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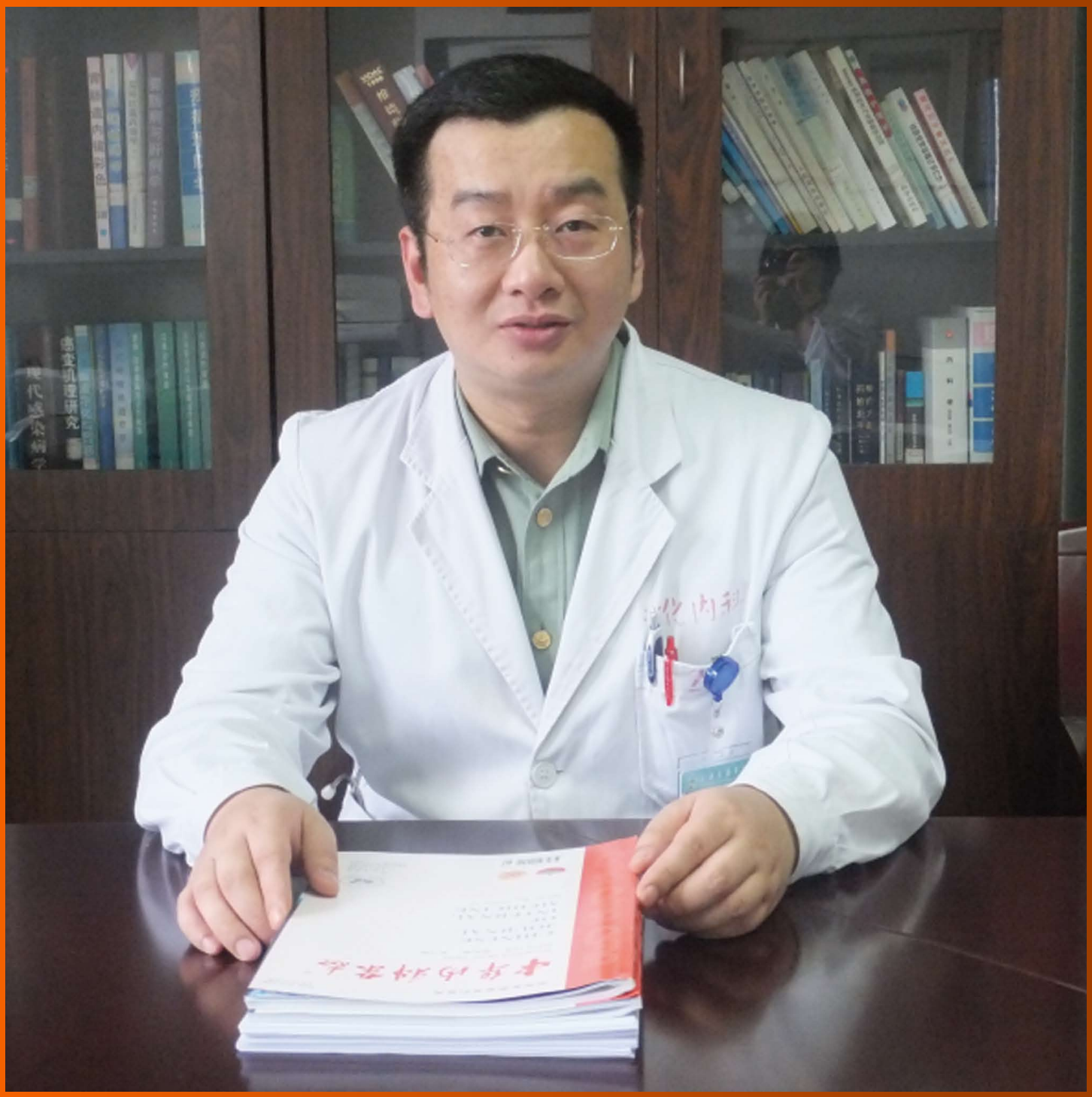

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World Journal of Gastroenterology

Contents

Weekly Volume 21 Number 19 May 21, 2015

EDITORIAL

5755 Sphincter of Oddi dysfunction Type Ⅲ: New studies suggest new approaches are needed
Wilcox CM

5762 Changes in the esophageal mucosa of patients with non erosive reflux disease: How far have we gone?
Triantos C, Koukias N, Karamanolis G, Thomopoulos K

5768 Hepatitis C: New challenges in liver transplantation
Filipec Kanizaj T, Kunac N

REVIEW

5778 Gastric cancer and the epoch of immunotherapy approaches
Niccolai E, Taddei A, Prisco D, Amedei A

5794 Pathophysiology after pancreaticoduodenectomy
Kang CM, Lee JH

5805 Incidence and treatment of brain metastasis in patients with esophageal carcinoma
Feng W, Zhang P, Zheng X, Chen M, Mao WM

MINIREVIEWS

5813 Liver involvement in pediatric celiac disease
Anania C, De Luca E, De Castro G, Chiesa C, Pacifico L

5823 Contribution of the IL-17/IL-23 axis to the pathogenesis of inflammatory bowel disease
Cătană CS, Berindan Neagoe I, Cozma V, Magdaş C, Tăbăran F, Dumitraşcu DL

ORIGINAL ARTICLE

Basic Study

5831 Magnetic resonance imaging of the pancreas in streptozotocin-induced diabetic rats: Gadofluorine P and Gd-DOTA
Cho HR, Lee Y, Doble P, Bishop D, Hare D, Kim YJ, Kim KG, Jung HS, Park KS, Choi SH, Moon WK

5843 Expression profile of microRNAs in gastrointestinal stromal tumors revealed by high throughput quantitative RT-PCR microarray
Tong HX, Zhou YH, Hou YY, Zhang Y, Huang Y, Xie B, Wang JY, Jiang Q, He JY, Shao YB, Han WM, Tan RY, Zhu J, Lu WQ

5856 Hepatocyte nuclear factor 4α induces a tendency of differentiation and activation of rat hepatic stellate cells
Liu K, Guo MG, Lou XL, Li XY, Xu Y, Ji WD, Huang XD, Yang JH, Duan JC

5867 MiR-451 inhibits proliferation of esophageal carcinoma cell line EC9706 by targeting CDKN2D and MAP3K1
Zang WQ, Yang X, Wang T, Wang YY, Du YW, Chen XN, Li M, Zhao GQ

5877 Effect of nuclear factor-κB and angiotensin Ⅱ receptor type 1 on the pathogenesis of rat non-alcoholic fatty liver disease
Tan DY, Shi HY, Li CP, Zhong XL, Kang M

5884 Effect of microRNA-1 on hepatocellular carcinoma tumor endothelial cells
Hu C, Shen SQ, Cui ZH, Chen ZB, Li W

Retrospective Cohort Study

5893 Validation of aspartate aminotransferase to platelet ratio for diagnosis of liver fibrosis and prediction of postoperative prognosis in infants with biliary atresia
Yang LY, Fu J, Peng XF, Pang SY, Gao KK, Chen ZR, He LJ, Wen Z, Wang H, Li L, Wang FH, Yu JK, Xu Y, Gong ST, Xia HM, Liu HY

Retrospective Study

5901 Prognostic value and clinical correlations of 18-fluorodeoxyglucose metabolism quantifiers in gastric cancer
Grabinska K, Pelak M, Wydmanski J, Tukiendorf A, d'Amico A

5910 Risk factors causing structural sequelae after anastomotic leakage in mid to low rectal cancer
Ji WB, Kwak JM, Kim J, Um JW, Kim SH

5918 Needle-knife fistulotomy *vs* double-guidewire technique in patients with repetitive unintentional pancreatic cannulations
Kim SJ, Kang DH, Kim HW, Choi CW, Park SB, Song BJ, Hong YM

5926 Risk scoring system and predictor for clinically relevant pancreatic fistula after pancreaticoduodenectomy
Chen JY, Feng J, Wang XQ, Cai SW, Dong JH, Chen YL

5934 Factors associated with early recurrence after curative surgery for gastric cancer
Kang WM, Meng QB, Yu JC, Ma ZQ, Li ZT

5941 Laparoscopic *vs* computerized tomography-guided radiofrequency ablation for large hepatic hemangiomas abutting the diaphragm
Gao J, Kong J, Ding XM, Ke S, Niu HG, Xin ZH, Ning CM, Guo SG, Li XL, Zhang L, Dong YH, Sun WB

5950 Endoscopic treatment for pancreatic diseases: Needle-knife-guided cannulation *via* the minor papilla
Wang W, Gong B, Jiang WS, Liu L, Bielike K, Xv B, Wu YL

5961 Novel immunological and nutritional-based prognostic index for gastric cancer
Sun KY, Xu JB, Chen SL, Yuan YJ, Wu H, Peng JJ, Chen CQ, Guo P, Hao YT, He YL

5972 Clinicopathologic features of remnant gastric cancer over time following distal gastrectomy
Zhang DW, Dong B, Li Z, Dai DQ

Contents

World Journal of Gastroenterology
Volume 21 Number 19 May 21, 2015

Clinical Trials Study

5979 Phase I trial of combination chemotherapy with gemcitabine, cisplatin, and S-1 in patients with advanced biliary tract cancer
Watanabe A, Kida M, Miyazawa S, Iwai T, Okuwaki K, Kaneko T, Yamauchi H, Takezawa M, Imaizumi H, Koizumi W

Randomized Clinical Trial

5985 Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis
Yoshimatsu Y, Yamada A, Furukawa R, Sono K, Osamura A, Nakamura K, Aoki H, Tsuda Y, Hosoe N, Takada N, Suzuki Y

5995 Efficacy and safety of a patient-positioning device (EZ-FIX) for endoscopic retrograde cholangiopancreatography
Lee S, Han JH, Lee HS, Kim KB, Lee I, Cha EJ, Shin YD, Park N, Park SM

Prospective Study

6001 Assessment of disease specific knowledge and health-related quality of life among United States military veterans with inflammatory bowel disease
Hou JK, Turkeltaub JA, McCarty III TR, El-Serag HB

6008 Risk of venous congestion in live donors of extended right liver graft
Radtke A, Sgourakis G, Molmenti EP, Beckebaum S, Cicinnati VR, Schmidt H, Peitgen HO, Broelsch CE, Malagó M, Schroeder T

6018 Vascular endothelial growth factor and tryptase changes after chemoembolization in hepatocarcinoma patients
Ranieri G, Ammendola M, Marech I, Laterza A, Abbate I, Oakley C, Vacca A, Sacco R, Gadaleta CD

META-ANALYSIS

6026 Association of type 2 diabetes mellitus and the risk of colorectal cancer: A meta-analysis and systematic review
Guraya SY

6032 Endoscopic submucosal dissection for early gastric cancer with undifferentiated-type histology: A meta-analysis
Bang CS, Baik GH, Shin IS, Kim JB, Suk KT, Yoon JH, Kim YS, Kim DJ, Shin WG, Kim KH, Kim HY, Lim H, Kang HS, Kim JH, Kim JB, Jung SW, Kae SH, Jang HJ, Choi MH

6044 Infliximab is superior to other biological agents for treatment of active ulcerative colitis: A meta-analysis
Mei WQ, Hu HZ, Liu Y, Li ZC, Wang WG

6052 Associations between *CD24* gene polymorphisms and inflammatory bowel disease: A meta-analysis
Huang XL, Xu DH, Wang GP, Zhang S, Yu CG

CASE REPORT

6060 Fulminant ulcerative colitis in a healthy pregnant woman
Orabona R, Valcamonico A, Salemme M, Manenti S, Tiberio GAM, Frusca T

6065 Fecal stream diversion and mucosal cytokine levels in collagenous colitis: A case report
Daferera N, Kumawat AK, Hultgren-Hörnquist E, Ignatova S, Ström M, Münch A

6072 Epstein Barr virus-positive mucocutaneous ulcer of the colon associated Hodgkin lymphoma in Crohn's disease
Moran NR, Webster B, Lee KM, Trotman J, Kwan YL, Napoli J, Leong RW

6077 Surgical repair of intractable chylous ascites following laparoscopic anterior resection
Ha GW, Lee MR

6082 Severe Henoch-Schönlein purpura with infliximab for ulcerative colitis
Song Y, Shi YH, He C, Liu CQ, Wang JS, Zhao YJ, Guo YM, Wu RJ, Feng XY, Liu ZJ

6088 Primary hepatic angiosarcoma: A report of two cases and literature review
Zhu YP, Chen YM, Matro E, Chen RB, Jiang ZN, Mou YP, Hu HJ, Huang CJ, Wang GY

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EDITORIAL

Sphincter of Oddi dysfunction Type Ⅲ: New studies suggest new approaches are needed

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Abstract

Sphincter of Oddi dysfunction (SOD) has been classified into three types based upon the presence or absence of objective findings including liver test abnormalities and bile duct dilatation. Type Ⅲ is the most controversial and is classified as biliary type pain in the absence of any these objective findings. Many prior studies have shown that the clinical response to endoscopic therapy is higher based upon the presence of these objective criteria. However, there has been variable correlation of the manometry findings to outcome after endoscopic therapy. Nevertheless, manometry and sphincterotomy has been recommended for Type Ⅲ patients given the overall response rate of 33%, although the reported response rates are highly variable. However, all of the prior data was non-blinded and non-randomized with variable follow-up. The evaluating predictors in SOD study - a prospective randomized blinded sham controlled one year outcome study showed no correlation between manometric findings and outcome after sphincterotomy. Furthermore, patients receiving sham therapy had a statistically significantly better outcome than those undergoing biliary or dual sphincterotomy. This study calls into question the whole concept of SOD Type Ⅲ and, based upon prior physiologic studies, one can suggest that SOD Type Ⅲ likely represents a right upper quadrant functional abdominal pain syndrome and should be treated as such.

Key words: Abdominal pain; Sphincter of Oddi dysfunction; Manometry; Sphincterotomy

Core tip: Prior observations suggest that biliary sphincterotomy may be of benefit in patients with sphincter of Oddi dysfunction (SOD) Type Ⅲ who have biliary type pain but no objective findings of bile duct obstruction. The prospective randomized blinded sham controlled trial termed evaluating predictors in SOD demonstrated no correlation between manometry and outcome and furthermore showed that patients receiving sham therapy had a better outcome than those receiving either biliary or dual sphincterotomy. Until other studies are available, patients with biliary type pain in the absence of objective findings

should not routinely undergo endoscopic retrograde cholangiopancreatography and do not benefit from sphincterotomy.

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INTRODUCTION

It has been almost 130 years since the muscular structure at the terminal end of the biliary and pancreatic ducts was first described by a young anatomist and physiologist Rugero Oddi[1]. His subsequent studies demonstrated that this sphincter muscle was under physiologic regulation. While bile duct pressures had been previously reported, it was not until 1980 that Geenen *et al*[2] described the use of a small catheter placed through the biliary sphincter into the bile duct at the time of endoscopic retrograde cholangiopancreatography (ERCP) whereby sphincter activity could be identified, measured and where its physiologic regulation was confirmed. At that time, they[2] and others[3] had postulated that perhaps disorders of the sphincter could result in clinical syndromes such as post-cholecystectomy abdominal pain.

DEFINITION

Sphincter of Oddi dysfunction (SOD) has been defined as an abnormality of either the biliary and/or pancreatic sphincter causing intermittent or fixed obstruction to flow of bile or pancreatic juice, respectively, associated with episodic biliary-type pain, recurrent pancreatitis, abnormal liver chemistry tests, or ductal dilatation. The ROME Ⅲ criteria defined biliary SOD as epigastric or right upper quadrant pain which included all of the following: episodes of pain lasting at least 30 min, symptoms occurring at different intervals but not on a daily basis; the pain was constant in nature and was of severity enough to alter or interrupt the patient's daily activities or lead to an emergency department visit; the pain was not relieved by postural changes, bowel movements, or antiacid therapy; and finally that the exclusion of other structural pancreaticobiliary diseases were excluded[4]. In contrast to the prior criteria, noninvasive methods were used to measure common bile duct diameter and contrast drainage times were not required. Manometrically, SOD is defined as a basal biliary or pancreatic sphincter pressure of > 40 mmHg which is greater than 3 standard deviations above normal[5]. Since the seminal observations of Geenen *et al*[2], many studies worldwide have reported on the use of both biliary and pancreatic manometry in symptomatic patients. In addition, a positive outcome of endoscopic therapy (sphincterotomy) has been reported for the treatment of abdominal pain or idiopathic pancreatitis of patients identified with SOD[6-11]. Indeed, SOD became a common place diagnosis for referral of patients to selected centers with biliary-type pain or idiopathic pancreatitis for sphincter manometry in the hopes of making the diagnosis and providing effective therapy. The results of the EPISOD (Evaluating Predictors and Interventions in Sphincter of Oddi Dysfunction) study[12] have now challenged the diagnosis of the most complex of these sphincter "disorders", SOD Type Ⅲ.

SOD TYPES

In 1987, these clinical syndromes all of which have in common biliary-type pain were classified into one of three types, often termed the Milwaukee classification[13]. Such a classification scheme takes into account the presence of objective findings (abnormal liver chemistry tests, ductal dilation) and based upon subsequent studies, such a classification system could help determine the likelihood of a positive manometric study thereby guiding therapy. As shown in Table 1, biliary Type Ⅰ SOD is defined as a dilated bile duct and abnormal liver tests. It was considered that these patients either have small stones, sludge, or true stenosis of the biliary sphincter leading to obstruction; some data suggests crystals are rare[14]. Nevertheless, even with these objective findings, biliary manometry can be normal in up to 35% of these Type Ⅰ patients[15]. Type Ⅱ patients have abnormal liver tests or biliary dilatation but not both potentially suggesting a sphincter disorder. In these patients manometric findings of sphincter hypertension can be found in up to 55%-65%[16-18]. Silverman *et al*[19] suggested a hybrid classification for type Ⅱ patients where these patients had pain and marginal (< 1.5 × ULN) elevation of liver enzymes with normal duct diameter[1]. They found no difference in prevalence of elevated sphincter pressures between the hybrid group and standard classification. Type Ⅲ patients have no objective findings of biliary obstruction and manometric findings of sphincter hypertension are less frequent, but were reported to be 59% in one study[17]. A similar classification scheme has also been proposed for those with pancreatic abnormalities[18].

ROLE OF MANOMETRY

While considered the gold standard, manometry is an imprecise technique and it is important that the number of normal patients evaluated has been small[5,20]. Manometric findings can be influenced by a number of both patient and technical factors[6,21,22]. Use of a guidewire through the catheter may alter pressure recordings. Likewise, if the catheter is up against the

Table 1 Classification of sphincter of Oddi dysfunction

	Abnormal liver or pancreatic chemistries	Biliary or pancreatic duct dilation on imaging
Type Ⅰ	Both	Both
Type Ⅱ	Either	Either
Type Ⅲ	Neither	Neither

Table 2 Randomised controlled trials's of Type Ⅱ sphincter of Oddi dysfunction *n* (%)

	Geenen ('89)	Toouli (2000)
Rome type 3	-	-
Concealed assignment	?	+
Blinded f/u	Yes	Yes
Type Ⅰ	0	0
Type Ⅱ	47 (100)	81 (100)
Type Ⅲ	0	0
Sham EBS	Yes	Yes
Mano Directed	No	Yes
Response		
+ mano (EBS *vs* S)	91%, 25%	85%, 38%
- Mano (EBS *vs* S)	33%, 42%	62%, 42%

F/U: Follow-up; EBS: Endoscopic biliary sphincterotomy; mano: Manometry; S: Sham.

bile duct wall, readings may be artificially elevated. The readings can also be influenced by where the baseline sphincter zone is interpreted to be located. Other factors include medications taken as well as those administered at the time of ERCP. One cannot use anti-motility agents such as hyoscyamine as the pressure may be reduced. Chronic opioid use can increase basal sphincter pressure; midazolam has been found to alter sphincter pressure whereas diazepam does not. Lastly, reproducibility may be an important issue. For example, in one study of over 5000 patients[23] a small group of patients (n = 30) in whom manometry was initially normal underwent repeat manometry. In these patients, 60% now had an abnormal tracing. From the original cohort, 80% were found to be positive on the initial study. If one then adds the number of patients in whom the study was positive initially, and then assumes 60% of all patients with an initially negative study would be positive, then this would suggest that up to 93% of patients from this cohort could have an initially positive manometry study. Such a high percentage is incredibly hard to believe.

OUTCOME AFTER SPHINCTEROTOMY

The correlation between SOD classification and clinical outcome after biliary sphincterotomy has been widely studied. Overall, a wealth of literature has suggested the efficacy of biliary sphincterotomy for sphincter of Oddi dysfunction[7]. For patients with types Ⅱ and Ⅲ SOD, there is a variable correlation between manometry findings and clinical outcomes after sphincterotomy[22,24]. Patients with SOD Type Ⅱ have shown improvement following biliary sphincterotomy in approximately 69% of patients, ranging from 60%-94%[7]. In contrast, for patients with biliary Type Ⅲ SOD, the clinical response is less ranging from 8%-62%[7]. The Indiana group has suggested that sphincterotomy of both the biliary and pancreatic sphincters (dual sphincterotomy) may further increase response[25].

It should be cautioned, however, that these positive studies were from selected centers, retrospective and non-blinded. We know from the pain literature that when pain is the primary outcome measure, non-blinding is a significant shortcoming[26,27]. We also appreciate that the placebo effect is strong in patients with pain and are well documented after interventions including endoscopic therapies[28]. For example, there have been numerous interventional trials in many disciplines where uncontrolled studies suggested efficacy, but when randomized blinded sham procedure trials were performed, no differences were found[29].

Prior to EPISOD, there were two prospective randomized studies which evaluated the role of biliary manometry and outcome after sphincterotomy, but these were limited to patients with SOD Type Ⅱ (see Table 2).

PROSPECTIVE STUDIES IN TYPE Ⅱ SOD

Geenen *et al*[30] randomized 47 patients with post-cholecystectomy abdominal pain and meeting criteria for sphincter of Oddi Type Ⅱ to either biliary sphincterotomy or sham biliary sphincterotomy. Prior to sphincterotomy, biliary manometry was performed in all patients although the results of manometry were not used to decide on therapy. Sphincterotomy resulted in improvement in pain scores at the one year follow-up in 10 of 11 patients in whom elevated biliary sphincter pressures were found. Conversely, only 3 of 12 patients with elevated basal sphincter pressure undergoing a sham procedure had improvement. In patients with normal sphincter pressure, no difference in outcome was observed whether sphincterotomy was performed. Thus, overall 17 of 18 patients with SOD documented manometrically benefitted clinically from biliary sphincterotomy.

Toouli *et al*[31] performed biliary manometry in 81 patients with SOD Type Ⅱ. The manometric findings were categorized as either sphincter of Oddi stenosis (elevated basal sphincter pressure > 40 mmHg), dyskinesia or normal. Following manometry, in contrast to the Geenen study[30], patients were randomized based upon the manometric findings to either biliary sphincterotomy or sham. Patients were followed up to 24 mo by an independent observer and manometry was repeated to assess the effect of sphincterotomy. Of the cohort, 32% had evidence of sphincter hypertension. Of these, 85% improved after sphincterotomy as compared to 38% after sham which was statistically significant. In contrast, patients

in whom dyskinesia was diagnosed, approximately 50% in the sham group as compared to 36% in the sphincterotomy group had symptomatic improvement. For those with normal biliary manometry, 42% in the sham group compared to 61% in the sphincterotomy group had improvement but both of these were statistically not significant. Several observations can be made from this study. Firstly, overall, the number of patients with SOD in Type Ⅱ patients was relatively low. Secondly, it appeared that patients in whom SOD was confirmed had a better symptomatic improvement compared to sham, but this was not seen in those with normal manometry or with dyskinesia. Of note, there was a high response rate in those with a normal manometry approaching 50%.

