platelets: 30 × 10^9/L; reticulocyte count: 3.3%; international normalized ratio: 1.23; prothrombin time: 16.5 s; prothrombin activity: 50.3%; Child-Pugh score: 8 and model for end-stage liver disease score: 18.53. Bone marrow biopsy results showed hyperplasia, a slight maturation disorder in granulocytes with a slightly increased proportion of phagocytes.

Imaging diagnosis

Abdominal computed tomography scan showed liver cirrhosis with portal hypertension and splenomegaly. Celiac computed tomography angiogram/digital subtraction angiography showed that there were two compensatory arteries supplying the spleen. The collateral arteries were confirmed as connected to the hilar splenic artery from the left gastro-epiploic artery and from the dorsal pancreatic artery on a celiac arteriogram.

Treatment

Partial splenic embolization (PSE) was performed through the compensatory arteries of the spleen.

Related reports

Very few cases about PSE for the treatment of hypersplenism after unsuccessful splenic artery ligation have been reported in the literature.

Term explanation

Both ligation of splenic artery (LSA) and PSE are minimally invasive surgical techniques. Special catheters, guide wires, and precision instruments are introduced with the guidance of medical imaging equipment to obtain a histological diagnosis and achieve local treatment.

Experiences and lessons

This case report not only describes a very rare presentation of hypersplenism after unsuccessful LSA, but also describes the successful retreatment of hypersplenism. PSE was performed through the compensatory arteries resulting in increased leukocyte and thrombocyte counts that remained within the normal range.

Peer review

This article reports successful management of hypersplenism after unsuccessful splenic artery ligation. PSE is an effective therapeutic modality for the retreatment of hypersplenism when other modalities have failed.

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