Given the high rates of documented abnormal manometry, favorable clinical outcomes and the fact that manometry is not widely available, several groups have studied empiric biliary sphincterotomy and outcome[11,32]. Such a practice was also adopted in a number of community settings. Results similar to the larger studies have been reported. These studies suggest that perhaps manometry may not be needed given the high response rate and as shown in some studies the lack of correlation between manometric findings and outcome after endoscopic therapy.

EPISOD

With that as a background, the EPISOD trial was conducted[12]. From initial planning to execution took approximately a decade[33]. Patients enrolled were ages 18-65 years who had significant post-cholecystectomy biliary type pain without evidence of prior pancreatitis or prior intervention of the biliary and/or pancreatic sphincter. Patients with a bile duct larger than 9 mm or were on daily narcotics were excluded as were those with significant psychological comorbidity. A number of questionnaires were also administered evaluating the burden of pain as well as psychological parameters. Overall, 214 patients underwent ERCP with manometry of both the biliary and pancreatic sphincter. Patients were then randomized in a 2 to 1 fashion irrespective of the results of manometry to sphincterotomy or to sham. Those in whom sphincterotomy was planned and who also had elevated pancreatic sphincter pressures were also randomized again to either biliary sphincterotomy alone or dual sphincterotomy. All patients received temporary pancreatic stents including the sham patients. The primary outcome measure was defined as a reduction in their pain score at 9 and 12 mo using the recurrent abdominal pain intensity and disability (RAPID) scale, without any sphincter reintervention and also without any additional use of narcotics. The RAPID scale assesses recurrent abdominal pain intensity and its effect on disability. It is a 90 d summary evaluation of the number of days where various daily activities were reduced because of episodes of abdominal pain. The instrument itself is quite similar to one used for headache related disability from migraines. The RAPID score was validated by study in two distinct groups of patients totaling over 100 patients[34].

The results were remarkable. The success rate for the sham-treated patients was in fact higher than those receiving endoscopic therapy. Thirty-seven percent of the sham treated patients were reported as a success as compared to only 23% in those receiving biliary sphincterotomy. Overall, 30% of those who received dual sphincterotomy responded clinically as compared to 19% for those undergoing biliary sphincterotomy alone which was not statistically significant. Overall, reinterventions occurred in 26% of treated and 34% of the control patients. As in many other studies, there was no correlation with the results of sphincter manometry and outcome. Likewise, 31% receiving dual sphincterotomy improved compared to 27% for biliary and 17% for sham had no improvement; these findings were not statistically significant. A group of patients who declined the randomization were also observed following sphincterotomy directed by the findings at manometry and the final results were similar. The data was analyzed in a number of ways using less stringent criteria for outcome and the results were unchanged.

Even if manometry is imperfect but some patients respond to therapy, why not still perform manometry or empiric biliary sphincterotomy on Type Ⅲ patients? The primary reason to avoid ERCP and manometry is the risk of pancreatitis as these patients have the highest risk for pancreatitis[35]. In addition, even when performed in expert hands such as by the investigators in EPISOD, the rate of pancreatitis despite use of pancreatic stents was 12% and in this group, 2 patients had a perforation and surgery was required in one; there were no deaths.

While not perfect, the EPISOD trial is the best study we have regarding efficacy- or lack thereof- for interventions in type Ⅲ SOD. The study has been criticized for the use of a new scoring system termed the RAPID system which had not been used previously but does measure the burden of intermittent pain. Approximately 1/3 of the patients had irritable bowel syndrome which could be a confounder. However, we recognize that many patients with SOD have other GI complaints. Regardless, given the quality of the study with the caveats as noted, the results really call into question whether SOD Type Ⅲ is even a disease.

If not the sphincter, then what is the cause of pain? Even in patients who report a response, pain is often still present suggesting that other causes must be considered[36]. Significant psychological comorbidity has been identified in these patients and could be a major contributor to or cause of pain. Indeed, a number of studies have suggested psychosomatic disorders, central sensitization, and even potentially visceral hyperalgesia[37-40]. As is common in many patients with functional gastrointestinal disorders, prior

sexual abuse, or other abuse, has been found[41]. Such psychological comorbidity is important to appreciate given the potential role for medical therapy as has been shown for other functional GI disorders[42].

OTHER MECHANISMS OF PAIN

An important physiologic study performed a decade ago suggests potential mechanisms for right upper quadrant or "biliary type" pain in SOD type Ⅲ[40]. These investigators studied 11 patients with post-cholecystectomy abdominal pain as well as ten controls with balloon distention studies of both the duodenum and rectum to evaluate this visceral pain perception. Psychological testing was also performed. They found that in patients referred with SOD Type Ⅲ, duodenal but not rectal hyperalgesia was shown as compared to controls. Furthermore, duodenal distention reproduced symptoms in all but one of the patients. Psychological testing showed high levels of somatization, depression, obsessive compulsive behavior, as well as anxiety. These provocative findings thus suggest that patients with SOD Type Ⅲ may have a functional abdominal pain syndrome related to visceral hypersensitivity.

ROLE OF MEDICAL THERAPY

A variety of medical therapies have been tried some of which have shown efficacy in uncontrolled studies[32,43]. Smooth muscle relaxers such as nitrates and nifedipine have been used with moderate success. Antidepressant medications like amitriptyline have been used most commonly and should be titrated to effect. When using such medical therapies, response rates similar to sphincterotomy have been reported from retrospective uncontrolled studies[32,43]. A novel therapy includes injection of botulinum toxin into the sphincter[44]. This could perhaps result in sphincter relaxation and in one study such a clinical response predicted a response to sphincterotomy. Nevertheless, given the results of the EPISOD study, the findings of any uncontrolled non-blinded study should be questioned. Thus, given the results of EPISOD, medications should be given and we typically would use antispasmodics - (hyoscyamine) for those in whom the abdominal pain has a crampy component. In addition, we use low dose antidepressants such as amitriptyline especially for those with chronic almost constant pain. Although not discussed, the use of psychological counseling may be appropriate as well given the frequent psychiatric issues in patients with functional abdominal pain.

CONCLUSION

Based upon EPISOD, at this juncture, sphincter of Oddi Type Ⅲ likely does not exist as a true pancreaticobiliary disease and these patients should be categorized as having functional abdominal pain[45] rather than a true pancreaticobiliary disorder[4]. Also, given the findings of EPISOD, the current classification system for SOD requires a reevaluation. When faced with such patients, medication trials and reassurance would be important in the Type Ⅲ patient. ERCP should be avoided given the low yield[46] and high potential for pancreatitis[35,47]. In such patients, EUS also has a relatively low yield[48]. For those in whom abnormal liver tests reproducibly occur during pain or in whom bile duct dilation is present (Type Ⅱ SOD), empiric biliary sphincterotomy may be appropriate taking into account the risk of pancreatitis, and measures to prevent post-ERCP pancreatitis must be followed[35,49]. Such an empirical approach to the Type Ⅱ patient may be cost effective[50]. Patients with abnormal liver tests and a dilated bile duct (Type Ⅰ) should undergo biliary sphincterotomy and manometry is not needed. Further work is necessary to better define other mechanisms for pain, the ideal methods to identify psychological issues which may require specific treatment, and to identify novel therapies for abdominal pain syndromes.

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EDITORIAL

Changes in the esophageal mucosa of patients with non erosive reflux disease: How far have we gone?

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Abstract

The normal esophageal mucosa creates a protective epithelial barrier that constrains the acidic reflux in the esophageal lumen. Microscopic findings and functional studies indicate that this barrier might be impaired in patients with non erosive reflux disease (NERD) but not in patients with functional heartburn (FH). Whereas endoscopy and pH monitoring are the most important diagnostic tools in the diagnosis of NERD, recent studies suggest that esophageal biopsies might have a complementary role. Particularly in the differential diagnosis between NERD and FH, the application of histological severity scores showed very promising results. Further evaluation of the scores could lead to routine application of histology in specific NERD populations.

Key words: Esophageal mucosa; Non erosive reflux disease

Core tip: The normal esophageal mucosa creates a protective epithelial barrier that might be impaired in patients with non erosive reflux disease (NERD). Whereas endoscopy and pH monitoring are the most important diagnostic tools in the diagnosis of NERD, recent studies suggest that esophageal biopsies might have a complementary role. Particularly in the differential diagnosis between NERD and functional heartburn, the application of histological severity scores showed very promising results. Further evaluation of the scores could lead to routine application of histology in specific NERD populations.

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INTRODUCTION

An increased prevalence of gastroesophageal reflux

disease (GERD) has been observed in developed countries and symptoms suggestive of GERD (heartburn and/or regurgitation) are a common reason for consultation[1,2]. The spectrum of GERD includes erosive reflux disease (ERD) characterized by the presence of esophagitis and non-erosive reflux disease (NERD) characterized by the absence of endoscopically visible lesions and the presence of abnormal pH monitoring. Patients with heartburn, normal endoscopy and normal pH monitoring are classified, according to the Rome Ⅲ criteria as functional heartburn patients (FH)[3].

NERD patients represent up to 60% of all patients with reflux symptoms, but the mechanisms involved in the pathogenesis of NERD are complex and multifactorial[3]. The effects of gastric reflux on the esophageal mucosa of NERD patients are still incompletely understood. It is well known that the normal esophageal mucosa creates an effective barrier that constrains the acidic refluxate in the esophageal lumen[4]. Microscopic changes in the esophageal mucosa indicate that this barrier might be impaired in patients with NERD suggesting a possible role in the pathogenesis of the disease[5].

The role of histology in the diagnosis of NERD is very limited, keeping in mind that individual histological markers related to GERD have shown poor diagnostic value[6]. However, recent studies indicated that a histological score, based on a combination of histological parameters might be significantly associated with patients' symptoms and esophageal acid exposure and could thus contribute not only to the diagnosis of NERD but also to the differential diagnosis between patients with NERD and patients with FH[7,8].

NORMAL MUCOSA

Esophageal epithelium acts as a barrier that constrains the noxious acidic refluxate into the esophageal lumen and separates it from the esophageal nociceptors. It is a multilayer, non keratinized, stratified squamous epithelium and is consisted of three layers: closest to the lumen is the stratum corneum, underneath lies the stratum spinosum and finally towards the serosa lies the stratum basale or stratum germinativum[4].

Between the cells of the esophageal epithelium there are strong intercellular junctions that create an effective mucosal barrier and limit the paracellular ion diffusion; the tight junctions, the adherens junctions and the desmosomes[4]. Tight and adherent junction proteins encircle the cells and seal the barrier that separates the lumen from the intercellular space. Mainly claudin proteins and occludin contribute to the formation of tight junctions while the main protein in adherent junctions is E-cadherin. Desmosomes contribute to structural integrity of the mucosa by keeping the close apposition of the adjacent cells[4,5].

ESOPHAGEAL MUCOSA IN NERD

The most extensively studied finding in the esophageal epithelium of NERD patients is the presence of dilated intercellular spaces (DIS). It has been proposed as a mechanism of impaired mucosal integrity and increased acid perception[9]. Acid perfusion in the esophagus of healthy volunteers caused dilation of intercellular spaces in initially normal epithelium[10]. Increased mucosal permeability due to DIS could permit the acidic fluid reach the sensitive esophageal nociceptors that terminate in the intercellular space[11-13]. Moreover, an experimental study showed that not only acidic but weakly acidic solutions containing bile acids could also provoke increased DIS[10]. It has been found that in NERD patients the mean intercellular space diameter in distal esophagus is threefold higher compared with controls[9]. PPI treatment resolves symptoms and normalize DIS[14], whereas DIS were still increased in refractory heartburn patients despite double PPI dose[15]. In parallel with DIS, an upregulation of specific desmosomal and tight junction proteins has been shown. This change could represent a mucosal reaction towards recovery of the epithelial barrier[16,17].

Hyperplasia of the basal layer of the epithelium and elongation of the papillae that are more prevalent in the mucosa of NERD patients compared to healthy controls and functional heartburn patients, are other interesting findings[6]. It has been proposed that these findings represent a regenerative response to reflux induced mucosal damage[6]. Comparing these markers to DIS, DIS shows higher sensitivity and specificity for the diagnosis of NERD, although it is found present in up to 30% of asymptomatic healthy subjects[6,12]. Thus, the lack of specificity and sensitivity make these markers of limited use for the diagnosis of NERD.

The functional integrity of the esophagus has been assessed *in vitro* and *in vivo.* In vitro assessment is made with the use of the Ussing chamber technique which includes the placement of an esophageal mucosa specimen in an aperture that separates two solutions. The transepithelial resistance (TER) is then calculated. TER is indicative for the functional integrity of the mucosal barrier that separates the luminal from the basal side of the epithelium[5]. When esophageal biopsies were exposed to acidic solutions the impairment in integrity as measured by TER was greater in NERD patients compared to controls, indicating a defective mucosal barrier[18]. *In vivo* functional integrity of the esophagus has been evaluated with the application of multichannel esophageal impedance catheter[19]. It has been shown that NERD patients had lower baseline esophageal impedance compared to FH patients and controls, thus supporting the hypothesis of increased mucosal permeability to ions and therefore increased sensitivity to acid[20,21] . These findings suggest that an easy

Table 1 Histological criteria for the assessment of microscopic lesions described by Yerian *et al*[28]

Criterion	Definition and method of assessment (magnification)	Severity score
Basal cell hyperplasia	Measure basal cell layer in μm and express as a proportion of total epithelial thickness (× 10)	0 (absent < 15%), 1 (15%-30%), 2 (> 30%)
Papillary elongation	Measure papillary length in μm and express as a proportion (%) of total epithelial thickness (× 10)	0 (absent < 50%), 1 (50%-75%), 2 (> 75%)
Dilated intercellular spaces	Include irregular round dilations and diffuse widening of the intercellular space (× 40) Small intercellular space= diameter < 1 lymphocyte Large intercellular spaces = diameter ⩾ 1 lymphocyte	0 (⩽ 5 small), 1 (⩾ 6 small and ⩽ 5 large) 2 (⩾ 6 large)
Intraepithelial eosinophils	Count cells in the most affected power field (× 40)	0 (0 cells in one high power field)
Intraepithelial neutrophils		1 (1-2 cells), 2 (> 2 cells)
Intraepithelial mononuclear cells	Count cells in the most affected power field (× 40)	0 (0-9 cells) 1 (10-30 cells), 2 (> 30 cells)
Erosions	Assess as presence of at least one of the following: necrosis, granulation tissue or fibrin with neutrophils (× 10)	0 (absent), 1 (present)
Healed erosions	Assess as presence of granulation tissue covered by thinned regenerative epithelium (× 10) in the absence of necrosis, fibrin, and neutrophils	0 (absent), 1 (present)
Combined severity score	Sum of lesion severity scores divided by the number of lesions assessed (excludes intraepithelial monuclear cells and neutrophils, and erosions/healed erosions) 0-0.25 normal mucosa, 0.5-0.75 mild esophagitis ⩾ 1 severe esophagitis	

Biopsies were taken from the Z-line and at 2 cm above it.

software aided assessment of baseline impedance could add diagnostic information in the routine application of pH-impedance measurements.

Finally, an immune mediated mechanism has also been investigated in the pathogenesis of NERD. It has been suggested that reflux might stimulate proinflammatory cytokine production (*e.g.*, interleukin 8) by the esophageal epithelium that mediates damage of the esophageal tissue[22]. IL-8 and IL-1β have been found upregulated in the esophageal mucosa of NERD patients when compared to controls[23,24]. Treatment with lansoprazole reduced the mucosal levels of both mRNA and protein IL-8 levels[25]. Additionally, upregulation of proteinase-activated receptor-2 (PAR-2) which has been demonstrated to induce proinflammatory and neuroinflammatory effects has also been found in NERD patients compared to controls[26] . In esophageal biopsies infiltration of the mucosa with inflammatory cells is more prevalent in NERD compared to FH patients and controls[6-8].

APPLICATION OF HISTOLOGICAL SCORES

The poor diagnostic value of individual histological markers has led to the application of histological scores in the diagnosis of NERD. These scores take into account a combination of histological parameters associated with extensive acid reflux and have opened new hopeful perspectives on the role of esophageal biopsies.

Recently a large international group of pathologists reached a consensus regarding the microscopic lesions in esophageal biopsies of patients with GERD that could provide the histological diagnosis of microscopic esophagitis. Individual lesions were assessed: basal cell hyperplasia, papillary elongation, DIS, intraepithelial eosinophils, neutrophils and mononuclear cells. After that, a combined histological severity score was obtained by summing up lesion scores for each of the above parameters (Table 1)[27,28]. Evaluation of the score showed good correlation with patients' reflux symptoms as well as good interobserver agreement[29].

Savarino *et al*[7] used light microscopy and applied the histological score in esophageal biopsies of pHmetry defined NERD and FH patients as well as in healthy controls (Table 2). Application of the score was able to differentiate patients with NERD from those with FH with an accuracy of 79%, a sensitivity of 74% and a specificity of 86%, whereas no difference was found in the prevalence of microscopic esophagitis between FH patients and healthy controls. Furthermore, in GERD patients refractory to PPIs application of a similar histological score was able to discriminate NERD and FH patients with sensitivity 0.85, specificity 0.64, positive predictive value 0.71 and negative predictive value 0.8 (Table 3). Overall patients with NERD were differentiated from patients with FH with high statistical significance ($P < 0.001$)[8].

Biopsy sampling and application of histological scores is a relatively safe and inexpensive procedure in a disease with a massive financial impact[30]. However, limitations for the use of histological scores do exist mainly regarding the position where the biopsies should be taken. It has been shown that the distribution of the microscopic findings is patchy and

Table 2 Histological score applied by Savarino *et al*[7]

Criterion	Definition and method of assessment (magnification)	Severity score
Basal cell hyperplasia	Measure basal cell layer in μm and express as a proportion of total epithelial thickness (× 10)	0 (absent < 15%), 1 (15%-30%), 2 (> 30%). Z line 1 (> 20%)
Papillary elongation	Measure papillary length in μm and express as a proportion (%) of total epithelial thickness	0 (absent < 50%), 1 (50%-75%), 2 (> 75%) Z line 1 (> 66%)
Dilated intercellular spaces	Include irregular round dilations or diffuse widening of the intercellular space (× 40) Small intercellular space= diameter < 1 lymphocyte Large intercellular spaces= diameter ⩾ 1 lymphocyte	0 (⩽ 5 small), 1 (⩾ 6 small and ⩽ 5 large) 2 (⩾ 6 large)
Intraepithelial eosinophils	Count cells in the most affected power field (× 40)	0 (0 cells in one high power field) 1 (1 cell), 2 (> 1 cells)
Intraepithelial neutrophils	Count cells in the most affected power field (× 40)	0 (absent), 2 (present)
Erosions/ necrosis	Assess as presence of at least one of the following: necrosis, granulation tissue or fibrin within neutrophils (× 10)	0 (absent), 2 (present)
Combined severity score	Sum of lesion severity scores divided by the number of lesions assessed. Erosions/necrosis are not counted for the global score Positive for microscopic esophagitis when the value was ⩾ 0.35	

Biopsies were taken from the squamous epithelium side of the squamocolumnar junction and at 2 cm above it.

Table 3 Histological score applied by Kandulski *et al*[8]

Type of Lesion	No changes	Mild changes	Moderate changes	Severe changes
Basal cell hyperplasia	0	1	2	3
Papillary elongation	0	1	2	3
Dilated intercellular spaces	0	1	2	3
Inflammation	0	1	2	3
Sum score	A cut-off value > 5 points was applied for discrimination between NERD and FH			

Biopsies were taken 3-5 cm above the gastro-oesophageal junction.

varies significantly according to the distance from the squamocolumnar junction and to the position of the biopsy. Mucosal changes occur more frequently closer to Z line and in the 3 o'clock quadrant[31]. Therefore a common biopsy protocol is necessary. Furthermore, the precise assessment of GERD related microscopic lesions severity which is necessary for the scoring could be troublesome or subjective although these lesions are often easily recognized. Hence adjustment to a consensus with strict detailed criteria is necessary[28].

Outcome studies in NERD patients investigating a possible association between histological score and the response to pharmacological therapies or to fundoplication would be noteworthy. A strong association could enhance the role of biopsies before therapeutic decisions for these patients. A multicenter study that included both patients with NERD and erosive esophagitis showed that baseline histological score could not be a predictor of treatment failure either for esomeprazole or fundoplication. However a subgroup analysis only for NERD patients has not been performed. An interesting result of this study suggesting a possible role of histology in the long term follow up of GERD patients is the significantly lower score found in patients with treatment induced remission compared to treatment failures[32].

Another application of histological score could be the evaluation of the natural history of NERD patients especially these with a high severity score. It has been hypothesized that chronic inflammation and continued epithelial injury could have important role in the pathogenesis of Barrett esophagus[33,34], thus a long term study including a second upper endoscopy of patients with a high severity score could estimate a possible higher incidence of Barrett esophagus among these patients.

Furthermore, histological findings could be of value in the differential diagnosis between NERD and FH especially in specific subgroups: NERD patients with borderline findings in 24 h pH metry, patients reluctant or unable to undergo 24 h pH monitoring, patients with suspicion that catheter intolerance has significantly influenced the diagnostic value of the test.

CONCLUSION

The normal esophageal mucosa creates a protective epithelial barrier that constrains the acidic reflux in the esophageal lumen. Microscopic findings and functional studies indicate that this barrier might be impaired in patients with NERD but not in patients with FH. Whereas endoscopy and pH monitoring are the most important diagnostic tools in the diagnosis of NERD, recent studies suggest that esophageal biopsies might have a complementary role. Particularly in the differential diagnosis between NERD and FH, the application of histological severity scores showed very promising results. Further evaluation of the scores could lead to routine application of histology in specific

NERD populations.

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EDITORIAL

Hepatitis C: New challenges in liver transplantation

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Abstract

In an era of great achievements in liver transplantation, hepatitis C viral infection (HCV) remains an unsolved problem. As a leading indication for liver transplantation in Western countries, HCV poses a significant burden both before and after transplantation. Post-transplant disease recurrence occurs in nearly all patients with detectable pretransplant viremia, compromising the lifesaving significance of transplantation. Many factors involving the donor, recipient and virus have been evaluated throughout the literature, although few have been fully elucidated and implemented in actual clinical practice. Antiviral therapy has been recognized as a cornerstone of HCV infection control; however, experience and success are diminished following transplantation in a challenging cohort of patients with liver cirrhosis. Current therapeutic protocols surpass those used previously, both in sustained viral response and side-effect profile. In this article we review the most relevant and contemporary scientific evidence regarding hepatitis C infection and liver transplantation, with special attention dedicated to novel, more efficient and safer antiviral regimens.

Key words: Hepatitis C; Liver transplantation; Treatment protocols; Pegylated interferon; Ribavirin; Direct acting antivirals

Core tip: Extensive and revolutionary new data are currently emerging in the field of hepatitis C viral (HCV) treatment. Knowledge is changing faster than ever, although the treatment of HCV infection remains the most challenging problem in transplantation. In this article we report new insights into the actual knowledge of treatment opportunities in the pre- and post-transplant periods.

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GLOBAL BURDEN OF HCV RECURRENCE

As the leading indication for liver transplantation (LT) in Western Europe and the United States, hepatitis C

viral (HCV) infection has captured the attention of both basic scientists and clinicians throughout the years[1]. In comparison to non-HCV transplant recipients, those with an HCV infection have higher death and allograft failure rates, mainly due to disease recurrence[2]. The deleterious recurrence of HCV infection universally occurs in patients with detectable viremia at the time of transplantation, leading to cirrhosis in up to 30% of patients within 5 years after LT[3,4]. Among those patients, approximately 50% experience decompensation within 1 year of follow-up, which is extremely high compared to non-transplant HCV patients[1].

A variety of factors influencing disease recurrence and graft fibrosis progression have been evaluated, with only a few reaching high enough significance to be at least partially implemented in routine clinical practice[1,5,6]. Using antiviral therapy to successfully prevent HCV recurrence and treat established graft infections has been recognized to improve patient and allograft survival[2,7].

Until 2011 and the arrival of direct acting antivirals (DAAs), boceprevir (BOC) and telaprevir (TVR), a combination of pegylated interferon (pegIFN) and ribavirin (RBV), was the basis of HCV therapy[8]. Although the SVR rates have improved with the new regimens, unsatisfactorily high rates of adverse events and serious drug-drug interactions have diminished clinicians' enthusiasm[9,10]. With the emergence of new DAA drugs, promising results have been obtained in the field of HCV infection therapy[11]. Although there are few studies on HCV liver waiting list and post-transplant patients, the results show improved rates of virus eradication along with acceptable side-effect profiles and negligible drug-drug interactions[12,13].

PATHWAY FOR DISEASE RECURRENCE

HCV RNA remains detectable in almost all patients after liver transplantation, with pretransplant levels being reached as early as a few days postoperatively[3]. In contrast to the natural course of HCV infection, disease progression is accelerated in post-transplant patients[1]. Of those with disease recurrence, 10%-30% develop cirrhosis within 5 years and have diminished survival rates of 41% and 10% at 1 and 3 years, respectively[2,14]. The most detrimental pattern of disease recurrence is fibrosing cholestatic hepatitis (FCH), occurring in 7%-15% of recipients and leading to early graft failure, decompensation and death[1,15]. As disease recurrence involves the majority of HCV liver recipients, the impact of various factors influencing the rate and severity of disease progression has been widely evaluated. Several potential factors concerning the donor, the recipient, and the hepatitis C virus have been proposed and linked to reinfection, although few have achieved universal consensus throughout the literature[14].

Advanced donor age has been shown to negatively influence graft and patient survival in many studies, with even relatively young donors (< 50 years of age) experiencing a substantial risk[5,16-18]. There has been much debate in the literature regarding the impact of donor type on disease recurrence and overall survival. Those in favor of living donor liver transplantation (LDLT) mention the overall younger age of donors, better organ quality and shorter cold ischemia time as factors with a positive impact, whereas those favoring deceased donor liver transplantation (DDLT) hypothesize that intense hepatocyte proliferation after LT and optimized donor-recipient HLA matching may negatively impact disease recurrence[19-22]. Recently, two large studies showed that there is no difference in patient or graft survival or HCV recurrence with regard to donor type[21,22]. Concerning pretransplant recipient variables influencing the post-transplant course, those found to have negative impacts include female gender, advanced recipient age and liver disease severity prior to LT[1,2,5].

The interplay between the recipient's suppressed immune response and the resulting "undisturbed" viral replication is the principal difference between post-transplant patients and those with native liver disease. An evaluation of the benefits of the two main calcineurin inhibitors (CNIs) used in liver recipients, cyclosporine (CsA) and tacrolimus (Tac), gave no clear recommendations in terms of the preferential use of one over another[15,17,23,24]. Although the antiviral and antiapoptotic properties of CsA have been demonstrated to inhibit liver fibrosis and decrease disease severity, recent studies have counterbalanced those findings and necessitated further investigations[1,24]. With respect to the effects of immunosuppression on disease recurrence, multiple studies have shown negative impacts of corticosteroid boluses used in the treatment of acute cellular rejection episodes on hepatitis C viremia and graft fibrosis progression[23,25].

Along with the well-proven effect of the interleukin 28B (IL-28B) polymorphism on antiviral therapy success, studies have evaluated its parallel impact on fibrosis progression and patient and graft survival. The impact of both donor and recipient IL-28B genotype on post-transplant outcome was discussed in a study by Charlton *et al*[26] suggesting that the IL-28B TT genotype in the recipient was associated with more severe disease recurrence. An appealing concept of IL-28B genotype donor and recipient matching was consequently investigated, but until now it has not reached practical implementation[27,28].

HCV genotype 1 has been shown to adversely affect post-transplant outcome in multiple studies, and advanced donor age has been proven to have the most negative impact on disease recurrence severity[6,17,29]. Studies evaluating the pre- and post-transplant viral load lack general conclusions, and despite undetectable viremia at the time of LT, 55% of patients develop HCV recurrence[15,16,23,29,30]. It is possible that HCV exists in the liver or peripheral mononuclear cells,

and therefore, even in cases of undetectable serum HCV RNA, recurrence can occur. In a study by Vasuri *et al*[31] patients with high serum and tissue HCV RNA levels were shown to have more severe and earlier disease recurrence, with significantly lower survival rates. The practice of obtaining protocol biopsies 1 year after transplantation has been established in many transplant centers, and it has been demonstrated that greater necroinflammatory activity and the presence of fibrosis are risk factors for the development of graft cirrhosis[18,29,32-34]. In addition to histological analysis of the liver graft, a study by Ghabril *et al*[16] evaluated the explanted liver inflammatory grade. It was found that greater inflammatory activity, mainly periportal and portal hepatitis, strongly correlates with post-transplant fibrosis progression.

APPROACHES IN BATTLING DISEASE RECURRENCE

Along with the impact of the previously mentioned factors on disease recurrence and overall patient and graft survival, antiviral therapy (AVT) success rates appear to be one of the most important factors[7,29,30,35-39]. Although more complicated and harder to achieve in patients with liver cirrhosis or after LT, SVR was proven in several studies to slow graft fibrosis progression with an impact on the overall disease course[7,35]. More importantly, in addition to slowing down the rate of disease progression, SVR could potentially contribute to clinical remission and prolongation or even the avoidance of the need for LT[37]. In a randomized controlled trial (RCT) by Carrión *et al*[36] that evaluated the impact of AVT on liver fibrosis progression, SVR was the only variable independently associated with fibrosis regression/stabilization.

The main reason for the generally lower SVR rates in patients with liver cirrhosis and post-LT patients is poor AVT tolerability with substantially higher rates of serious adverse events (SAEs), leading to dose reductions and therapy discontinuation. Accordingly, patients with higher grades of liver cirrhosis [Child turcotte pugh (CTP) class B or C] experience the lowest SVR rates with frequent complications[38-40]. Multiple studies have confirmed that patients with more severe liver disease obtain lower SVR rates, with many of the studies noting that HCV genotype 1 is an additional negative contributor[1,30,37-40].

An issue specific to post-transplant patients is the effect of immunosuppression, possibly "blunting" the response to standard interferon based therapy[14]. In a recent meta-analysis, Rabie *et al*[41] found slightly higher SVR rates with the use of CsA compared to Tac, yet the heterogeneity of the studies and the need for larger well-established trials limited their ability to draw clear conclusions. Another predictor of SVR that was recently intensively evaluated was the IL-28B polymorphism, both donor and recipient genotypes of which were shown to affect the AVT success rate[26,42].

Attempts to minimize disease recurrence with pretransplant AVT first utilized the pegIFN and RBV combination, which was the main HCV therapeutic option until very recently. The frequent presence of pancytopenia and other manifestations of liver disease were the main obstacles to even initiating therapy in some patients[1,14,37,40]. The concept of a low accelerating dose regimen (LADR) was presented by Everson *et al*[37]. They treated 124 patients with a mean CTP score of 7.4 ± 2.3 with interferon alfa-2b or peginterferon alfa-2B plus RBV, achieving an end of treatment (ETR) response of 46% and SVR of 24%. Importantly, they found that 80% of patients who were HCV RNA negative at the time of LT lacked post-transplant recurrence, whereas those who were HCV RNA positive at the time of LT experienced universal infection recurrence. Overall disease recurrence was avoided in 26% of patients. Side effects, mainly cytopenias and complications of advanced liver disease, were commonly encountered, thus highlighting the need for caution and close supervision of the treated population.

In the first RCT of pretransplant treatment of HCV infection using pegIFN and RBV to prevent disease recurrence after LT, post-transplant clearance of HCV was achieved in 25% of patients, similar to the results found in previous studies[38]. The relapse rate of 50% was higher than that observed in previous studies, accentuating the need for therapy of adequate duration because those who received fewer than 8 wk of treatment universally relapsed. In contrast, the early virologic response (EVR) (undetectable serum HCV RNA or a 2 $\log_{10}$ or greater drop in HCV RNA at week 12 of therapy) was able to predict the likelihood of recurrence prevention. Although SAEs occurred with similar frequencies in the treated *vs* untreated groups (68% *vs* 55%, $P = 0.003$), the numbers of SAEs and infections were higher in the treated population, once again showing the detrimental effect of this therapeutic regimen in patients with advanced liver disease.

In 2011, with the arrival of new DAAs, the protease inhibitors boceprevir and telaprevir, optimism emerged regarding the treatment of patients with liver cirrhosis and those on liver transplant waiting lists. Despite the considerably higher SVR rates when combined with pegIFN-RBV in patients with genotype 1 and cirrhosis, further studies halted the wave of enthusiasm[1,8,10,43,44]. A large study including a cohort of patients with compensated cirrhosis and evaluating the safety profiles of DAAs showed an SAE rate of 40% and a 6.4% rate of death and severe complications, with a platelet count $\leqslant 100000/mm^3$ and serum albumin concentration < 35 g/L as indicators for high risk patients[45]. Currently, the general opinion is that triple therapy should be used only in patients with compensated cirrhosis and in well-experienced transplant centers[8,43,44].

A promising SVR rate of 69.6% in genotype 1b patients with advanced liver disease treated with TVR

Table 1 Data on new therapeutic protocol efficacy and safety in post-liver transplantation period

	Charlton *et al*[13]	Forns *et al*[68]	Pellicelli *et al*[69]
Patients (*n*)	40	104	12
Regimen	SOF + RBV	SOF + RBV + pegIFN	SOF + DCV
Patients with cirrhosis	40%	50%	75%
End of treatment response	100%	87%	100%
Sustained viral response 12	70%	62%	NA
Sustained viral response 24	70%	NA	NA
Serious adverse events	15%	33%	30%
Deaths	0	12.5%	25%

SOF: Sofosbuvir; RBV: Ribavirin; pegIFN: Pegylated interferon; DCV: Daclatasvir; NA: Not available.

was presented by Ogawa *et al*[46]. Indicators for the likelihood of achieving SVR included prior response to therapy, rapid viral response (RVR) (defined as undetectable HCV viral load at 4 wk of therapy) and favorable IL-28B genotype, as SVR was obtained in only 12.5% of patients with a prior null-response and the IL-28B TC/CC genotype. Almost all of the patients required RBV dose reductions due to anemia, which was the main adverse effect in addition to leuko/thrombocytopenia and dermatological disorders, leading to therapy discontinuation in 12.7% of cases.

In a multicenter study of 160 patients with liver cirrhosis treated with BOC and TVR, Saxena *et al*[9] analyzed the overall efficacy and SAE rate with regard to disease severity. SVR12 was achieved in 35% of patients with Child-Pugh (CP) $\geqslant$ 6, compared to 54% of those with CP = 5 (P = 0.02), with RVR and genotype 1b identified as predictors for SVR. An encouraging rate of 67% post-transplant SVR was achieved, mostly (80%) in patients who were HCV RNA negative for at least 5.5 wk prior to LT. SAEs subsequently leading to IFN dose reduction, growth factor use and transfusions were more frequent in the CP $\geqslant$ 6 group, thus requiring treatment discontinuation in 42% of patients (Table 1).

Recent results from the CUPIC study group of 511 patients with compensated cirrhosis revealed relatively high SVR rates of 74.2%, 40% and 19.4% in patients with a relapse, partial response and null response, respectively[47]. However, the high number of SAEs (49.4%), infections (10.4%) and deaths (2.2%) once again demonstrated the need for caution, even with the possibility of attaining positive SVR rates.

In addition to treating patients in the pretransplant period, two post-transplant strategies have evolved for preventing HCV disease recurrence[1,8,14,43,44]. Although there is the possibility of treating liver recipients in this phase with lower HCV RNA levels and in the absence of significant graft injury, the preemptive/prophylactic regimen has not yet achieved clinical implementation[8,43,44,48,49]. One of the reasons accounting for the lack of wider use of early post-transplant therapy is low patient eligibility, mainly due to cytopenias, renal impairment and severe debilitation. Even when treatment initiation is possible, patients in this vulnerable period experience frequent SAEs, leading to dose reductions, discontinuation and unsatisfying SVR rates[48,49].

The results obtained from the PHOENIX study verified the lack of benefit from prophylactic treatment[49]. With only 65% of patients able to complete therapy, SVR was achieved in 22.2% in the prophylactic group and in 21.4% of patients in the observation group, where treatment was started upon significant HCV recurrence (histological activity index $\geqslant$ 3 and/or fibrosis score $\geqslant$ 2). The results showed no clear benefit regarding HCV recurrence or patient or graft survival, thus lending no support to that strategy, at least until enough experience has been gained with these new regimens.

The first attempts to treat recurrent HCV infection after liver transplantation were made using a standard combination of interferon/pegylated interferon and ribavirin, with SVR rates reaching up to 40%[36,50-52]. A significant number of patients were not able to sustain full doses of the antivirals, and adverse events, mainly cytopenias, occurred frequently[14,36,50-52]. In a study by Angelico *et al*[50] in which 35% of patients required IFN dose reductions and only a minority tolerated full doses of RBV, significant anemia occurred in almost all of the patients. The importance of careful patient selection for both the AVT success rate and the minimization of adverse events was accentuated in a study by Carrión *et al*[36]. They grouped 81 patients into categories according to the liver fibrosis stage, showing that patients with severe recurrence (fibrosis stage 3-4, FCH) responded much worse (SVR 18.5%) compared to patients with mild recurrence (SVR 48%). In that study, AVT was shown to be the only independent variable associated with fibrosis improvement/stabilization (OR = 3.7, P = 0.009). A comprehensive multicenter study by Gordon *et al*[52] once again highlighted the importance of sustaining the full dose and duration of treatment. Of 125 patients treated with pegIFN-alfa-2b and RBV, only 58.4% completed 48 wk of therapy, achieving 55% SVR. The overall SVR rate was 28.8% and was significantly higher in patients with genotype 2/3 (55%) than with genotype 1 (23.8%) and in those who achieved RVR (83.3% *vs* 25.7%, P = 0.0098). Despite attaining a relatively high SVR in those who were able to complete the full treatment duration, adverse events occurred in almost all of the patients, with 65% of patients requiring either dose reduction or discontinuation.

To improve the relatively low SVR rates in genotype 1 patients with HCV recurrence, the protease inhibitors BOC and TVR were added to the standard dual therapy regimen[1,8,10,14,43,44]. A remarkable SVR rate increase (from 45% to 75%) was obtained, although a high

incidence of SAEs and significant drug-drug interactions necessitated careful patient selection and precise treatment supervision[43,44].

In a retrospective study of a cohort of patients treated with TVR, Werner *et al*[53] reported SVR24 in 5 of 9 patients treated, although the overall benefit diminished, with two-thirds of patients experiencing severe anemia requiring transfusions and growth factor administration.

A study of 60 patients treated with BOC and TVR published by Pungpapong *et al*[54] showed undetectable HCV RNA at week 24 of therapy in 67% of patients treated with TVR and 45% treated with BOC. Limited treatment efficacy was found in patients with HCV genotype 1a and IL-28B polymorphism CT or TT, but interestingly, no correlation existed between the on-treatment virological response and either the fibrosis stage or baseline HCV level. A major concern during the treatment was the universal need for dose reductions of pegIFN and RBV and the administration of hematologic growth factors and transfusions in more than half of the patients. The incidence of acute cellular rejection (5%) was similar to the rates in the published studies of dual antiviral therapy[55]. Frequent drug-drug interactions between BOC, TVR and CNIs were demonstrated, necessitating immunosuppressive dose reductions. With both treatments being substrates and inhibitors of CYP3A4/5 and the efflux pump P-glycoprotein, pharmacokinetic studies showed a 70-fold and 4.6-fold increase in the exposure to Tac and CsA, respectively, when they were administered with TVR, and a 17-fold and 2.7-fold increase in Tac and CsA exposure when administered with BOC[56,57].

A recent multicenter study by Coilly *et al*[58] presented ETR rates of 72% and 40% for patients treated with BOC and TVR, respectively, with an impressive ETR of 33% in patients with FCH. Although limited by the low number of enrolled patients (n = 37), EVR was shown to be the principal factor in achieving SVR. With EVR rates of 89% and 58% in patients treated with BOC and TVR, respectively, SVR12 was obtained in 71% of BOC- and 20% of TVR-treated patients.

Even more encouraging results were presented by Burton *et al*[59] demonstrating an SVR rate of 63% in patients treated with BOC plus TVR, proving EVR to be highly predictive of SVR. One-fifth of patients experienced a decline in hemoglobin to < 8 g/dL, with erythropoietin and packed red blood cells used in 81% and 57% of patients, respectively. Overall, 27% of patients required hospitalization, with death occurring in 9% of cases. Along with significant and potentially dangerous interactions with CNIs, adverse events were the main factor compromising the achievement of relatively high SVR rates, adding to the non-establishment of triple therapy in post-transplant disease recurrence.

NEW EFFICACIOUS AND SAFE THERAPEUTIC REGIMENS

With the approval of the NS5B nucleotide polymerase inhibitor sofosbuvir (SOF) in 2013, a brighter perspective finally appeared for HCV infected patients, especially for liver cirrhosis and post-LT patients[11]. Owing to its high efficacy, pangenotypic activity, high barrier to genetic resistance, rare drug-drug interactions and acceptable side-effect profile, sofosbuvir rapidly emerged as a savior in the treatment of patients with advanced liver disease[60-62].

In an open-label phase 2 study by Curry *et al*[12] the combination of SOF and RBV was assessed in preventing HCV recurrence after LT. They enrolled 61 patients with HCV of any genotype and cirrhosis on the LT waiting list due to hepatocellular carcinoma. SOF and RBV were administered for 48 wk, with 43 patients achieving undetectable HCV RNA at the time of LT. Of those patients, 70% achieved pTVR12 (defined as undetectable HCV RNA at 12 wk post-transplant in patients who had undetectable HCV RNA at their last assessment prior to LT), which led to an overall pTVR12 of 49%. It was demonstrated that the removal of the infected liver with the achievement of undetectable HCV RNA led to a low risk of recurrence, thus diminishing the significance of extrahepatic viral reservoirs. Nevertheless, a 23% rate of recurrence raised questions about the adequate duration of viral suppression prior to LT and the possibility of extending treatment to the post-transplant period[63,64]. Proving the safe side-effect profile of SOF, the adverse events most frequently encountered were fatigue (38%), headache (23%) and anemia (21%), and the discontinuation rate was low.

Jacobson *et al*[61] presented the results of 2 RCTs in which they evaluated the efficacy of SOF and RBV in the treatment of patients with HCV infections of genotypes 2 and 3. In the POSITRON trial, a blinded placebo-controlled study in patients (n = 207) for whom IFN antiviral regimen was not an option, an SVR12 of 78% was obtained after 12 wk of therapy. The second study, a blinded active-control FUSION trial of previously treated patients (n = 201), showed an SVR12 rate of 50% with 12 wk of therapy and 73% with 16 wk of therapy (P < 0.001). Both studies revealed lower SVR rates for genotype 3 patients and those with cirrhosis, with additional benefits achieved after treatment prolongation. Adverse events associated with RBV therapy (fatigue, insomnia, anemia) appeared more frequently in the group that received SOF and RBV, whereas other common adverse events occurred similarly in the treatment and placebo groups. There was no difference in the frequency of adverse events with regard to treatment duration or the presence of liver cirrhosis.

In contrast to IFN-free sofosbuvir regimens, Lawitz

et al[65] published the results of pegIFN-RBV plus SOF therapy in patients with genotype 2 or 3 HCV infections and liver cirrhosis. With an encouraging SVR12 rate of 89%, that combination appeared to be an effective option for treatment-experienced patients with liver cirrhosis who were able to receive IFN. Again, better SVR rates were obtained in genotype 2 than in genotype 3 patients (96% and 83%, respectively), with no significant difference in patients with *vs* without cirrhosis.

The combination of a second-wave NS3/4A protease inhibitor, simeprevir (SMV), plus SOF was evaluated in the COSMOS randomized trial[62]. A total of 167 patients were grouped according to their previous therapy experience and liver disease severity, and they were administered 150 mg of SMV and 400 mg of SOF once daily for 24 wk with or without RBV. A promising AVT success rate with an acceptable adverse event frequency was achieved because the SVR12 was 90% in patients with no or mild fibrosis (F0-2) and 94% in those with advanced fibrosis and cirrhosis.

Another two new DAAs, the NS5A replication complex inhibitor daclatasvir (DCV) and the NS3 protease inhibitor asunaprevir, were assessed in an all-oral therapy HCV genotype 1b study (HALLMARK-DUAL). Including patients with cirrhosis, the combination of 60 mg of daily DCV and 100 mg of twice daily asunaprevir for 12 wk produced an SVR12 rate in 82%-90% of patients, according to previous treatment experience and tolerability. Adverse events occurred in up to 7% of patients, leading to a negligible discontinuation rate, thus proving this IFN-free therapeutic regimen to be safe and effective in a difficult-to-cure patient population[66].

Data regarding the treatment of HCV recurrence after liver transplantation with new DAAs were scarce until Charlton *et al*[13] published a study of SOF and RBV treatment for patients with compensated infection recurrence. That prospective multicenter study enrolled 40 patients; 83% of the patients had a genotype 1 infection and 40% of patients had liver cirrhosis. On an intention-to-treat basis, after 24 wk of SOF and RBV therapy, SVR12 was achieved in 70% of patients, with undetectable HCV RNA observed in 97-100% patients at week 4 of treatment. Fatigue, diarrhea or headache occurred in approximately one-third of patients, and despite a slow dose escalation protocol, anemia precluded full ribavirin dosing in the majority of patients. No death, graft loss or rejection episodes occurred in the studied population. In addition to the safe administration of SOF, its exposure was only minimally altered by CNIs, and no net directional change in the trough levels of CsA or Tac were observed. Despite these findings, vigilant monitoring of the CNI concentration during and after treatment is recommended[13,67]. Possible limitations on the general acceptance of the highly effective and safe administration of SOF in patients with HCV recurrence may relate to the fact that, as in most pretransplant series, the studied population consisted of patients with well-compensated liver disease. In a compassionate use program providing SOF for patients with a severe recurrent HCV infection and FCH, Forns *et al*[68] evaluated 104 patients in which half of the patients had compensated or decompensated cirrhosis and the other half had FCH and early disease recurrence. An overall rate of treatment discontinuation of 30% and a high occurrence of death (12.5%) diminished the significance of the relatively high SVR12 rate of 62%.

Simeprevir and daclatasvir have also been evaluated in post-transplant HCV recurrence treatment, although until now only case reports and small patient series have been published. Pellicelli *et al*[69] treated 12 patients with severe HCV recurrence (n = 9) and FCH (n = 3) with a combination of SOF and DCV, with or without ribavirin. ETR was achieved in all 9 patients who completed the treatment, and undetectable HCV RNA proof was available for 5 patients at week 8 (n = 2) or week 4 after treatment (n = 3). Confirming the lack of significant drug-drug interactions, no adjustment of immunosuppressive drug dosage was necessary during the treatment. With 4 patients experiencing SAEs and 3 who died (25%), the authors strongly recommend that treatment be started at an early stage of HCV recurrence, thus avoiding the frequent complications of advanced liver disease.

In two case reports by Fontana *et al*[70,71] SVR was achieved in patients with FCH with either SOF plus DCV or pegIFN-RBV plus DCV therapy. Favorable safety profiles of SOF plus DCV were observed, along with negligible interactions with CNIs.

Successful DCV-based treatment of a patient with BOC triple therapy failure after liver transplantation was described by Reddy *et al*[72]. Although he responded to triple therapy, the patient remained HCV RNA positive, experienced serious adverse events and required immunosuppression dosage adjustment. After 2 wk of DCV and pegIFN/RBV therapy, he became HCV RNA negative and remained so for 12 wk after therapy completion.

Concerning experience with simeprevir use in post-transplant HCV recurrence, Campos-Varela *et al*[73] presented two HIV-HCV co-infected patients with dual (pegIFN-RBV) plus BOC based triple therapy failure after transplantation, respectively. SOF and SMV plus RBV therapy produced SVR12 in both patients, and the treatment was well-tolerated and no adjustment of immunosuppression was needed. More importantly, as demonstrated by CTP and the model for end stage liver disease (MELD) scores, the overall condition of the patients improved.

In a pilot study by Tanaka *et al*[74] 5 patients underwent 12 wk of SMV, pegIFN and RBV therapy as part of a preemptive dual therapy course. All of the patients completed the course without significant adverse events and with minimal CNI dose modifications. RVR was observed in 3 out of 5 patients, creating a positive basis for future larger

studies establishing simeprevir for post-transplant HCV recurrence therapy.

Compared to IFN-based regimens, except for their greater efficacy and shorter treatment duration, new IFN-free regimens have the most favorable side-effect profiles. Excellent treatment results with these new regimens are challenged by scarce data on the treatment of minimally decompensated liver transplant candidates (CTP C) due to unfavorable drug metabolism in hepatic failure and renal insufficiency.

CONCLUSION

With the evolution of new antiviral drugs and more precise and clear knowledge of HCV disease recurrence, promising results have begun to emerge in the complex field of liver transplantation. A substantial proportion of patients who are either ineligible for or poorly tolerate interferon-containing regimens experience rapid deterioration in their natural HCV infection course and upon HCV recurrence after transplantation. Highly effective and safe antiviral therapy regimens that have been extensively evaluated have the potential to prevent many HCV patients from undergoing the burden of transplantation and may provide benefits for liver recipients.

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REVIEW

Gastric cancer and the epoch of immunotherapy approaches

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Abstract

The incidence of gastric cancer (GC) fell dramatically over the last 50 years, but according to IARC-Globocan 2008, it is the third most frequent cause of cancer-related deaths with a case fatality GC ratio higher than other common malignancies. Surgical resection is the primary curative treatment for GC though the overall 5-year survival rate remains poor (approximately 20%-25%). To improve the outcome of resectable gastric cancer, different treatment strategies have been evaluated such as adjuvant or perioperative chemotherapy. In resected gastric cancer, the addition of radiotherapy to chemotherapy does not appear to provide any additional benefit. Moreover, in metastatic patients, chemotherapy is the mainstay of palliative therapy with a median overall survival of 8-10 mo and objective response rates of merely 20%-40%. Therefore, the potential for making key beneficial progress is to investigate the GC molecular biology to realize innovative therapeutic strategies, such as specific immunotherapy. In this review, we provide a panoramic view of the different immune-based strategies used for gastric cancer treatment and the results obtained in the most significant clinical trials. In detail, firstly we describe the therapeutic approaches that utilize the monoclonal antibodies while in the second part we analyze the cell-based immunotherapies.

Key words: Gastric cancer; Immunotherapy; Monoclonal antibodies; T cells; Dendritic cells; NK cells

Core tip: The overall 5-year survival rate of gastric cancer after surgery resection remains poor (approximately 20%-25%) also adopting different treatment strategies,

such as adjuvant chemotherapy, adjuvant chemo-radiotherapy and perioperative chemotherapy. Several data support the idea that anti-gastric cancer (GC) specific immunotherapy could be an interesting therapeutic strategy. In this review, we provide a panoramic view of the various immune-based approaches adopted and the results obtained in the most significant clinical trials with GC patients.

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INTRODUCTION

The incidence of gastric cancer (GC) fell dramatically over the last 50 years, but according to IARC-Globocan 2008, GC is still the third most frequent cause of cancer-related deaths after lung and liver cancer in male, and after breast and lung cancer in female[1,2]. Interestingly, there is a marked geographical variation in GC incidence, with the highest rate in Japan, China and Eastern Europe and the lowest in North America, India, Philippines and Australia. Histologically, there are three subtypes of gastric adenocarcinoma: intestinal, diffuse and indeterminate (mixed type). Of those, intestinal subtype influences the changes in the epidemiological incidence[3].

Worldwide, the well known epidemiological observation in gastric cancer includes: (1) If migrants from high risk areas move to low risk areas (China to North America), the incidence rate shows a remarkable reduction reaching to almost equal rates as in low risk countries[4]; and (2) preventable by lifestyle modification such as reduced salt intake and increased vegetable and fruit consumption, together with avoidance of smoking and countermeasures against *Helicobacter pylori* (*H. pylori*) infection, reduce the risk of gastric cancer[5].

Case fatality GC ratio is higher than other common malignancies, such as colon, breast and prostate cancer[6]. Cancer excision is the principal remedy for GC though the 5-year survival rate is not high (approximately 20%-25%). To improve the outcome of resectable gastric cancer, different treatment strategies have been evaluated such as adjuvant chemotherapy, adjuvant chemo-radiotherapy, and perioperative chemotherapy.

The US Intergroup 0116 trial reported the benefit of postoperative-chemo-radiotherapy using 5-fluorouracil (5-FU)/leucovorin in a US population. In this study, only 10 % of patients received D2 resection. In Korean patients after D2 resection, the ARTIST trial failed to show any benefit from adding radiotherapy to adjuvant chemotherapy in terms of 3-year disease-free survival.

The MAGIC trial compared perioperative chemotherapy with surgery alone and reported a prolonged 5-year overall survival in the perioperative chemotherapy arm. In resectable gastric cancer, the benefit of adjuvant chemotherapy compared with surgery alone has been clearly demonstrated. After D2 dissection, S-1 adjuvant chemotherapy improved the overall survival (ACTS-GC trial) and capecitabine/oxaliplatin combination chemotherapy improved 3-year disease-free survival (CLASSIC trial). To date, for resectable gastric cancer, the use of chemotherapy in addition to surgery has proved to be beneficial in decreasing the rate of recurrence and improving overall survival. The optimal sequence of chemotherapy and surgery, as well as the development of new optimal chemotherapeutic agents, are future goals for research. In D2-resected gastric cancer, the addition of radiotherapy to chemotherapy does not appear to provide any additional benefit[7-11].

First-line chemotherapy raises the overall survival (OS) of patients with advanced GC and the association of two drugs (generally fluorouracil and cisplatin) was more effective than monotherapy[12]. Moreover, the addition of a third drug (*e.g.*, anthracycline or docetaxel) to a platinum-fluoropyrimidine association further increases the OS[13,14]. Lastly, both capecitabine and oxaliplatin had similar results to fluorouracil and cisplatin, respectively, about the OS and progression-free survival (PFS)[14,15].

In metastatic GC patients, chemotherapy is the column of palliative treatments with a median OS of 8-10 mo and objective response rates (ORRs) of merely 20%-40%[16].

Therefore, the potential for making key beneficial progress is to investigate the molecular biology of tumors to realize innovative therapeutic strategies, such as specific immunotherapy[17,18].

The behavior of immune response is centered on a task partition involving the innate (especially macrophages, dendritic and NK cells) and specific immune response (T and B lymphocytes), that often cooperate to obtain an efficacious anti-cancer response. Up to now, various strategies (vaccines, T cells infusion or cytokines) have been proved to be able to stimulate the immune system, exploiting essentially two principal mechanisms: (1) strengthening the anti-tumor response (by raising the amount of effective cells and/or cytokines/chemokines); or (2) increasing the immunogenicity and/or susceptibility of cancer cells.

However, the neoplastic cells are able to develop various strategies to evade immune surveillance: decrease of tumor antigen or MHC expression, modulation of Fas-L, secretion of inhibitory cytokines [interleukin (IL)-10 and/or TGF-β] and generation of regulatory cells such as Tregs (regulatory cells) and myeloid-derived suppressor cells (MDSC)[19,20].

In other words, the prerequisite for an effective anti-tumor immune-based treatment is the stimulation of a successful cancer-specific immune response, able

to crack the immunological tolerance of tumor cells. The aim of this review is to provide a panoramic view of the different immune-based strategies used for GC management (Figure 1) and discuss the data of the most significant clinical trials. In detail, firstly we describe the therapeutic approaches that utilize the monoclonal antibodies while in the second part we analyze the cell-based anti-GC treatments.

MONOCLONAL ANTIBODIES DIRECT TO MOLECULAR AND CELLULAR GASTRIC CANCER TARGETS

The typical paradigm of drug development, especially for targeted therapies, has primarily been in the metastatic setting, followed by introduction into chemoradiation, and finally, subsequent evaluation in a randomized trial.

In this paragraph, we will focus on three of the most widely studied therapies with monoclonal antibodies (moAb) in gastric cancer: anti-epidermal growth factor receptor (anti-EGFR), anti-HER2 and anti-vascular endothelial growth factor (anti-VEGF); as they have been evaluated by this paradigm. The second part will focus on pathways and drug targets currently under evaluation for gastric cancer.

Anti-epidermal growth factor receptor (cetuximab/panitumumab/matuzumab)

EGFR (ERBB1) is an element of the ERBB trans-membrane growth factor receptor family, that promotes and modulates, *via* a receptor-associated tyrosine kinase (TK), various cell processes such as apoptosis or proliferation[21].

The EGFR hyperexpression shows a relationship with augmented invasion and more unfavorable prognosis of patients with esophago-gastric cancers (EGC)[22-25]. In addition, the anti-EGFR MoAb therapy is ineffective in colorectal cancer (CRC) patients that have K-ras mutations[26-28].

Lately, Janmaat *et al*[29] showed mutated K-ras in 8.7% patients with EGC; but the prognostic role of K-ras status in the anti-EGFR therapy is practically indefinite.

Existing anti-EGFR treatments in EGC patients consist of oral TK inhibitors (TKIs; erlotinib, gefitinib) and moAb (cetuximab, panitumimab and matuzumab).

Cetuximab obstructs the lignad junction with the EGFR[30], promotes EGFR internalization[31] and also, can start the immune-mediated cytotoxicity[32,33].

Due to the better ORR and time-to-progression (TTP) for the cetuximab/irinotecan association compared with the irinotecan monotherapy[34] , the FDA (Food and Drug Administration) has been approved the cetuximab use in irinotecan-refractory CRC.

In addition, the FDA has been authorized the Panitumumab therapy of chemo-refractory EGFR-positive CRC, because a recent study showed an amelioration in ORR and PFS over best current treatment[35]. Besides, a phase Ⅰ study reported a stable disease (SD), for 7 mo, in one refractory EGC patient, treated with panitumumab[36].

Lastly, a recent study showed that one patient, with esophageal cancer (EC), cured with Matuzumab (the last anti-EGFR moAb) had a durable six-month PR[37]. Also, the combination of matuzumab with the ECX regimen (epirubicin/cisplatin/capecitabine) registered encouraging results as first-line therapy in patients with $EGFR^+$ gastric cancer. The ORR in 20 evaluable patients was 65% with a median TTP of 5.2 mo[38].

Metastatic results: Numerous phase Ⅱ studies have been performed with cetuximab in combination with chemotherapy in advanced EGC. One of the first trials[39] evaluated cetuximab with FOLFIRI in thirty-eight patients with untreated advanced gastric or GE junction adenocarcinoma. Cetuximab was given with an initial loading dose of 400 mg/m^2 followed by weekly doses of 250 mg/m^2. The overall response rate was 44.1%, with a median survival of 16 mo. In another randomized phase Ⅱ study, cetuximab was added to 3 chemotherapy regimens: ECF (epirubicin, cisplatin, 5-FU), IC (irinotecan/cisplatin), and FOLFOX[40]. The response rates were 58%, 38%, and 51% in the 3 arms, respectively.

The role of anti-EGFR therapy in advanced EGC was tested in a phase Ⅲ study evaluating the efficacy of panitumumab with combination chemotherapy in the REAL 3 study[41]. Patients with inoperable/metastatic esophageal, gastric, or GE junction cancer were randomized to receive EOC (epirubicin, oxaliplatin, capecitabine) with or without panitumumab. An early planned interim analysis showed that the panitumumab arm was statistically inferior after 553 (76%) patients were enrolled. Median survival was 11.3 mo in the chemotherapy-alone arm *vs* 8.8 mo for chemotherapy plus panitumumab [hazard ratio (HR) = 1.37, P = 0.013). Although patients with rash in the panitumumab arm did better than those without rash, the subgroup of patients with rash still had a numerically worse median survival than the entire chemotherapy-alone group.

Chemoradiation results: Chemoradiation with cetuximab has been extensively studied in the phase Ⅱ setting. One clinical study evaluated 60 patients treated with cetuximab, paclitaxel, and cisplatin in combination with radiation therapy. A pathologic complete response rate of 27% was seen with this regimen[42]. In the Swiss Group for Clinical Cancer Research phase Ⅰb/Ⅱ trial (SAKK 75/06), 28 patients with adenocarcinoma or squamous cell carcinoma were treated with induction cisplatin, docetaxel, and cetuximab followed by radiation therapy to 45 Gy along with concurrent cisplatin and cetuximab. A pathologic

complete response (pCR) rate of 32% was seen with this regimen. Neither of these studies demonstrated excess risk with the addition of cetuximab[43].

In contrast, ECOG 2205 evaluated a neoadjuvant regimen of cetuximab in combination with infusional 5-FU, oxaliplatin, and radiation therapy. The study was closed after an excessive number of early deaths. Four of 18 patients died postoperatively of the acute respiratory death syndrome (ARDS) despite compliance with strict radiation lung dosimetry guidelines. This high rate of ARDS, not seen in other studies of 5-FU with oxaliplatin and radiation, raised the possibility that cetuximab may have added to the risk of postoperative pulmonary complications[44].

Evolution of chemoradiation: Radiation Therapy Oncology Group (RTOG) 0436 is a randomized phase Ⅲ trial of cisplatin, paclitaxel, and radiation therapy to 50.4 Gy with or without cetuximab in inoperable esophageal cancer. In the spring of 2012, the study underwent a planned interim analysis to document superiority of the cetuximab arm as measured by clinical complete response rate. The study failed to meet this end point and closed to further patient enrollment.

The SCOPE1 study from the United Kingdom is a similarly designed 2-arm randomized phase Ⅱ/Ⅲ study comparing cisplatin/capecitabine/radiation with or without cetuximab[45]. This study will also undergo a planned analysis after the phase Ⅱ portion to document a freedom from treatment failure rate exceeding 75% at 24 wk in the cetuximab containing arm.

Given the negative results of the REAL 3 trial, and RTOG 0436 closing enrollment to adenocarcinoma due to a lack of efficacy with cetuximab, it is unlikely that anti-EGFR strategies will be further developed in the United States in unselected patients.

Anti-HER2/ERBB2 (trastuzumab)

HER-2/neu (ERBB2) is part of the ERBB TK receptor family. The ligand of these receptors leads to homo/hetero-dimerization of the receptors and with their formation displaying a distinct hierarchy. In this system, HER-2/neu has a key role because each receptor with a specific ligand promotes the association with Her-2/neu. This predilection is more influenced by Her-2/neu hyperexpression, as seen in numerous types of human tumor cells[46].

About EGCs, HER-2/New hyperexpression has been shown in esophageal cancer and GE junction carcinoma[47,48]. HER-2/neu hyperexpression has been connected with increased invasion and poor response to neo-adjuvant chemotherapy[49] or overall reduced survival[50].

The anti-HER2/neu moAb treatment that has been tested in EGC patients is Trastuzumab, that exercises its role by different ways: blocking HER-2 receptor dimerization, favoring the receptor demolition and promoting the cytotoxicity[51]. Currently, it has been used in association with chemotherapy for HER-2/neu$^+$ and node$^+$ breast cancer[52-56].

Metastatic results: The proof of the therapeutic benefit of HER2-directed therapy in gastric and GE cancer comes from the trastuzumab for gastric cancer trial, a large randomized trial of trastuzumab added to standard chemotherapy in HER2$^+$ advanced gastric cancer[57]. In this study, patients with HER2$^+$ gastric or GE cancer were randomized to either trastuzumab and chemotherapy or chemotherapy alone.

Chemotherapy consisted of (5-FU or capecitabine in combination with cisplatin given every 3 wk for 6 cycles. Trastuzumab was continued until disease progression. HER2 positivity was defined as 3$^+$ staining by immunohistochemistry (IHC). Tumors with IHC 2+ staining had to be confirmed by the evidence of amplification by fluorescence in situ hybridization.

Tumors from 3807 patients were centrally tested for HER2 status, of which 22.1% were HER2$^+$. These 594 patients were randomized to 1 of the 2 treatment groups, with well-balanced clinical characteristics. A planned interim analysis was performed after 75% of the events had occurred, and the independent data monitoring committee recommended release of the data because the prespecified boundary had been exceeded, with a median follow-up of 17.1 mo. Median survival was improved with the addition of trastuzumab to chemotherapy from 11.1 to 13.5 mo (P = 0.0048; HR = 0.74; 95%CI: 0.60-0.91). The overall response rate was also improved from 34.5% to 47.3% with the addition of trastuzumab (P = 0.0017). The toxicity was similar in both arms.

Specifically, there was no difference in congestive heart failure. Asymptomatic decreases in left ventricular ejection fraction were similar in both arms (4.6% with trastuzumab, 1.1% without). Based on this study, trastuzumab was approved in the setting of HER2$^+$ advanced gastric and GE cancer.

Chemoradiation results: Recently it has been performed a pilot study of trastuzumab added to chemoradiation in patients with locally advanced esophageal carcinoma[58].

Patients were required to have HER2 positivity (HER2 2$^+$/3$^+$ expression). Chemoradiation was delivered with a dose of 50.4 Gy along with concurrent weekly cisplatin (25 mg/m^2) and paclitaxel (50 mg/m^2). In cohort 1, 3 patients received a 2-mg/kg bolus dose in week 1 followed by a weekly dose of 1 mg/kg. In cohort 2, 3 patients received a 3-mg/kg bolus dose in week 1 followed by a weekly dose of 1.5 mg/kg. In the third cohort, 13 patients received a 4-mg/kg bolus dose in week 1 followed by a weekly dose of 2 mg/kg. Maintenance trastuzumab was given for 1 year at a dose of 6 mg/kg every 3 wk. Despite the advanced

disease in many patients, such as celiac adenopathy (37%) or retroperitoneal (37%), a striking 3-year overall survival of 47% was observed, although a lot of patients did not undergo surgery owing to extensive adenopathy or medical morbidities. Additionally, there were no observed increases in adverse events from the addition of concurrent or maintenance trastuzumab. Because surgery was not required, there was no meaningful pCR data.

Trastuzumab emtansine, or T-DM1, is an antibody-drug conjugate linking trastuzumab to a highly potent anti-microtubule agent. Preclinical data on human GC cells and xenografted tumors suggested that T-DM1 is more effective than trastuzumab. Recently, a phase Ⅲ study evaluating T-DM1 *vs* lapatinib and capecitabine in HER2$^+$ trastuzumab-refractory breast cancer demonstrated an improvement in median survival favoring T-DM1 (not reached *vs* 23.3 mo; HR = 0.621; 95%CI: 0.475-0.813; *P* = 0.0005)[59]. Additionally, T-DM1 had a higher response rate (43.6% *vs* 30.8%) and duration of response (12.6 mo *vs* 6.5 mo). Furthermore, T-DM1 showed antitumor effects even in xenografted tumors that had developed resistance to trastuzumab. Based on this evidence, an international phase Ⅱ/Ⅲ trial in second-line advanced EGC will open randomizing between T-DM1 *vs* a taxane (weekly paclitaxel or q3w docetaxel).

Evolution of chemoradiation: Based on the positive results observed in the metastatic setting with the addition of trastuzumab as well as the encouraging safety and efficacy data from the Brown group, the RTOG has initiated a randomized trial, RTOG 1010, studying the addition of trastuzumab to chemoradiation. In this study, patients with operable locally advanced adenocarcinoma of the esophagus and GE junction are centrally screened for HER2 positivity. If the tumor is found to be HER2$^+$, patients are randomized to concurrent and maintenance trastuzumab in addition to chemoradiation. Chemo-radiation consists of a dose of 50.4 Gy along with weekly carboplatin (AUC 2) and paclitaxel (50 mg/m^2). The results of this ongoing trial will inform the future use of trastuzumab in localized HER2-overexpressing esophageal and GE junction cancer[60].

Anti-vascular endothelial growth factor (bevacizumab)

The action of most powerful angiogenic factor, VEGF, is started by linking to various high-affinity trans-membrane receptors, most remarkably VEGFR types 1 and 2[61].

VEGF is over-expressed in different cancers[62] and besides, in esophageal and gastric cancer the hyperexpression correlates with cancer stage, bad prognosis and reduced survival[63-70]. Also, the moAb bevacizumab is an anti-VEGF monoclonal antibody, that associated with the chemotherapy increases the ORR and TTP in patients with CRC[71] NSCLC[72] and breast cancer[73]. It seems that bevacizumab have a double anti-cancer effect: as anti-angiogenic factor and also increasing chemotherapy drug delivery, favoring the decrease of interstitial fluid pressures[74,75].

Metastatic results: Multiple phase Ⅱ studies evaluated bevacizumab in combination with a variety of chemotherapy regimens in esophagogastric cancer. In a phase Ⅱ study[76], the addition of bevacizumab to cisplatin and irinotecan showed a response rate of 65% and a median survival of 12.3 mo. In another phase Ⅱ study, Shah *et al*[77] evaluated bevacizumab in combination with docetaxel, cisplatin, and 5-FU. This regimen yielded a response rate of 67% and an impressive median survival of 16.8 mo. Similarly, a high response rate of 68% was observed when bevacizumab was combined with docetaxel, cisplatin, and irinotecan[78].

With these higher obtained response rates, a randomized phase Ⅲ trial was performed evaluating the efficacy and safety of bevacizumab in combination with chemotherapy. The Avastin in Gastric Cancer (AVAGAST) trial randomized patients with inoperable locally advanced or metastatic gastric or GE junction adenocarcinoma with no previous therapy to bevacizumab or placebo in combination with capecitabine (or 5-FU) and cisplatin. 774 patients were randomized, with 95% of patients having metastatic disease[79]. The median survival was 10.1 mo for chemotherapy alone *vs* 12.1 mo for chemotherapy plus bevacizumab (HR = 0.87; *P* = 0.1002). Although this result did not reach statistical significance, there was an improvement in progression-free survival from 5.3 to 6.7 mo (HR = 0.80; *P* = 0.0037), and the overall response rate increased from 29.5% to 38% (*P* = 0.0121). However, despite this negative trial, some of the trends in the secondary endpoints have led to further evaluation of bevacizumab in the metastatic setting.

Chemoradiation results: An interesting study[80] demonstrated that bevacizumab could change tumor physiology of rectal cancer and theoretically potentiate the effects of radiation therapy. In localized esophageal cancer, a similar approach was used in a phase Ⅱ trial evaluating bevacizumab with erlotinib in a neoadjuvant chemoradiation study[81]. Patients with stage Ⅰ-Ⅲ esophageal or GE junction cancer were enrolled. Ninety-five percent of patients had adenocarcinoma, and 93% of patients had stage Ⅱ or Ⅲ disease. Bevacizumab was added to a regimen consisting of carboplatin (AUC 5, days 1 and 22), paclitaxel (200 mg/m^2, days 1 and 22), and continuous infusion of 5-FU (225 mg/m^2 per day, from day 1 to 35) in combination with radiation therapy to 45 Gy. Of sixty patients enrolled, a pathologic complete response rate of 30% was observed.

Another phase Ⅱ study, reported results of pre-operative chemoradiation with cisplatin, irinotecan, and bevacizumab. Patients with Siewert Ⅰ/Ⅱ adenocarcinoma

of the esophagus received induction chemotherapy with cisplatin, irinotecan, and bevacizumab. This was followed by concurrent chemotherapy with cisplatin, irinotecan, and bevacizumab in combination with radiation therapy to 50.4 Gy. Surgery was followed by adjuvant bevacizumab. A pathologic complete response was seen in 4 of 33 patients (12%). Progression-free survival and overall survival were 14 and 30 mo, respectively[82].

Evolution of chemoradiation: The negative primary result of the AVAGAST study has mitigated some of the enthusiasm for bevacizumab in the context of chemoradiation for esophageal cancer. Given the lack of improvement in the pathologic complete response rate in the phase Ⅱ study discussed earlier in the text compared with historical control groups, further development of bevacizumab with chemoradiation for esophageal cancer is currently not being pursued in a phase Ⅲ study[60].

Anti-hepatocyte growth factor/mesenchymal-epithelial transition factor (rilotumumab)

The cell surface receptor c-MET [mesenchymal-epithelial transition factor (MET)] and its ligand hepatocyte growth factor (HGF) are potential therapeutic targets in esophagogastric cancer. Physiological MET tyrosine kinase activation is mediated by binding of HGF, leading to signal transduction down multiple downstream pathways, including those involving Ras, PI3K, mTOR, STAT3, and NF-κB[83,84]. Additionally, the HGF/MET axis can stimulate tumor endothelial cells, thereby altering the tumor microenvironment and promoting angiogenesis[85,86]. Physiological MET signaling can be altered by ligand/receptor overexpression or gene amplification as well as *MET* gene mutations[85]. Specifically, *MET* gene amplification is a driver in some esophagogastric cancers[87-90].

Additionally, *MET* gene mutations have been documented in hereditary and sporadic renal carcinoma, esophagogastric cancer, hepatocellular cancer, head and neck cancer, ovarian carcinoma, small-cell lung cancer, and glioma[85,91,92]. Strategies to inhibit the HGF/MET axis include blocking both the ligand and the receptor.

Rilotumumab is a human moAb to HGF. In a randomized phase Ⅱ study, patients with newly diagnosed GC were randomized to receive 1 of 2 doses of rilotumumab (15 mg/kg or 7.5 mg/kg) in combination with ECX (epirubicin, cisplatin, capecitabine) chemotherapy or chemotherapy alone[93]. Tumors that were IHC^+ in > 50% of cells were defined as MET high. In the MET-high subgroup, representing approximately half of the patients, the 2 rilotumumab arms had a median survival superior in the chemotherapy-alone arm (11.1 mo *vs* 5.7 mo; HR = 0.29; 95%CI: 0.11-0.76; *P* = 0.012). In contrast, the MET-low patients in the 2 rilotumumab-containing arms had a trend toward a worse survival than the MET-low patients in the chemotherapy-alone arm (HR = 1.84; 95%CI: 0.78-4.34). In the chemotherapy- alone arm, patients with MET-high tumors had a worse overall survival (HR = 3.22; 95%CI: 1.08-9.63) than those with MET-low tumors. This study suggested that expression, as opposed to amplification, may be a reasonable biomarker. In this study, MET expression was both predictive (good) for anti-HGF antibody therapy and prognostic (poor). Based on these data, a phase Ⅲ study has been planned in the first-line setting for EGC patients with MET-high tumors.

CELL-BASED IMMUNOTHERAPY APPROACHES

Therapies with T cell

The central anti-cancer role of T cells has been highlighted by the documented cancer incidence in immunodeficient disorders[94] and by evidence that the intra-tumoral T cell infiltration is associated with better patient survival[95].

Currently, there isn't FDA-approved adoptive T-cell therapy, but the growing new acquisitions on the cancer nature and lymphocyte role do hope that, shortly, the adoptive T-cell therapy can become a clinical cancer practice. Topical information obtained from adoptive transfer in lymphodepleted hosts[96], the immunosuppressive capacity of Tregs[97] and the utilize of better culture systems[98] have not yet been tested in clinical studies.

Essentially exist two different therapeutic protocols of T cell-based anti-cancer treatment: (1) cytotoxic T lymphocytes (CTL); (2) tumor infiltrating lymphocytes (TIL) (Figure 1).

Cytotoxic T lymphocytes: Improved CTL cell culture technology has permitted the first clinical tests for adoptive transfer of CTLs and this technique[99,100] seems to result in substantial activity in melanoma patients: 40% of patients showed an anti-tumor immune responses[101]. Similar results were obtained by Yee *et al*[102] in an independent trial in which engraftment of the CTLs was detectable up to two weeks after T-cell transfer in all patients.

Survivin has been demonstrated to be an excellent target for immunotherapy in various cancer types and recent data suggest a role also in gastric cancer[103]. In this study, elevated efficiency was obtained upon inducing survivin-derived peptide-specific CTL from mononuclear cells isolated from blood of healthy donors. The induced CTLs showed specific lysis against tumor cells *in vitro*, and *vs* primary cell cultures isolated from GC patients. These data suggest that survivin epitope peptide could be a promising vaccine candidate for GC immunotherapy.

Instead, another recent study[104] examined the possibility of using cancer-specific immunotherapy based upon mitotic centromere-associated kinesin (MCAK), a new cancer antigen. To evaluate the

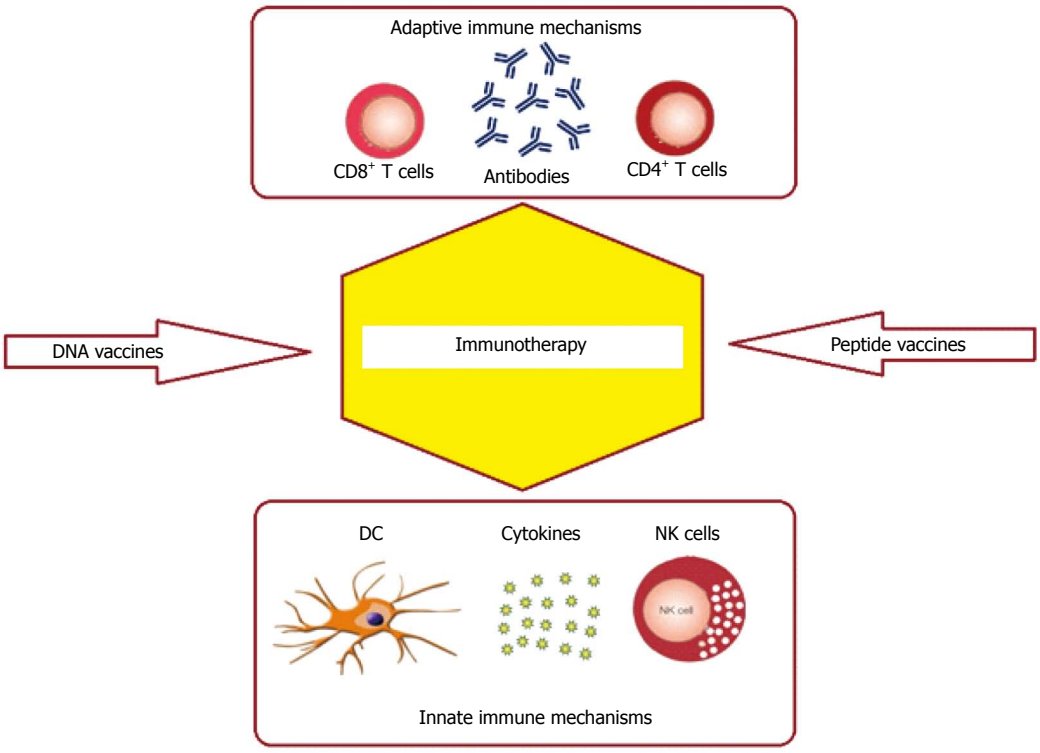

Figure 1 Different immunotherapy approaches. The figure illustrates the two different vaccination approaches (using DNA or peptides) and the cellular and molecular elements used in anti-cancer immunotherapy. The adopted specific immune mechanisms are monoclonal antibodies and T cells: $CD8^+$ (CTL) or $CD4^+$ (T helper). About innate immune mechanisms, cytokines, dendritic cells (DCs) and natural killer cells (NKs) represent the most exploited components.

feasibility of developing cancer immunotherapy using MCAK peptides, Kawamoto *et al*[104] studied HLA-A^{1}0201 and ^{1}2402 as targets for CTLs.

The CTLs killed HLA-A-^{1}0201/^{1}2402 colon and gastric cancer $MCAK^+$ cells, as well as the peptide-pulsed target cells, in an HLA-I restricted manner. These results prospect the opportunity of designing peptide-based immunotherapeutic treatments for patients with $MCAK^+$ gastric cancer.

Of late, Kim *et al*[105] demonstrated the anti-gastric cancer power of cytokine-induced killer (CIK) cells (essentially T $CD80^+$ cells), that were isolated from the human peripheral blood mononuclear cells (PBMC), cultured in medium with IL-2 and anti-CD3 antibody. The CIK cells were able to destroy, *in vitro*, the MKN74 cells (a human gastric cancer cell line) and to inhibit the MKN74 tumor growth in nude mouse model. These results suggest the potential use of CIK cells as adoptive GC immunotherapy patients, as described in different studies[106,107]. In fact, the CTLs from GC patients are capable to attack the autologous cancer cells, recognizing specific tumor-associated antigens[107,108], such as MG7-antigen, that shows a big potential for starting immune responses to gastric cancer[109,110]. In addition, the use of HLA-A-restricted allogeneic GC cells to stimulate tumor-specific CTLs could be a different immunotherapeutic approach for GC patients[111].

Of note, different studies suggest that the association of CIK cells with chemotherapy can be functional in advanced GC patients[112,113]. In fact, the patients cured with the combined therapy showed a significant decrease of serum levels of the cancer markers and a marked improvement of life quality, in comparison to patients treated only with chemotherapy.

In summary, preclinical/clinical evidence supports the idea that CIK cell immunotherapy can be a successful anti-GC treatment, but it is still unclear what is the injection *via* which guarantees the best distribution of effector cells.

In a mouse model of gastric cancer Du *et al*[114] observed the distribution of CIK cells injected by three different *via* of infusion: peritumoral (pt), intravenous (iv) and intraperitoneal (ip).

They demonstrated that the pt injection produced a considerable tumor infiltration of CIK cells for 48 h and induced the most tumor inhibition in comparison to the ip or iv infusion, that caused a very small CIK intratumoral accumulation and a short *in vivo* inhibition of tumor growth only following injection. In conclusion, the pt injection of CIK can be an effective and minimally invasive approach of adoptive cellular immunotherapy for GC patients.

Adoptive transfer therapy with TILs: The use of TILs as adoptive transfer therapy is a "not immediate" therapeutic approach because it requires about six weeks before the T cells would be ready for infusion. In fact, the protocol necessitates firstly the T cell isolation from neoplastic tissue, after the *in vitro*

expansion and finally the selection of tumor-specific T cells. In addition, only 30%-40% of the biopsies yield acceptable T-cell populations and the whole process[115]. So, the adoptive transfer of TILs has been promising in preclinical models[116] but not in clinical trials[117,118], except for the melanoma patients for easy surgical availability of the tumor tissue. However, should technical limitations of current tissue culture approaches are overcome; new data indicate that the presence of TILs positively correlates with patient survival in ovarian and colorectal cancer[95,119] and have a important role in pancreatic cancer[120] , thus prompting the enforcement of this protocol for other usually encountered epithelial cancers.

In the past, we have demonstrated, in GC patients, the functional role of TILs reactive *vs* different peptides of GC-associated antigens[121]. We have documented a peptide-specific T-cell response in 17 out of 20 enrolled patients and the majority of specific TILs had an effective role showing a T helper 1 (Th1) cytokine profile with high cytotoxic activity. In other words, in most of GC patients, a specific type-1 T-cell response to GC antigens was detectable and would have the potential of killing the cancer cells. But, in order to get "*in vivo*" tumor cell destroying, the the quantity and quality of tumor-specific T cells almost certainly need to be enhanced by vaccination with the appropriate cancer antigens/peptides or by injection of the autologous cancer-specific T cells, previously expanded *in vitro*.

It is remarkable to note that not always the lymphocytic infiltrate has an anti-cancer role and often the TILs can promote the expansion of tumor cells. Recently, we have investigated the functional profile of HP0175-specific TILs in GC patients, infected with *H. pylori*. The TILs cells were able to produce IL-17 and IL-21 in response to HP0175 but showed poor cytolytic activity and high helper activity for monocyte MMP-2, MMP-9 and VEGF production. In a nutshell, these data suggest that HP0175 drives gastric Th17 response and promoting pro-inflammatory low cytotoxic TIL response, so providing a link between *H. pylori* and gastric cancer[122].

In addition, different studies highlight that most of GC TILs show a Treg profile. Recently, Shen *et al*[123] demonstrated that that $CD4^+$ and $CD8^+$ TILs were not associated with the OS of GC patients and that in the tumor sites, higher Tregs/$CD8^+$ ratio was an independent factor for worse OS (P = 0.037). The 1-year, 2-year and 3-year OS rates were 90%, 77.5% and 70% for the group with intratumoral high Tregs/$CD8^+$ ratio, compared with 100%, 94.3% and 90.5% for the group with intratumoral low ratio. So, intratumoral high Tregs/CTLs ratio was a prognostic factor for GC patients. Accordingly, an independent study showed that a higher Tregs/Th cell ratio is associated with an unfavorable prognosis and loco-regional recurrence pattern in gastric cancer[124].

It can be inferred that a combination of deletion of Tregs and stimulation of effector T cells may be a successful immunotherapy to prolong survival of GC patients.

Dendritic cell-based vaccination

Antigen presentation by dendritic cells (DCs) is essential to start the cellular immune responses required for tumor immunotherapy[125,126] (Figure 1). In addition, in mouse models *ex vivo* generated DCs can provoke antigen-specific T-cell responses[127], supporting the use of DC-based anticancer vaccines in clinical studies[128].

In GC patients the number of DCs correlates with the clinical stage and prognosis: patients with abundant DCs infiltration showed a better 5-year survival rates than patients with smaller amount of DCs[129,130]. Moreover, it has been documented that the use of adjuvant immunotherapy enhances the survival in resected GC patients with small tumor DCs infiltration[131].

Of the 325 trials reported in ClinincalTrials. Gov on DC therapy, six studies involve GC patients (Table 1)[132-134] but only three have been terminated (Table 1) and only two have published their results.

Kono *et al*[132] reported a phase- I clinical study of GC patients treated with DCs pulsed with HER-2/neuro-peptides. After the vaccination, one (out of 9 patients HER-2/neu^+) showed decreased levels of CEA and CA19-9 while two registered a significative cancer regression (> 50%). Of note, the vaccine protocol did not register considerable side effects.

Recently Sadanaga *et al*[133] published the results of a phase-I trial, where twelve patients, with advanced gastrointestinal carcinoma, were immunized with DC pulsed with MAGE-3 peptides without significant side effects. After vaccination, in four patients was registered the presence of peptide-specific CTL while in seven was observed the serum decrease of cancer markers. In addition, small cancer regressions were highlighted in three patients.

Finally, Galetto *et al*[135] described that memory T cell specific to GC antigens could be activated by cancer-loaded autologous DCs, isolated from blood mononuclear cells and activated by stimulation with apoptotic autologous tumor cells.

Nevertheless, the clinical application of DC vaccines has been limited for the the short lifespan of DCs, and one of the factors threatening DC survival is antigen-specific $CD8^+$ that acquire cytolytic activities after activation by DCs[136].

In recent times, Kim *et al*[98] ameliorated the efficiency of a DC vaccine with a small interfering RNA (siRNA) targeting phosphatase and tensin homolog (PTEN), that has a key role as a negative regulator in the signal transduction of the PI3K/AKT pathway[137].

The PTEN downregulation in DCs resulted in AKT-dependent maturation, which generated a considerable

Table 1 List of the anti-gastric cancer clinical trials using dendritic cells

Ref.	Title	Sponsor/collaborator	Status	Duration
Kono *et al*[132], *Clin Cancer Res* 2002	Dendritic cells pulsed with HER-2/neu-derived peptides can induce specific T-cell responses in patients with gastric cancer	Yamanashi Medical University Japanese Clinical Oncology Fund and from the Public Trust Haraguchi Memorial Cancer Research Fund	Completed published	NA
Sadanaga *et al*[133], *Clin Cancer Res* 2001	Dendritic cell vaccination with MAGE peptide is a novel therapeutic approach for gastrointestinal carcinomas	Medical Institute of Bioregulation, Kyushu University Japan Society for the Promotion of Science, Grant-in-Aid for Scientific Research (A) (08557074)	Completed published	Study started: January 1997 Completed: August 2000
http://www.clinicaltrials.gov/	A phase I study of active immunotherapy with carcinoembryonic antigen RNA-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen	Duke Cancer Institute NCI (NCT00004604)	Unknow[1]	Study first received: May 2, 2000 Last updated: December 13, 2011
http://www.clinicaltrials.gov/	A pilot study of active immunotherapy with HER2/neu intracellular domain protein-pulsed, autologous, cultured dendritic cells in patients with no evidence of disease after standard treatment for HER2/neu expressing malignancies	Duke Cancer Institute NCI (NCT00005956)	Unknow[1]	Study first received: July 5, 2000 Last updated: December 13, 2011
http://www.clinicaltrials.gov/	A phase I study of active Immunotherapy with autologous dendritic cells infected with CEA-6D expressing fowlpox -tricom in patients with advanced or metastatic malignancies expressing CEA	Duke Cancer Institute NCI (NCT00027534)	Completed	Study first received: December 7, 2001 Last updated: December 13, 2011
http://www.clinicaltrials.gov/	A Phase I clinical trial of mTOR inhibition with rapamycin for enhancing intranodal dendritic cell vaccine induced anti-tumor immunity in patients with NY-ESO-1 expressing solid tumors	Roswell Park Cancer Institute (NCT01522820)	Not yet recruiting	Study first received: January 25, 2012 Last updated: February 3, 2012

[1]Indicates status has not been verified in more than two years. NA: Not availiable.

surface hyperexpression of costimulatory molecules and the chemokine receptor, CCR7, leading to an increased T cell activation *in vitro* and a migration to a draining lymph node *in vivo*, respectively. In addition, the PTEN siRNA transfected DCs (DC/siPTEN) showed an augmented survival and most remarkably, DC/siPTEN generated a major number of cancer-specific Tc cells and a stronger anticancer response in vaccinated mice compared to the controls.

In short, these data suggest that manipulation of the PI3K/AKT pathway with the siRNA system could improve the efficacy of a DC-based tumor vaccine, such as in GC treatment.

Immunosuppressive factors, such as IL-10, secreted by DCs (or other regulatory cells) downregulates the Dcs functionality by specific surface receptors (*e.g.*, IL-10R). Recent data showed that the targeting IL-10 receptor with siRNA, can increase the effectiveness of DC-based vaccine, suggesting a potential clinical use of siRNA[138].

In addition, very interesting are the results of He *et al*[139] concerning the opportunity of increasing the anticancer immunity through *GM-CSF* gene-modified DCs. After *GM-CSF* gene modification, DCs are able to secrete elevated levels of GM-CSF and have a major propensity to be maturated. In this way, the DCs increase their ability of activating the proliferation of T cells. In addition, *in vitro* the dendritic cells with *GM-CSF* gene modified can stimulate specific CTL to kill the cancer cells.

Finally, in comparison with the vaccination alone, DCs vaccination and the preventive removal of Tregs substantially enhances the activation of tumor-specific T-cell responses[140].

Treatments by using NK cells

The NK cells are able to arrest the metastatic spreading of human cancers[141] and also, the intra-cancer infiltration of NK cells is associated with a better prognosis of GC patients[142]. The key function of NK cells in anti-tumor response gives us the possibility to counteract the cancer progression by manipulating the NK cell "arms". However, some obstacles make it difficult to the therapeutic use of NK cell-based treatments: (1) unfinished characterization of the specific function of the different NK cell subpopulations;

(2) little knowledge of the mechanisms involved in NK functionality; (3) the exiguous amount of blood NK cells; and (4) the troubles about a massive production in good manufacturing practices (GMP) of NK cells[143].

About gastric cancer, a recent study evaluated the NK number in 72 patients with gastric adenocarcinoma and its correlation with patient survival. The conclusions are that patients with high concentration of NK cells showed a higher survival rate when compared to the low concentration, especially in the advanced stage[144].

Moreover, very interesting data have been obtained by Saito *et al*[145] which demonstrated that the frequency of apoptotic NK cells in GC patients was significantly higher than in normal controls. Moreover, their frequency was related to the GC progression. Fas^+ NK cells were significantly more common in GC patients compared with normal controls and Fas expression was closely related to the frequency of NK cell apoptosis. Also, the frequency of tumor-infiltrating NK cell apoptosis was significantly higher than that of circulating NK cell apoptosis. Furthermore, apoptotic circulating NK cells significantly decreased after surgery compared to before surgery.

Finally, Voskens *et al*[146] showed that numerous cytotoxic NK cells can be obtained from cancer patients co-culturing autologous PBMC with K562 cells. Of note, the *ex vivo* development increased the cytotoxic activity of NK cells *vs* the autologous derived, suggesting a future clinical application, as cell-based immunotherapy, for autologous expanded NK cells (as alone as and in association with specific monoclonal antibodies).

Very interesting are the recent results about lupeol, a triterpene that has curative action *vs* various diseases. Recently, Wu *et al*[147] showed that lupeol is able to favor the proliferation of NK cells, increasing also their killing action *vs* the GC cells. Moreover, lupeol inhibit the proliferation of different GC cell lines. These data suggest that lupeol could serve as a potential agent against gastric cancer alone or with adoptive transfer of NK cells.

FUTURE DIRECTIONS

We have used this review to provide a panoramic view about the current immunotherapeutic anti-GC approaches, some of which have been used in clinical trials with fairly good results about the tumor regression and patient survival. But, the role of immunotherapy for gastric cancer continues to evolve. As the current development suggests, gastric cancer therapy has suffered from a relative lack of gastric-specific biological exploration. The most common developmental path to date has been limited to the study of immune-based therapies that have demonstrated efficacy in other somewhat similar diseases, and then have been tested in gastric cancer.

Moreover, an additional great challenge in the field is to develop randomized clinical trials validating the medical benefits to justify the logistics and especially the costs of these personalized cell treatments.

Usually the clinical trials enroll patients with advanced GC, this factor could determine an unfavorable result, because the anti-cancer battle of current immune response is already a lot compromised. For this purpose, it would be strategic to recruit early-stage GC patients, that being in the early stages of tumor development, may better react to the immunotherapy strategies.

Finally, to set up successful anti-GC immunotherapy approaches, it is necessary to understand the "fine" immune escape mechanisms, adopted by the cells of gastric cancer.

Past and recent studies have supplied new insights about the thick crosstalk between tumor and immune cells. Comprehending this operative dialogue and the hierarchic grade of the various cancer-immune evasion mechanisms at distinct steps of neoplastic evolution, will guide the development of innovative curative strategies aiming to demolish the "tumor fortress".

So, it will be remarkable to evaluate the pathways of the various components that modulate the growth and mobility of Tregs, MDSCs and tolerogenic DCs within cancer-draining lymph nodes and the cancer surroundings.

Another important target for the future anti-GC immunotherapy treatment could be the immune checkpoints[148] that are inhibitory pathways hardwired into the immune system. The immune checkpoints play critical roles for physiological homeostasis because they are essential for maintaining self-tolerance and modulating the duration and amplitude of physiological immune responses in order to minimize collateral tissue damage. But, these checkpoints may also allow immune escape in cancer.

Checkpoint pathways are regulated by ligand/receptor interactions. For example, programmed death-1 receptor (PD-1) and CTL-associated antigen 4 (CTLA-4) are inhibitory molecules whose presence on lymphocytes signifies a blunted immune response. PD-1 negatively regulates T cell responses and downregulation and eventually apoptosis is initiated following binding of a PD-1 ligand with PD-1. PD-1 ligands, PD-L1 or PD-L2, are frequently expressed on tumor cells and can thus thwart the immune response. One approach to overcome this inhibition of the immune response has been to target immune checkpoints with blocking moAb. For example, PD-1 moAb binds to the PD-1 receptors on T cells and inhibits their binding to the ligands on tumor cells thus preventing the tumors from down regulating the cytotoxic lymphocyte response. This approach has been successful clinically in advanced melanoma[149,150]. and phase I clinical trials of anti-PD-L1 moAb are under investigation for gastric cancer[148].

It is realistic to declare that, in the future effective anti-GC immunotherapy strategies must include combined approaches, which should use both systemic radio/chemotherapy and transplantation, to diminish

the burden or to remove immune suppressive cells, and tailored immunotherapies customized to each single patient.

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REVIEW

Pathophysiology after pancreaticoduodenectomy

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Abstract

Pancreaticoduodenectomy (PD) will result in removal of important multiorgans in upper intestinal tract and subsequently secondary physiologic change. In the past, surgeons just focused on the safety of surgical procedure; however, PD is regarded as safe and widely applied to treatment of periampullary lesions. Practical issues after PD, such as, effect of duodenectomy, metabolic surgery-like effect, alignment effect of gastrointestinal continuity, and non-alcoholic fatty liver disease were summarized and discussed.

Key words: Pancreaticoduodenectomy; Delayed gastric emptying; Metabolic surgery; Exocrine insufficiency; Fatty liver; Postoperative pancreatic fistula

Core tip: In the past, pancreaticoduodenectomy (PD) should be avoided because of its extremely high morbidity and mortality. With the advance of surgical techniques and perioperative management, PD has been regarded as good choice for the treatment of periampullary pathologic conditions. In this moment, turning our interest to potential physiological change following PD may be necessary, because PD always results in removal of important internal organs in upper gastrointestinal tract and altering normal path of gastrointestinal flow. Well awareness of these "internal" changes will be helpful for proper management of the patients with PD.

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INTRODUCTION

In the past, it was thought that pancreaticoduodenectomy (PD) should be avoided because of its extremely high rates of morbidity (greater than 70%) and mortality (greater than 30%)[1]. More recently, many surgeons have focused on technical innovation to reduce postoperative severe morbidity after PD. Based on advancements in surgical experiences, perioperative management and interventional radiology, it is thought that most

Table 1 Experimental study showing the relationship between motilin and duodenectomy

Ref.	Year	Study design and model	Primary end point	Observations
Tanaka *et al*[74]	1987	Normal dog *vs* Duodenectomized dog	Phase Ⅲ contraction, plasma level of motilin	All control dogs showed characteristic MMC Duodenectomized dog showed non-typical, irregular and non-cyclic pattern of contraction Duodenectomized dog showed low plasma concentration of motilin without cyclical variation
Tanaka *et al*[75]	1988	Normal dog *vs* Duodenectomized dog	Inter-digestive gastric and small intestinal MMC plasma level of motilin and Polypeptide Y	MMC was abolished in duodenectomized dogs (3 out of 4 dogs) The other dogs showed intermittent cyclic, but markedly abnormal characteristics of gastric contraction Jejunal MMC appeared with short interval Duodenectomy abolished cyclic variation of plasma motilin and polypeptide Y
Suzuki *et al*[76]	2001	Conscious dog *vs* Duodenectomized dog	Phase Ⅲ contraction, plasma level of insulin, and motilin	Duodenectomy resulted in no phase Ⅲ contraction in upper GI tract Duodenectomy resulted in no fluctuation of plasma motilin (low level of motilin) Exogenous administration of motilin resulted in comparable response of phased Ⅲ as shown in control
Malfertheiner *et al*[77]	1989	Normal dog *vs* Duodenectomized dog	Pancreatic trypsin GI motility plasma motilin, PPY	In duodenectomized dog Trypsin secretion was not coordinated with inter-digestive motility, motilin, and PPY Inter-digestive motility was altered Plasma level of motilin and PPY were reduced, and showed no cyclic pattern
Itoh *et al*[78]	1976	Normal dog	GI motility plasma motilin	Gastrointestinal contractile activity in the conscious dog, Digestive states: motilin had no influence upon the motor activity Inter-digestive states: had influence upon the motor activity
Vantrappen *et al*[79]	1979	Human	GI motility plasma motilin level	The effect of exogenous motilin on interdigestive migrating motor complex Plasma motilin levels is one of the factor involved in the production of the activity front of the MMC in man
Sarna *et al*[80]	1983	Normal dog	Plasma motilin levels Migrating myoelectric complexes (MMCs)	Cause and effect relationship between plasma motilin levels and migrating myoelectric complexes Endogenous motilin does not initiate spontaneous mmcs MMC contractions release motilin

GI: Gastrointestinal.

complications related to PD can be managed in a conservative way. Based on the literature, mortality after PD is now considered to be 2%-5% and morbidity is reported to be 33%-64%[2-4]. PD recently has gained wide acceptance as a safe surgical method of choice for the treatment of periampullary pathological conditions.

PD consists of two surgical components: (1) resection phase: removal of pancreatic head, common bile duct, gallbladder, and duodenum along with some part of the proximal jejunum. Partial gastrectomy can be included; and (2) reconstruction phase: gastrointestinal continuity is created by pancreatico-enterostomy [pancreaticogastrostomy (PG) or pancreaticojejunostomy (PJ)], hepaticojejunostomy, and duodeno-or, gastro-jejunostomy.

When surgical technique is largely standardized, potential physiological changes following PD need to be concerned because PD results in the removal of important internal organs in the upper gastrointestinal tract and alters the normal path of the gastrointestinal flow. Therefore, surgeons who perform PD should be well aware of these "internal" challenges for proper management of patients with PD. Herein, the following issues will be discussed to understand the practical pathophysiological changes that occur after PD.

EFFECTS OF DUODENECTOMY

The duodenum is a source of various peptide hormones. Among them, motilin is a 22 amino acids peptide that is primarily localized in enterochromaffin cells of the duodenum and proximal jejunum[5], which is known to be responsible for phase Ⅲ activity of the gastroduodenal migrating motor complex (MMC)[5]. It was found that exogenous motilin could induce premature phase Ⅲ contraction in the upper gastrointestinal tract. Moreover, reduced plasma concentrations of motilin were associated with gastroparesis (Table 1). Therefore, PD can lead to the inevitable removal of the duodenum, which can reduce plasma levels of motilin, resulting in delayed gastric emptying (gastroparesis) by reducing coordinated stomach, duodenum and proximal jejunum movements.

Motilin is not yet available for clinical use. However, there is some clinical evidence to support these experiments and hypotheses. Naritomi *et al*[6] evaluated the first occurrence of MMC and motilin in patients with pylorus-preserving pancreaticoduodenectomy (PPPD) and duodenum-preserving pancreatic head resection (DPPHR). They found that the PPPD group required a longer amount of time for initial gastric phase Ⅲ

recovery, and the plasma levels of motilin were lower. Yeo *et al*[7] performed a prospective randomized placebo-controlled trial and found that erythromycin could significantly accelerate gastric emptying after PD and reduce the incidence of delayed gastric emptying (DGE) by 37%. Indeed, erythromycin can act as a motilin agonist by binding motilin receptors, and its clinical benefit to improve gastric emptying has been demonstrated in diabetic gastroparesis[8] and postvagotomy gastroparesis[9]. Matsunaga *et al*[10] also showed manometric evidence of improved early gastric stasis by erythromycin after PPPD. Administration of saline caused no changes in gastric or jejunal motility; however, erythromycin could induce phase Ⅲ-like gastric contraction and reduce the amount of gastric juice output in all patients.

Duodenectomy also influences on the secretion of other gastrointestinal hormones. Malfertheiner *et al*[11] showed that plasma levels of pancreatic polypeptide (PP) were altered with no cyclic pattern in duodenectomized dogs. Müller *et al*[12] evaluated changes in CCK, PP, and gastrin in PPPD and DPPHR patients. They found that PP was significantly reduced in both PPPD and DPPHR, and cholecystokinin (CCK) was reduced in an early postoperative period after PPPD. Tangoku *et al*[13], and Kingsnorth *et al*[14] evaluated plasma gastrin and CCK responses between standard PD and PPPD. Basal plasma levels of gastrin and CCK were significantly higher in controls compared with patients with standard PD ($P < 0.05$), suggesting that preservation of the stomach and part of the duodenum (pylorus-preserving) appeared to be a more physiological procedure for performing PD.

Regarding reduced gastrin levels following PD, it has been proposed that postoperative atrophic changes in the remnant pancreas after PD can be derived from removal of the duodenum and distal stomach because these organs are a source of gastric stimulation[15]. Jang *et al*[16] investigated the effects of induced hypergastrinemia on the prevention of pancreatic atrophy after PPPD. They performed a randomized control study and successfully demonstrated that induced hypergastrinemia by Lansoprazole could prevent postoperative volume change of the remnant pancreas and preserve long-term exocrine and endocrine function in patients with PPPD. This study is a good example to show how potential physiological changes can be translated into clinical practice for proper management of patients who undergo PD.

Furthermore, Chung *et al*[17] investigated the role of vagal and efferent adrenergic innervation to coordinate the gastric and small intestinal MMCs after removing the pylorus, duodenum, and upper jejunum in three dogs. They concluded that duodenectomy could reestablish gastric MMC-like activity without motilin, showing a peak after 1-4 mo, and it appeared to require extrinsic innervation. PD sometimes (depending on the surgeons' preference and disease extent) requires extensive soft tissue dissection around a major arterial system, including the celiac axis, common hepatic artery, and superior mesenteric artery for margin-negative resection. Too much dissection of soft tissue (for example, extended PD) can result in surgical denervation of visceral autonomic nerves and can be one of the reasons for transient delayed gastric emptying in a clinical setting[18,19].

Based on this brief review of the literature, it can be noted that duodenectomy not only disrupts the coordination of gastric and intestinal MMC but also disrupts the coordination between inter-digestive motility and pancreatic secretion and abolishes the inter-digestive cyclic variations in plasma gastrointestinal hormones, such as motilin, CCK, gastrin, and pancreatic polypeptide (PP). Additionally, extensive soft tissue dissection-induced disconnection of neural stimulation and secondary postoperative inflammatory insults can cause pathophysiological changes after PD, which can be attributed to a clinical delay in postoperative recovery.

METABOLIC SURGERY-LIKE EFFECTS

The bariatric surgical procedures were attempted to promote weight loss by restricting food intake and promoting malabsorption. The most commonly performed procedures were Roux-en-Y gastric bypass (46.6%), vertical sleeve gastrectomy (27.8%), adjustable gastric banding (17.8%), and biliopancreatic diversion with duodenal switch (2.2%)[20]. Interestingly, when looking at schematic figures showing PD, it could be noted that PD is somewhat similar in appearance to Roux-en-Y gastric bypass (Figure 1). The food passage after PD could be similar to that after Roux-en-Y gastric bypass, bypassing duodenum and passing directly into distal jejunum. Natural bile and pancreatic flow can be thought of as a Roux-en-Y loop in PD. Therefore, PD might cause the physiological changes that appear after bariatric surgery.

Notably, glucagon-like peptide-1 (GLP-1) is an interesting gastrointestinal hormone. After Roux-en-Y gastric bypass, GLP-1 is secreted by L cells of the small bowel, with higher concentrations in the distal ileum and colon. This peptide is produced in response to a meal and decreases food intake through its effects on the hypothalamus and brainstem. Additionally, GLP-1 is known to slow gastric emptying, inhibit glucagon release and stimulate the pancreas to secrete insulin (incretin effect)[21,22]. Recently, You *et al*[23] showed that about 30% of patients with PD were found to have hypertrophic changes in the remnant pancreas, and Wu *et al*[24] also reported resolution of diabetes after PD. They observed resolution of long-standing diabetes after PD in patients with (3, 9.1% of 33 patients, $P = 0.005$) and without (6, 9.8% of 61 patients) pancreatic cancer, suggesting that PD-associated anatomical changes might play an important role in the resolution of DM after PD.

Despite conflicting observations about GLP-1 levels

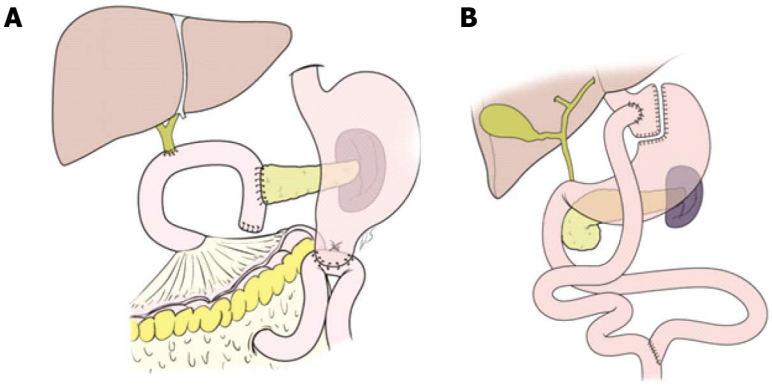

Figure 1 Schematic diagrams of pancreaticoduodenectomy and Roux-en-Y gastric bypass.

after PD[25], several studies have investigated changes in plasma GLP-1 levels after PD. Ohtsuka *et al*[26] previously showed that improved glucose metabolism after PD was mainly influenced by improved insulin resistance. They observed significantly increased plasma GLP-1 levels after PD; however, even after removal of the pancreatic head (reduced pancreatic volume), β-cell function did not change. Muscogiuri *et al*[27] evaluated the effect of duodenectomy on GLP-1 secretion after PD. They found that PPPD was associated with a remarkable increase in GLP-1 levels, which reached levels comparable with those observed after gastric bypass[28]. Harmuth *et al*[29] reported that conventional PD was associated with accelerated gastric emptying, enhanced postprandial GLP-1 release, and improved insulin sensitivity. The rapid transport of unabsorbed nutrients to the distal bowel triggers enhanced release of GLP-1, resulting in improved glycemic control.

Notably, GLP-1 agents used to control diabetes have been associated with an increased risk of pancreatic cancer in patients with type 2 diabetes[30]. However, a recent study demonstrated that GLP-1 could harbor anticancer properties against pancreatic cancer. GLP-1 receptor activation has anti-tumor effects on human pancreatic cancers *via* inhibition of the PI3K/Akt pathway[31]. Additionally, activation of the GLP-1 receptor was found to inhibit growth and promote apoptosis of human pancreatic cancer cells[32]. PD-induced GLP-1 release can be used for future treatment of resected pancreatic head cancer, although further investigations are warranted.

ALIGNMENT EFFECT OF GI CONTINUITY

In addition to the direct effects of removing organ by resection, pathophysiological changes after PD will also be influenced by how the gastrointestinal alignment is rearranged in the reconstructive phase. Various methods for reconstruction, similar to gastrointestinal alignment, have been reported in PD, such as Billroth Ⅰ (the Imanaga method)[33], Billroth Ⅱ (the Whipple and/or Child method)[34], Roux-en-Y loop fashion[35], an additional Braun anastomosis[36], and retrocolic/antecolic reconstruction[37]. In clinical practice, DGE appears to represent the pathophysiological changes that occur after PD. Conflicting observations have been reported about the incidence of DGE, and the exact mechanisms to explain the occurrence of DGE according to different reconstruction method remain to be determined. However, robust evidence is accumulating about the incidence of DGE according to different gastrointestinal reconstructive methods following PD (Table 2).

Short-term perioperative outcomes, such as postoperative complications, length of hospital stay, and resuming of acceptable diet, are the main concerns after PD. Miyakawa *et al*[38] demonstrated that fat absorption after Billroth Ⅰ PG (PG-Ⅰ) is superior to that after Billroth Ⅱ PJ (PJ-Ⅱ) in patients with disordered exocrine function of the pancreatic remnant, suggesting that PG-I allows for more effective utilization of the exocrine enzymes of the pancreatic remnant because of elimination of the blind loop characteristic of the PJ-Ⅱ. Ohtsuka *et al*[39] evaluated nutritional status and quality of life after PD, and compared these data between 18 patients with end-to-end (Imanaga) and 13 patients with end-to-side (Traverso) gastrointestinal reconstruction. They found that the scores of psychosocial conditions remained low, even over a long-term, in both groups. However, the values of nutritional parameters showed no significant difference between the two groups at each time point, suggesting that the postoperative quality of life and nutritional status were not different between Imanaga and Traverso reconstructions after PPPD. However, a paucity of high-level evidence exists about long-term outcomes, including nutritional outcomes and quality of patients' life, which could be influenced by potential pathophysiological changes after PD according to reconstruction methods.

Some recent trials showed that removal of the pylorus could result in a lower incidence of DGE. Matsumoto *et al*[40] performed a prospective randomized comparison between PPPD and modified classical PD, and assessed the effects stomach-preserving PD on

Table 2 Incidence of delayed gastric emptying according to different gastrointestinal reconstructive methods following pancreaticoduodenectomy

Ref.	Year	Study design	Primary end point	Observations
Eshuis *et al*[81]	2014	In PPPD Antecolic (n = 125) *vs* Retrocolic (n = 121)	DGE	No differences in DGE (45 patients (36%) *vs* 41 (34%), absolute risk difference: 2.1% (95%CI: -9.8-14.0) No differences in need for postoperative nutritional support, other complications, hospital mortality, and median length of hospital stay
Tamandl *et al*[82]	2014	In PPPD, antecolic (n = 36) *vs* retrocolic (n = 28)	DGE	No differences in DGE (17.6% *vs* 23.1%, P = 0.628) No differences in length of hospital stay [13.0 (10.0-17.5) *vs* 12.5 (11.0-17.0) days; P = 0.446], time to regular diet [5 (5-7) d *vs* 5 (4-6) d, P = 0.353], and NG tube requirement [4 (3-7) d *vs* 3 (3-5) d, P = 0.600]
Imamura *et al*[83]	2014	In PPPD, antecolic (n = 58) *vs* vertical retrocolic (n = 58)	DGE	No difference in DGE (12.1% *vs* 20.7%, P = 0.316) At postoperative 6 mo, DGE was accelerated in antecolic group At postoperative 12 mo, better postoperative weight recovery in vertical retrocolic group (93.8% ± 1.2% *vs* 98.5 % ± 1.3%, P = 0.015)
Tani *et al*[84]	2014	In PD, Conventional (n = 76) *vs* Isolated Roux-en-Y (n = 77)	POPF/DGE	No differences in DGE and POPF POPF: conventional (34%) *vs* isolated Roux-en-Y (33%), P = 0.909 DGE: conventional (12%) *vs* isolated Roux-en-Y (15%), P = 0.609
Shimoda *et al*[85]	2013	In SSPPD, Billroth Ⅱ (n = 52) *vs* Roux-en-Y (n = 49)	DGE	Lower DGE in Billroth Ⅱ: (5.7% *vs* 30.4%, P = 0.028) Shorter hospital stay in Billroth Ⅱ (31.6 ± 15.0 d *vs* 41.4 ± 20.5 d, P = 0.037) Significant association between POPF and DGE (P = 0.037)
Ke *et al*[86]	2013	In PD Continuous loop (n = 109) *vs* Roux-en-Y (n = 107)	DGE/POPF	No differences in DGE and POPF POPF: continuous loop (17.6%) *vs* Roux-en-Y (15.7%), P > 0.05 DGE: continuous loop (25%) *vs* Roux-en-Y (23%), P > 0.05
Gangavatiker *et al*[87]	2011	In conventional PD and PPPD Antecolic (n = 32) *vs* Retrocolic (n = 36)	DGE	No difference in DGE (34.4% *vs* 27.8%, P = 0.6)
Kurahara *et al*[88]	2011	In SSPPD, Antecolic (n = 24) *vs* retrocolic (n = 22)	DGE	Lower incidence of DGE in the antecolic group [20.8% *vs* 50%, P = 0.0364, especially in the incidence of DGE grade B/C (4.2% *vs* 27.3%, P = 0.0234)] Significantly shorter time to full resumption of diet in antecolic group No significant difference in other postoperative complications
Chijiiwa *et al*[89]	2009	In PPPD, Antecolic (n = 17) *vs* retrocolic (n = 18)	DGE	No difference in DGE DGE: 6% *vs* 22%, P = 0.34

PPPD: Pylorus-preserving pancreaticoduodenectomy; DGE: Delayed gastric emptying; PD: Pancreaticoduodenectomy; POPF: Postoperative pancreatic fistula.

Table 3 Definition of postoperative pancreatic fistula

	Postoperative pancreatic fistula		
Grade	A	B	C
General appearance (clinical condition)	Well	Often Well	Ill appearing, Bad
Medical or interventional approach	No	Yes or No	Yes
Postoperative radiologic finding (US/CT)	Negative	Negative or Positive	Positive
Long-time drainage (≥ 21 d)	No	Usually Yes	Yes
Reoperation	No	No	Yes
Mortality related to POPF	No	No	Possibly yes
Sign of infection	No	Yes	Yes
Sepsis	No	No	Yes
Readmission	No	Yes or No	Yes or No

US: Ultrasonography; CT: Computed tomographic scan; POPF: Postoperative pancreatic fistula.

postoperative DGE occurrence and long-term nutritional status. They observed that the incidence of DGE, as assessed by the International Study Group of Pancreatic Surgery, was similar (20% *vs* 12%, P = 0.414), and long-term nutritional status indicated by serum albumin levels, serum total cholesterol levels, and body mass index during the 3-year follow-up) were also comparable between the two groups. Similarly, Kawai *et al*[41] reported their prospective, randomized, controlled study comparing PPPD and Pylorus-resecting PD (PrPD), showing that PrPD was associated with a low incidence of DGE; however, during a 6-mo follow-up period, comparable outcomes for quality of life, weight loss, and nutritional status between the two groups were observed.

REMNANT PANCREATIC FUNCTION

Previously, most concerns after PD were postoperative pancreatic fistula, because it was one of the main causes of significant morbidity and mortality related to PD. However, with advances in surgical techniques,

Table 4 Recent clinical studies about fatty liver after pancreaticoduodenectomy

Ref.	Year	Patient number	Follow-up period (mo)	Definitions of NAFLD	Incidence of fatty liver, *n* (%)	Risk factors/observation
Song *et al*[90]	2011	228	16	When CTS-L was equal to or less than 10 HU When CTL/S was equal to or less than 0.9 HU	15 (7.8)	In multivariate analysis, Pancreatic fistula (HR = 3.332, P = 0.037) External pancreatic duct stent (HR = 4.530, P = 0.017)
Sato *et al*[91]	2014	110	6	Hepatic CT value of less than 40 HU	44 (40)	In multivariate analysis, Younger age (OR = 1.079, P = 0.002), Female (OR = 6.102, P < 0.001) Small remnant pancreatic volume (< 10 mL), OR = 4.109, P = 0.009 Suspicion infection on POD7-28 (OR = 3.109, P = 0.027)
Kato *et al*[92]	2010	54	7.7 ± 2.1	Hepatic CT value of less than 40 HU a	20 (37.0)	In multivariate analysis, Pancreatic adenocarcinoma (P < 0.05) Pancreatic resection line (left side of SMA, SMA/PV) (P < 0.01) Diarrhea (P < 0.05)
Nagai *et al*[71]	2014	361	6	When CTL/S was equal to or less than 0.9 HU	30 (8.3)	In patients with NAFLD, CTL/S ratio was significantly improved by pancrelipase treatment Nutritional status by total protein, albumin, and cholesterol was significantly improved by pancrelipase treatment Severe diarrhea was improved Malnutrition after PD might be cause for postoperative NAFLD
Ito *et al*[93]	2014	100	NA	When CTL/S was equal to or less than 0.9 HU	12 (12)	In multivariate analysis, Blood loss (HR = 1.001, P = 0.016)
Nakagawa *et al*[94]	2014	104	Median 7.7 (2.5-23.6)	When CTS-L was equal to or less than 10 HU	26 (25)	In multivariate analysis, Postoperative pancreatic exocrine insufficiency (HR = 4.16, P = 0.02)
Tanaka *et al*[72]	2011	60	12	When CTL/S was equal to or less than 0.9 HU When CTL/S was equal to or less than 0.9 HU	14 (23)	In multivariate analysis, Pancreatic head cancer (OR = 12.0, P = 0.006) *De novo* NAFLD after PD was associated with body weight loss and decreases in serum levels of albumin, cholinesterase, and total cholesterol After administration of pancreatic enzyme, body weight and serum concentrations of albumin, cholinesterase, and total cholesterol were markedly increased In addition, hepatic steatosis and serum AST and ALT levels were also significantly improved by treatment *De novo* NAFLD after PD was primarily caused by pancreatic exocrine insufficiency

NAFLD: Non-alcoholic fatty liver disease; NA: Not available; AST: Aspartate transaminase; PD: Pancreaticoduodenectomy.

perioperative management, and interventional radiology, most PD-related complications can now be managed by conservative methods, and surgeons have begun to focus on long-term functional outcomes after PD.

Several reports have shown a potential relationship between morphologic changes (pancreatic atrophy, stricture, and main pancreatic duct dilatation) and remnant pancreatic function after PD[42-46]. Notably, Lemaire *et al*[47] evaluated pancreatic function, pancreatic atrophy, and main pancreatic duct dilation in the remnant pancreas after PD. They found a significant reduction in pancreatic parenchymal thickness and increased dilation of the main pancreatic duct in remnant pancreas. Finally, pancreatic atrophy tended to develop over time, and all patients were reported to have reduced levels of fecal-1 elastase. Nakamura *et al*[48] also demonstrated reduced pancreatic parenchymal thickness (atrophy), which indicated pancreatic exocrine insufficiency after PD. Therefore, this morphological change can indirectly show the some aspects of exocrine function in the remnant pancreas remain after PD. Tomimaru *et al*[49] reported a significant atrophy of the pancreatic parenchyma that occurred postoperatively in the PG and PJ groups (P < 0.0001), but these changes were more severe in the PG group than in the PJ group (P = 0.0018), suggesting that PJ was preferable to PG after PD. Fang *et al*[50] evaluated the long-term morphological and functional outcomes of the remnant pancreas after PD. The pancreatic duct diameter in the remnant pancreas usually increased, but there was no

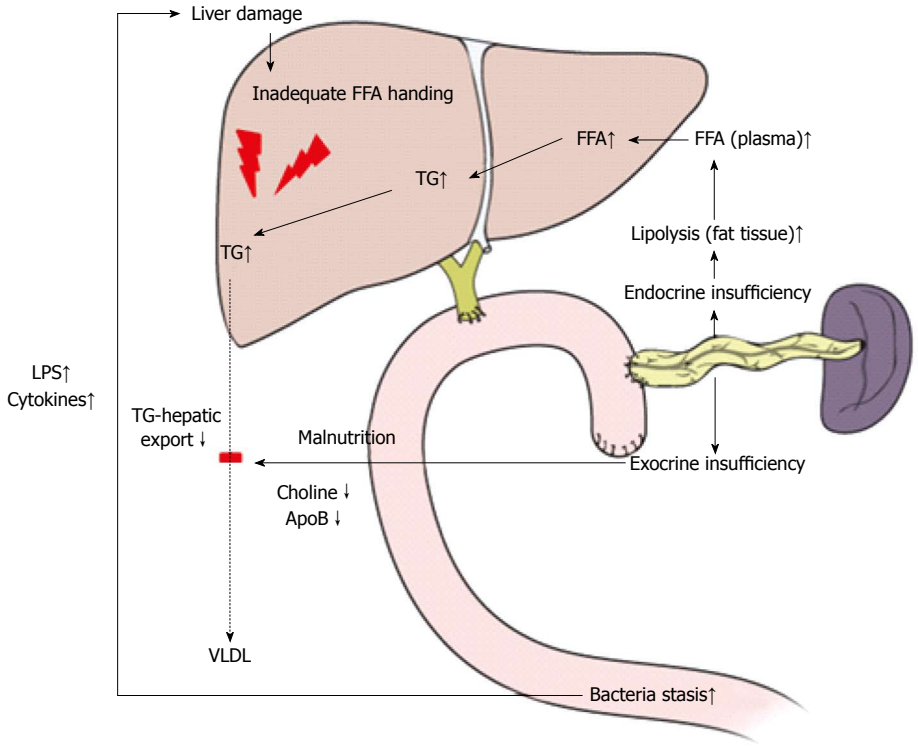

Figure 2 Mechanisms underlying non-alcoholic fatty liver disease after pancreaticoduodenectomy. FFA: Free fatty acid; TG: Triglyceride.

significant difference in the pancreatic duct diameter in both the PJ and PG groups, indicating that there was no significant difference in pancreatic exocrine or endocrine insufficiency, or pancreatic morphological changes. This evidence strongly suggests that the remnant pancreas following PD will have a chance to undergo atrophic changes and deteriorating exocrine pancreatic function after a long period of time.

Generally, there are two methods for remnant pancreatic reconstruction; PJ and PG. Several theoretical concerns exist regarding the functional outcome of the remnant pancreas following PD, which are as follows: (1) because of the absence of ampullary function, the remnant pancreas is thought to be vulnerable to regurgitation of gastrointestinal fluid into the main pancreatic duct. Most notably in PG, reflux of ingested food and low pH-gastric juice to the pancreatic duct can result in chronic inflammation, stenosis, and inactivation of pancreatic enzymes, leading to insufficiency of the remnant pancreas[51,52]; (2) in PJ, the easy activation of pancreatic enzymes can occur by intestinal enterokinase and an alkaline pH, resulting in irritating the remnant pancreas and clinically relevant pancreatic fistula[53]; and (3) reduced plasma levels of gastrin resulting from removal of the duodenum and distal part of stomach can affect atrophic changes of the remnant pancreas[15,16].

Interestingly, no significant difference in postoperative morbidity has been observed, even for postoperative pancreatic fistula[54] (POPF, Table 3), between PG and PJ[55-58]. However, a recent meta-analysis[59] demonstrated that PG was associated with lower postoperative pancreatic and biliary fistula rates in PD. One RCT dataset[60] showed that PG was related not only to a lower POPF rate but also to lower weight loss and better exocrine pancreatic function compared with PJ, suggesting that the "battle" between PG and PJ is ongoing. Most available reports on the functional outcome of the remnant pancreas following PD were based on retrospective study designs and limited numbers of patients. Most RCTs that tested PG and PJ focused on short-term perioperative outcomes, such as morbidity and mortality. Therefore, further evidence-based clinical investigations about remnant pancreatic function following PD should be performed.

NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is thought to be associated with excessive nutrition and is one of the most common forms of chronic liver disease[61]. This disease started to be reported in late 1980[62], and a few clinical investigations correlating fatty liver and PD reported that PD can influence hepatic fat content, which was associated with frequent hepatic steatosis[63,64]. In severe cases, even steatohepatitis leading to hepatic decompression can develop because of malnutrition after PD[65]. Therefore, surgeons need to be concerned about this condition, especially in patients expecting long-term survival following PD. Recent clinical studies of fatty liver after PD are summarized in Table 4.

The mechanisms underlying NAFLD after PD (Figure 2) might differ from usual NAFLD associated

with metabolic syndrome because NAFLD after PD was related to non-obese status, malnutrition, and a lack of hyperlipidemia or insulin resistance[66]. Most studies listed in Table 4 directly and indirectly suggest that malnutrition resulting from exocrine pancreatic insufficiency might cause NAFLD after PD. Pancreatic exocrine insufficiency induced malabsorption of essential amino acids, such as choline, which might result in the development of NAFLD after PD[67]. It has been shown that choline deficiency reduces plasma levels of apoprotein B[68], a major component very-low-density lipoprotein (VLDL), suggesting impaired hepatic export of TG in the form of VLDL. Insufficient secretion of insulin could play another role in the development of NAFLD after PD, which can enhance peripheral lipolysis and increase hepatic FFA uptake, and liver could have some difficulty in handling hepatic fat secretion by coupling triglyceride to apoprotein B[69], which plays an important role in secreting triglycerides from hepatocytes as VLDL particles. Overgrowth of small intestinal bacteria and hepatic stimulation of LPS[70] because of intestinal motor dysfunction and stasis can reduce the secretion of gastric juices and blind loops can also play an important role in NAFLD after PD. Therefore, NAFLD after PD represent the nutritional status of patients and is clinical reflection of the pathophysiological changes that occur after PD. Interestingly, NAFLD after PD is known to be associated with pancreatic cancer[71,72] and chemotherapy[73], so it will be interesting to investigate the potential correlation between the degree of post-hepatic steatosis and oncologic outcomes in resected pancreatic head cancer.

CONCLUSION

Previously, surgical techniques and safety were the only concerns regarding PD. This technique was regarded as one of the most complex and risky surgical procedures. However, as a consequence of advances in surgical experiences, techniques, and perioperative management, PD has become safer and the gold standard for treating periampullary pathologies. PD accompanies the removal of important organs and rearrangement of flow in the upper gastrointestinal tract, which can result in altered normal physiology and distinct clinical manifestations. In addition to proper surgical techniques, pancreatic surgeons need to understand these potential pathophysiological changes that can occur after PD for proper patients care in clinical practice. Further studies to link these potential pathophysiological changes with clinical outcomes will yield new insights to better understand how PD affects the lives of patients.

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REVIEW

Incidence and treatment of brain metastasis in patients with esophageal carcinoma

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Abstract

Brain metastasis from esophageal carcinoma (BMEC) is very rare, but its incidence has increased in the United States, Japan, China and other counties. Reports on BMEC have largely been focused on examining whether adjuvant therapy for esophageal cancer influences the survival duration of BMEC patients and on the imaging characteristics of BMEC determined using new medical equipment. The difference between different pathological types of esophageal cancer, especially adenocarcinoma and squamous cell carcinoma, is one important factor used to assess the influence of BMEC. Adjuvant therapy, including radiotherapy and chemotherapy, for esophageal cancer with different characteristics in different countries may affect BMEC treatment outcomes. The degree of popularization of advanced medical equipment is a major concern related to the prevalence of BMEC. Furthermore, targeted BMEC treatment is under development in developed countries. In this article, we reviewed the debate surrounding BMEC and analyzed BMEC studies from different perspectives.

Key words: Brain metastasis; Esophageal carcinoma; Magnetic resonance imagin; Computed tomography

Core tip: The incidence of brain metastasis from esophageal carcinoma (BMEC) is extremely low but has increased in recent years. The relevant reports on this unique disease have primarily focused on issues such as whether the auxiliary treatment of esophageal cancer promotes BMEC, the correlation of survival duration with different treatment methods, and imaging characteristics determined using various

imaging approaches. We reviewed the different perspectives of BMEC and compared BMEC studies performed in different countries.

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INTRODUCTION

Metastatic brain tumors are the most common type of brain tumor in adults. Brain metastasis (BMs) occur in approximately 25%-35% of malignancies[1]. Lung cancer (48%) and breast cancer (15%) are the primary metastatic brain tumors[2,3]. However, BM from esophageal carcinoma (BMEC) is extremely rare. According to Bartelt and colleagues, the incidence of BM from gastrointestinal tumors, including esophageal, gastric, and colorectal carcinomas, is less than 4%[4]. As the incidence of esophageal cancer has been reported to have gradually increased in the United States[5], the number of reports on BMEC has also increased in recent years[6]. Advances in neuroimaging such as computed tomography (CT) scanning and magnetic resonance imaging (MRI) have contributed to the early detection of BMEC. Esophageal tumor pathology can be classified as adenocarcinoma, squamous cell carcinoma, and small cell carcinoma, all of which are likely to metastasize to the brain. We searched the literature in PubMed and found 346 articles from 13 countries on BMEC, 321 of which were original articles (United States 144, Japan 103, China 31, China and Japan 6, Germany and Austria 21, and Germany 16) and 25 of which were case reports (Japan 5, United States 8, Turkey 1, Italy 4, Ireland 1, Australia 1, Iran 1, India 1, United Kingdom 1, Canada 1, China 1). The literature discusses the incidence, clinical characteristics, survival and some unusual phenotypes of BMEC.

It is generally thought that BM only occurs with esophageal cancer recurrence or in the advanced disease stage. However, BM also occurs in the early stage of the primary esophageal lesion[7]. Regarding the treatment of BM from lung cancer, BMEC is treated *via* surgery, radiotherapy (RT), and chemotherapy. Generally, the therapeutic approaches for BM are determined based on the type of metastasis, *i.e.*, single, limited or multiple BMs. If a patient has a limited number of metastatic brain tumors (generally 1-3 tumors or a small number of tumors that are close to each other) and if the primary cancer is under control, surgery is performed to confirm the diagnosis and to remove the tumor, followed by RT. RT may involve whole-brain RT (WBRT), stereotactic radiosurgery, or both. However, if the primary cancer is not well controlled, treatment includes WBRT and possibly chemotherapy. In general, the primary treatment for multiple metastatic brain tumors (or multiple tumors that are not close to each other) is WBRT. If no primary cancer site is found, surgery may be performed to obtain a tissue sample, which is likely followed by WBRT. There is growing interest in the efficacy of chemotherapy for metastatic brain tumors. However, many unresolved issues regarding BMEC diagnosis and treatment merit further exploration. This review summarizes the issues concerning the diagnosis and treatment of BMEC and discusses possible solutions for these issues.

INCIDENCE OF BMEC

In the 20th century, the most accurate method for confirming BMEC was autopsy; physicians were surprised by the initial discovery of BMEC based on autopsy. Since then, different groups have reported varying BMEC incidence rates: some have reported incidence rates of 1%-5%[8-11], whereas other studies based on autopsies of over 200 BMEC cases reported rates of 0%-0.9%[12-14]. However, it is difficult to determine the reliability of such reports because from the current perspective, it appears that finding tiny or single BMEC lesions during an autopsy is impossible. Therefore, the reliability of the incidence rates obtained during autopsy is debatable.

Historically, Dunlap was the first to report BMEC in one patient with esophageal cancer in 1932[15]. Since then, few new cases have been reported. In 1978, Irie and colleagues[16] stated that only four BMEC cases had been reported in the previous 50 years. This situation subsequently changed, and additional BMEC cases have been reported since CT imaging technology was applied in clinical practice in the 1970s. However, the BMEC cases initially detected on CT were considered as accidental events and not worthy of further exploration; thus, no relevant reports were published. In the 1980s, MRI provided a popular, powerful tool for diagnosing BM. It was difficult to detect small lesions in the brainstem and cerebellum using CT, but such lesions could be detected *via* MRI. Until recently, most BM cases were detected *via* MRI or CT. Of the BMs detected, only those treated surgically were pathologically confirmed.

Similar to the debate concerning the incidence of BMEC detected during autopsy, the incidence of BMEC based on CT or MRI remains controversial due to historical and technical issues. Regardless of the method used to determine the BMEC incidence, we cannot deny that there is an increasing number of BMEC patients in the clinic.

CHARACTERISTICS OF PATIENTS WITH BMEC IN DIFFERENT COUNTRIES

In previous years, most BMEC reports came from the

Table 1 Comparison of the clinical characteristics of brain metastasis from esophageal carcinoma patients in different countries *n* (%)

Clinical characteristic	Japan[19] *n* = 36 2003-2010	United States[20] *n* = 27 1993-2001	China[18] *n* = 31 2000-2012
Pathology			
Squamous cell carcinoma	23 (64)	2 (7)	26 (83.8)
Adenocarcinoma	5 (14)	22 (82)	3 (9.7)
Small cell carcinoma	5 (14)	0	2 (6.5)
Other	3 (8)	3 (11)	0
RPA class			
Ⅰ	3 (8)	2 (7)	9 (29)
Ⅱ	11 (31)	21 (78)	19 (61.3)
Ⅲ	22 (61)	4 (15)	3 (9.7)
Number of BMEC lesions			
Single	19 (53)	13 (48)	18 (58.1)
Multiple	17 (47)	14 (52)	13 (41.9)
Location of BMEC			
Cerebral hemisphere	25 (69)	5 (18)	47 (75.8)
Cerebellum	8 (22)		15 (24.2)
Other	3 (8)	22 (82)	
Other metastatic sites			
Yes	25 (69)	19 (70)	16 (51.6)
No	11 (31)	8 (30)	15 (48.4)
Treatment for BMEC			
Chemotherapy alone	3 (8)	0	3 (9.6)
RT alone	9 (25)	15 (56)	9 (29)
Surgery alone	12 (33)	6 (22)	
Surgery + RT	5 (14)	6 (22)	2 (6.5)
Surgery + chemotherapy	1 (3)		
Chemotherapy + RT	1 (3)		11 (35.5)
Surgery + RT + chemotherapy	0		4 (12.9)
Palliative care	5 (14)		2 (6.5)

BMEC: Brain metastasis from esophageal carcinoma.

United States and Japan, whereas fewer reports have come from China, where more cases of this disease have been found[17] but where the investigation has been focused on the diagnosis and treatment of BMEC[18]. The United States was the first to attach great importance to BMEC; this emphasis was followed by substantial research worldwide on the diagnosis and treatment of BMEC. However, one issue arose from those studies: the repeated submission of case reports by diligent Japanese doctors resulted in a large number of repeated cases being reported. Ogawa and colleagues[19] believed that excluding the 36 cases that they had reported, there were only 61 cases of BMEC in Japan prior to 2002, of which 35 cases were published in 13 English journals and the remaining 26 cases were published in 14 Japanese journals. If this is the case, retrospective studies on the diagnosis and treatment of BMEC based on previous literature would be very difficult. Thus, in this review, only the 36 cases reported in the Japanese literature after 2002 were included for comparison. As improved chemotherapy and novel chemotherapeutic agents have been used in the clinic in China, the overall survival of esophageal cancer patients has been prolonged to a certain extent, and more BMEC patients have sought clinical assistance. BMEC cases confirmed by autopsy in China remain rare because of local customs; Chinese patients are typically unwilling to permit autopsy after their death. A comparison of the clinical characteristics of BMEC cases in China[18], the United States[20], and Japan[19] is shown in Table 1; 31 Chinese patients were diagnosed and treated at our hospital. We have also included published reports from the MD Anderson Cancer Center in the United States in this review.

As shown in Table 1, it is evident that the pathological characterization of BMEC varies between the three countries. Adenocarcinoma was primarily detected in the United States, which is a Western country; squamous cell carcinoma was more common in Japan and China, which are Asian countries. Small cell esophageal cancer was identified in patients from both Japan and China, whereas more patients from the United States had multiple lesions, of which adenocarcinoma was the most frequent. In terms of therapy, standard treatment was commonly used in the United States, whereas additional therapeutic approaches were provided in Japan and China. For squamous cell carcinoma cases, Chinese patients were generally in good condition, whereas 61.1% of Japanese patients were in recursive partitioning analysis class Ⅲ.

The Japanese study records of 36 patients (1.4%) with BMEC who were treated between 1986 and 2000 were reviewed. A total of 2554 patients with esophageal carcinoma were treated at three different hospitals. An American study conducted at The University of Texas M. D. Anderson Cancer Center identified 1588 patients with primary esophageal carcinoma between June 1, 1993 and July 31, 2001. Of these patients, 27 (1.7%) were diagnosed with BMEC. A Chinese study was based on more than 10 000 patients with esophageal carcinoma from 1953 to 2003 at Zhejiang Cancer Hospital. Only 31 patients (approximately 0.3%) with BMEC were found. As mentioned above, autopsy performance and historical and technical factors are the key factors that explain the different incidences between the three countries.

AUXILIARY TREATMENT OF ESOPHAGEAL CANCER PROMOTES BMEC

Thomas *et al*[21] first proposed the hypothesis that the auxiliary treatment of esophageal cancer promotes BMEC in 2006. In their study, they included 403 patients with esophageal cancer who received only

esophageal radical resection and 369 patients who received adjuvant therapy in addition to radical resection from 1985 to 2002; the latter group included 118 patients who received preoperative adjuvant therapy, 124 patients who received postoperative adjuvant therapy, and 127 patients who received both forms of adjuvant therapy. The risk of BM occurrence between the two groups was compared. Years later, they found that 29 patients (6 from the control group and 23 from the intervention group) developed BM, 20 of whom developed BM within one year. The risk of BM occurrence was 2.5%, 4%, 7.0%, and 18.4% in the control, preoperative intervention, postoperative intervention, and preoperative and postoperative intervention groups, respectively. Thus, preoperative and postoperative adjuvant therapy and distant metastasis were identified as risk factors affecting the survival of BMEC patients. In that study, the overall median survival of BMEC patients was only 3.5 mo. Moreover, some authors proposed that not only the disease itself but also the adjuvant chemo-radiation therapy affects the development of BMEC. In 2007, Kawabata *et al*[22] reported a retrospective study of 254 esophageal cancer patients who received either surgery alone or surgery and adjuvant chemotherapy from 1984 to 2004. They showed that of the 73% of patients who received chemotherapy, 11 patients developed BM. The clinical stage and the performance of chemotherapy treatment during surgery were closely related to BMEC development. Furthermore, it is unclear whether patients receiving trimodal therapy should be screened for BM. In 2013[23], 518 esophageal adenocarcinoma patients receiving trimodal therapy were retrospectively analyzed. In that study, distant metastasis was found in 188 cases (36.3%), including 20 cases of BM (3.9%). Most patients (90%) with BM were diagnosed within 24 mo of surgery. Although 17 of these 20 patients received therapy for BM, their median overall survival was only 10.5 mo (95%CI: 6.6-14.0). It is difficult to detect BM by screening EC patients who receive adjuvant RT or chemotherapy because the incidence of BM after adjuvant therapy is relatively low. This conclusion should be considered with caution because patients who receive chemotherapy and adjuvant treatment are often in the very late stages of esophageal cancer (Ⅲ-Ⅳ), in which immune function may be impaired by the progression of the disease, resulting in tumor metastasis.

MISDIAGNOSIS OF BMEC THAT OCCURS EARLIER THAN THE DIAGNOSIS OF ESOPHAGEAL CANCER

BM typically occurs after treatment[4,6,7,18,20-23], although concurrent BM and EC have been rarely reported (less than 10 cases in the literature). In some individual case reports[7,24-26], especially in misdiagnosed cases[7,25,26], BM was found before the primary lesion of the esophagus was detected. In those cases, BM was often misdiagnosed as meningitis, a pituitary tumor, or glioma. BM predominantly occurs after treatment[4,6,7,18,20-23] based on the results of BM occurrence at 5[27], 6.7[17], 8.4[28], or 10 mo[29] after treatment, ranging from 4 to 57 mo. Clinical staging and adjuvant therapy are considered to be associated with BMEC, but the factors influencing the interval from treatment to BMEC occurrence have not been clarified.

UNCERTAIN VALUE OF THE UNIQUE IMAGING CHARACTERISTICS OF BM

Although there has been an increase in the number of reports on BMEC due to the clinical application of CT, few studies have analyzed the CT data from BMEC patients. In 1995, Gabrielsen *et al*[30] reported 334 esophageal cancer cases, which included 230 cases of adenocarcinoma (male:female ratio = 202:28) and 104 cases of squamous cell carcinoma (male: female ratio = 61:43). BM ultimately developed in only 10 cases of adenocarcinoma and two cases of squamous cell carcinoma. Therefore, it was believed that the incidence rate of BMEC in that study was low (only 3.6%). At that time, CT was not recommended as a routine examination tool due to its cost. Since their report was published nearly 20 years ago, many changes have been made in clinical diagnosis because of the fast-growing economy. In recent decades, additional CT findings of BM have been reported. Among those reports, that by Takeshima and colleagues[28] of CT data from eight BMEC cases is considered as a classic report even today. Their study reported that CT displayed enhanced thin-walled cystic lesions in four of six squamous cell carcinoma cases and in both cases of small cell carcinoma. They stated that the enhancement of thin-walled cystic lesions in the CT images was the primary characteristic of BMEC. Although many have cited this report in the past decade, the number of cases in these reports is smaller than the number of cases in the original report. Moreover, the MRI characteristics of BM from adenocarcinoma remain unknown. Thus, further investigations exploring the imaging characteristics of BM are needed. Additional imaging characteristics of BM aside from the thin-walled cystic contents and the enhanced thin-wall cystic lesions reported by Takeshima and colleagues are anticipated because BM is often mistaken for other diseases, as some BM cases do not exhibit any symptoms to support the diagnosis of BM.

VARIED SURVIVAL DURATION AFTER THE TREATMENT OF BMEC

The efficacy of BM treatment is an interesting topic. Until recently, there were no prospective randomized controlled clinical trials or comprehensive analyses

Table 2 Outcomes of different treatment methods

Study	Number of patients	Treatment	Median survival (mo)	Factors influencing prognosis
Ogawa *et al*[19]	12	SR + WBRT	9.6	Treatment plan
	24	WBRT	1.8	KPS score
Total	36 (MOS)		3.9	
Weinberg *et al*[20]	4	SR + WBRT	9.6	Liver metastasis
	6	SR	3.8	RPA class
	15	WBRT	3.9	
	2	SRT	2.5	
Total	27 (MOS)		3.8	
Wadhwa *et al*[23]	20 (MOS)		10.5	None
Kanemoto *et al*[27]	6	WBRT	1.6	None
	2	SR + WBRT	4.1	
	2	SRT	4.0	
	1	SRS	18.2	
	1	None	0.4	
Total	12 (MOS)		2.1	
Yoshida[36]	3	SR + WBRT	65.5	None
	7	SR	17.7	
	4	SRT	9.5	
	3	WBRT	27.1	
	(No MOS)			

MOS: Overall median survival; KPS: Karnofsky performance status; SR: Surgical resection; SRS: Stereotactic radiosurgery; SRT: Stereotactic radiotherapy; RPA: Recursive partitioning analysis; WBRT: Whole-brain radiation therapy.

involving a large cohort of BMEC cases demonstrating the effectiveness of different treatments. Moreover, many publications have reported varying results for different BM treatment approaches. In general, the BM treatment approaches include simple surgery, chemotherapy, RT, or a combination of two or all of these approaches. There are three types of surgery for BM: punch partial excisional biopsy[26], subtotal tumor removal[31], and single lesion resection[21-23]. Combined 5-fluorouracil (5-FU) and platinum is the most commonly used chemotherapy for BM. Other drugs such as doxorubicin, irinotecan, and cetuximab are also used[23]. RT includes WBRT, partial brain irradiation combined or not combined with WBRT, conformal RT, intensity-modulated RT, and stereotactic body RT[23]. The use of local heavy ion and proton irradiation has been reported in Japan. Chemotherapy can be administered before surgery, after surgery, or both. The primary tumor and metastatic lesions can be treated simultaneously in some patients in whom BM and esophageal cancer are detected simultaneously[26].

Some BM patients may abandon treatment for various reasons, leading to a short survival duration that ranges from 10 d to 2 mo[27]. Survival duration as a treatment outcome in several reports is summarized in Table 2. However, because of the limited number of cases, we were unable to compare the effectiveness of different treatments. There are a few reports of patients with esophageal cancer with longer survival durations, *e.g.*, exceeding 6 years and 11 years[32-36]. We treated a patient with squamous cell carcinoma who had survived for almost 14 years and who had undergone brain surgery and chemotherapy for BMEC that was discovered 15 mo after postoperative chemotherapy for esophageal cancer. At present, this is likely the longest survival duration among BMEC patients[18]. Prolonged survival confirms the hypothesis that surgery combined with RT and chemotherapy leads to better treatment effects for patients with a single brain lesion. The difference in the survival duration of patients with adenocarcinoma-associated BMEC and those with squamous cell carcinoma-associated BMEC has recently been debated[6]. This issue should not be overlooked. Regarding the mechanism underlying tumor metastasis, the primary tumor may develop simultaneously metastatic tumors in the brain and in other organs. Therefore, targeted therapy may also be effective for BMEC. Data from the Shizuoka Cancer Center in Japan[27] showed that BMEC patients with squamous cell carcinoma had a median survival duration of only 2.1 mo; the majority of these patients had multiple lesions, double primary cancers, and low Karnofsky performance status scores. That study also reported that the examined patients died soon after treatment, indicating the association of survival with the time at which MRI was performed. In that study, MRI was performed after the BM patients had developed symptoms from the lesion, *i.e.*, in the very late stage of BM.

Small cell carcinoma is the most common type of esophageal cancer in regions such as China and Japan. Currently, there is a great deal of information regarding this disease in China[37]. Small cell esophageal carcinoma is more aggressive than esophageal adenocarcinoma or squamous cell carcinoma[38]. Among the 31 patients with BMEC at our hospital[18], two patients with small cell esophageal cancer had multiple lesions in the brain and metastatic tumors in other organs. One patient in particular had multiple metastatic tumors in the cerebrum, the cerebellum, the brainstem, and the spinal cord. These patients exhibited neurological symptoms during treatment of their esophageal lesion. RT displayed a higher treatment efficacy in the patients with small cell esophageal cancer. After treatment, the cerebral spinal lesions disappeared from CT or MRI scans. This finding indicates the primary difference between small cell esophageal cancer and esophageal adenocarcinoma or squamous cell carcinoma. Given its many cystic lesions, esophageal adenocarcinoma and squamous cell carcinoma do not respond to RT.

POTENT TARGETED THERAPY FOR BMEC TREATMENT

As the majority of BM of gastroesophageal junction cancer is adenocarcinoma, it is possible to use targeted therapy to treat BMEC in the same manner as it is used to treat lung cancer. For example, Geldart

and Astras[39] reported in 2011 that one case of adenocarcinoma BM in which lesions developed rapidly did not respond to chemotherapy. However, after using trastuzumab combined with chemotherapy, the lesion in the brain shrank and was controlled.

In 2012, Abu *et al*[40] performed a retrospective study of 142 cases of esophageal cancer in the past 10 years. In that report, the overexpression of human epidermal growth factor receptor type 2 (HER2) was detected *via* immunohistochemistry in five (56%) out of nine patients with BM. The authors suggested that HER2 overexpression correlated with postoperative BM. In 2013, Preusser *et al*[41] reported a similar study involving 21 cases of esophageal cancer and BM in which only one case was squamous cell carcinoma and all of the others were adenocarcinoma. Among these tumors, three (14%), seven (33%), nine (43%), 18 (86%), and 0 were positive for HER2, epidermal growth factor receptor (EGFR), phosphorylated signal transducer and activator of transcription 3 (pSTAT3), hypoxia-inducible factor-1α (HIF1-α), and BRAF V600E, respectively. Moreover, the median Ki-67 index was 59%, and the microvessel density was 20/21 (95%). That study also showed that HER2 and EGFR expression correlated with the primary tumor and brain lesions, suggesting that HER2 and EGFR might be angiogenic factors that can be used as targets for the treatment of BMEC. In 2014, Niu *et al*[42] reported a HER2-positive esophageal adenocarcinoma patient who was treated with trastuzumab and lapatinib; brain metastasis occurred after the liver metastases responded well to treatment. Negative HER2 expression was detected in the brain lesion. Another study reported a young patient (33-year-old) with Williams Syndrome, a multisystem neurodevelopmental disorder[43], and concomitant esophageal cancer who ultimately developed BM[24]. The patient was diagnosed based on hemizygosity for 7q11.23, as assessed by FISH. The report proposed the hypothesis that a genetic abnormality may cause BM.

CONCLUSIVE EVIDENCE FOR EC METASTASIS TO THE BRAIN

The development of BM occasionally results in unusual phenotypes that warrant investigation. In the report by Santeufemia *et al*[32], a 51-year-old female patient with stage Ⅱ esophageal cancer (PT2N0M0) developed metastatic tumors, including a 3 cm × 3 cm nodule in the left breast and a 1 cm × 1 cm nodule in the right frontal cortex six months after surgery. After one cycle of chemotherapy, the left breast nodule shrank, and excision of the intracranial and breast metastatic lesions was subsequently performed. The patient had subsequently survived for 11 years when the case was reported. An increasing incidence of cancer of unknown primary (CUP) has been observed. For most CUP patients with BM, the primary tumor is difficult to detect, but evidence can be found in some cases. In a case of BM with initial neural symptoms, the postoperative pathological examination of brain lesions showed that they were poorly differentiated adenocarcinoma and ganglioneuroma. Subsequently, the primary esophageal lesions were identified[44]. This case may have been the first example of BM coincident with ganglioneuroma. As another example, a 72-year-old male patient with both prostate and esophageal cancer[45] underwent brain lesion resection, and the pathological findings comprised a 2.5-cm cerebellar lesion containing both esophageal and prostate cancer components.

In conclusion, studies on BMEC are limited, and these studies have primarily been performed in Japan and the United States (a total of 260 cases). Moreover, studies in this field are not as thorough as those of lung cancer. Due to the issue of inconsistent data, it is difficult to compare the results provided by different hospitals in different countries. In recent years, additional attention has been focused on BMEC development and its diagnosis and treatment. In 2014, the median survival of thirty BMEC patients who underwent gamma knife radiosurgery was only 4.2 mo[46]. We expect that more joint projects, including basic and clinical studies, on BMEC will be conducted by researchers in different countries. We believe that further investigation of the diagnosis and treatment of BMEC will benefit many patients with esophageal cancer.

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Liver involvement in pediatric celiac disease

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Abstract

Celiac disease (CD) is an intestinal inflammatory disease that manifests in genetically susceptible individuals when exposed to dietary gluten. It is a common chronic disorder, with a prevalence of 1% in Europe and North America. Although the disease primarily affects the gut, the clinical spectrum of CD is remarkably varied, and the disease can affect many extraintestinal organs and systems, including the liver. The hepatic dysfunction presenting in CD ranges from asymptomatic liver enzyme elevations or nonspecific reactive hepatitis (cryptogenic liver disorders), to chronic liver disease. In this article, we review the clinical presentations and possible mechanisms of CD-related liver injury to identify strategies for the diagnosis and treatment of these disorders in childhood.

Key words: Celiac disease; Cryptogenic hypertransaminasemia; Autoimmune liver disease; End-stage liver disease; Fatty liver

Core tip: Celiac disease (CD) is increasingly reported in children who are symptomless or present atypical symptoms and signs. Liver abnormalities are common extraintestinal manifestations in patients with CD and range from mild hepatic injury to severe liver disease. Awareness of this may help clinicians to improve strategies for the diagnosis and treatment of these disorders in childhood.

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INTRODUCTION

Celiac disease (CD) is a chronic intestinal inflammatory disease that manifests in genetically susceptible individuals when exposed to dietary gluten[1]. The prevalence of CD is high in the European and North American population (1%), reaching 10% to 15% in patients who have first-degree relatives with this disease[1,2]. Genetic predisposition plays an important

role in the development of CD. Ninety percent of affected individuals carry the HLA-DQ2 (*e.g.*, DQA1*0501-DQB1*0201) haplotype, 5% the DQ8 haplotype (*e.g.*, DQA1*0301-DQB1*0302), and the remaining 5% carry at least one of the two DQ2 alleles (frequently the DQB1*0201)[1,3]. Ingestion of gluten is necessary for the disease to develop[4]. Immunogenic peptides, created by deamidation of food-derived gliadin peptides by small intestinal tissue transglutaminase, are presented by antigen-presenting cells, mostly dendritic cells bearing HLA-DQ2 and DQ8 molecules, to proinflammatory $CD4^+$ T cells, activating them[4]. Upon activation, the T cell produces a variety of cytokines like interferon-gamma as part of a Th1 response which results in clonal expansion of activated T cells, stimulation of cytotoxic T cells and B cell recruitment with subsequent production of anti-gliadin (AGA) and anti-transglutaminase antibodies (tTGA)[4]. Thus, intolerance to gluten is responsible for an immune-mediated damage of the intestinal mucosa, which resolves after a gluten-free diet (GFD)[4].

CD diagnosis still relies on serology and small intestinal biopsy. tTGA and anti-endomysial antibodies (EMA) of the immunoglobulin A (IgA) class have the highest diagnostic accuracy with a sensitivity of 98% and a specificity ranging from 90% to 99%. Deamidated gliadin peptide antibodies (DGP) of IgG class are a valuable diagnostic tool for identifying CD in patients with IgA deficiency and in children aged less than 2 years. Small bowel biopsy remains in adults the diagnostic gold standard, whereas in children and adolescents, as recently recommended, CD diagnosis can be accepted without the need for duodenal biopsy in symptomatic cases showing tTGA at high titer (> 10-times upper normal limit), backed up by EMA and HLA-DQ2 and/or positive DQ8[3].

Although CD primarily affects the gut, the clinical manifestations of the disease are remarkably wide, with many extraintestinal organs and systems, including the liver, affected[5,6]. Liver changes in patients with CD have been reported since 1977 by Hagander *et al*[7] who demonstrated that transaminases were often increased in untreated CD, normalizing upon a strict GFD. More recently, studies performed after CD was identified as an autoimmune disease, have underlined the strong relationship between CD and autoimmune liver disorders. In this article, we review the clinical presentations and possible mechanisms of CD-related liver injury in order to identify strategies for the diagnosis and treatment of these disorders in childhood.

CRYPTOGENIC LIVER DISORDER (CELIAC HEPATITIS)

An association between CD and cryptogenic liver damage was first reported in 1977 by Hagander *et al*[7] who found that 40% of adults with incipient CD had increased serum concentrations of transaminases, which returned to normal upon GFD in the majority of patients. One year later, Lindberg *et al*[8] reported elevation of serum aminotransferases in about one-third of pediatric patients with CD. Approximately one decade later, a mild to moderate hypertransaminasemia was observed in about 60% of symptomatic Italian children aged less than 2 years with newly diagnosed CD[9]. Prevalence studies have reported that transaminases are elevated in 39% to 47% of celiac adults[10-12] and in 26% to 57% of children at diagnosis of CD (Table 1)[9,13-15]. Frequently, elevation in transaminases is mild, and is not associated with hepatomegaly or splenomegaly. In those patients who had undergone liver biopsy[10,16-18], histological changes such as Kupffer cell hyperplasia, mononuclear cell infiltration, steatosis, and mild fibrosis have been reported. In most cases, transaminase values normalized upon a 1-year GFD.

Conversely, CD is present in patients investigated because of chronic unexplained hypertransaminasemia. Volta *et al*[18] for the first time reported that adults with elevated concentrations of aminotransferases of unknown origin were affected by symptomless CD. Five of the 55 study patients with cryptogenic elevation of transaminases fulfilled the criteria for CD diagnosis. Other common causes of liver disease were excluded. Three of these patients showed histologically a picture of reactive hepatitis typical of CD patients with elevated transaminases. The importance of these findings has been confirmed by other investigators, who found a similar prevalence of CD in large patient populations with cryptogenic hypertransaminasemia[19].

Recently, Sainsbury *et al*[20] conducted a meta-analysis to estimate the prevalence of CD in adults with cryptogenic hypertransaminasemia, as well as the prevalence of hypertransaminasemia in those with incipient CD. The combined proportion with positive celiac serology and biopsy-proven CD in unexplained hypertransaminasemia were 6% (95%CI: 3%-10%) and 4% (1%-7%), respectively. However, there was significant heterogeneity between studies ($P < 0.001$). This is about four times the risk of CD, in the general population (about 1%)[20]. The combined proportion with abnormal serum aminotransferases in incipient CD was 27% (13%-44%). A 12-mo GFD normalized serum transaminase values in 63%-90% of patients. Discordant results were reported by Korpimäki *et al*[21] in a large population-based study including celiac patients with minor or atypical symptoms, and with or without GFD, as well as subjects without CD. The authors estimated that only 11% of the untreated celiac patients had elevated transaminase values. This prevalence was about the same as was found in treated CD cases and controls without CD. Variation in the CD clinical presentation and severity, as well as definition of the upper normal limits for serum transaminases may account for such discrepancies.

Table 1 Studies reporting the prevalence of cryptogenic hypertransaminasemia in children and adolescents with celiac disease

Ref.	Study design	Study population with CD	Diagnosis of CD	Number of patients with elevated transaminases	Effect of GFD	Comment
Bonamico *et al*[9], 1986	Observational	65 untreated symptomatic children aged 6-mo to 18 yr	Intestinal biopsy	37 (56.9%) had elevated (> 45 U/L) ALT (3.1%) or AST (29.2%) or both (24.6%)	Only 5 cases had a follow-up for 3-4 wk after GFD: normalization of transaminases was achieved in all	Excluded were Hepatitis A and B, but not other causes of liver disease
Farre *et al*[13], 2002	Prospective	114 untreated symptomatic children aged 9-mo to 17 yr	Serology (EMA IgA or IgG and tTGA IgA) and/or intestinal biopsy	37 (32.0%) had elevated[1] ALT-or- AST (14.9%) or both (14.9%)	35 of 37 had a follow-up for 9-18 mo after GFD: normalization of transaminases was achieved in all	
Arslan *et al*[14], 2005	Observational	27 untreated symptomatic children with a mean age of 6 (SD 5) years	Serology (EMA IgA and AGA IgA/IgG) and/or intestinal biopsy	7 (25.9%) had elevated ALT (> 45 U/L)	All patients had normalization of transaminases after 2-11 mo of GFD	
Di Biase *et al*[15], 2010	Prospective	350 untreated children with suspected CD aged 1 to 16 yr	Serology and intestinal biopsy according to the ESPGHAN criteria	140 (40.0%) had elevated AST (≥ 38 U/L) and/or ALT (≥ 41 U/L); four with values > 5 times upper normal levels	Normalization of transaminases after 6 mo of GFD was achieved in 133 (97.8%) of 136 children with transaminase values < 5 times upper normal levels	The four children with transaminase values > 5 times upper normal levels as well as the 3 children with persistent elevated transaminases had further laboratory investigation and were found to be affected by autoimmune hepatitis

[1]Normal reference values for AST < 50 U/L from 1 to 6 years, < 38 U/L from 6 to 18 years; for ALT < 31 U/L from 1 to 18 years. CD: Celiac disease; GFD: Gluten-free diet; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IgA: Immunoglobulin A; IgG: Immunoglobulin G; EMA: Anti-endomysial antibodies; tTGA: Anti-tissue transglutaminase antibodies; AGA: Anti-gliadin antibodies; ESPGHAN: European Society for Paediatric Gastroenterology, Hepatology and Nutrition.

Also in children, hypertransaminasemia may represent the only manifestation of CD. In 1986 an 11-year-old girl with a chronic and unexplained elevated aminotransferases was reported. Liver histology evidenced slight inflammation of the portal tract[22]. CD was diagnosed on the basis of antireticulin antibodies and subsequently by intestinal biopsy. Seven years later six children with chronic hypertransaminasemia and histologic findings ranging from reactive hepatitis to moderately active chronic hepatitis, were reported[23]. They were asymptomatic and had jejunal histology consistent with CD diagnosis. In all subjects, transaminases normalized on a GFD. Resolution of hepatic histologic lesions occurred in two children, whereas amino-transferases increased in three children upon a gluten challenge[23]. Finally, in a prospective study involving 425 children and adolescents with isolated hypertransaminasemia, Iorio *et al*[24] found 166 patients with persistently (more than 6 mo) elevated transaminases of whom three (1.8%) were identified as having CD. Therefore, routine screening for CD is to be recommended in children with otherwise unexplained hypertransaminasemia.

AUTOIMMUNE LIVER DISORDERS ASSOCIATED WITH CELIAC DISEASE

Autoimmune liver disorders (AILD), including autoimmune hepatitis (AIH), primary sclerosing cholangitis (PSC), and primary biliary cirrhosis (PBC) have been shown to be associated with CD[25-28].

AIH is a progressive inflammatory liver disorder and is more common among females. It is associated serologically with high levels of aminotransferases and IgG, the presence of autoantibodies, and histologically with interface hepatitis in the absence of known etiology[29]. Hepatitis at the portal-parenchymal interface ("interface hepatitis") is typical. The picture is characterized by a lymphoplasmacytic infiltrate crossing the limiting plate and invading the liver parenchyma. Other associated lesions are hepatocyte swelling and pycnotic necrosis. Fibrosis is found in all forms of the disease except the mildest ones[30]. Two types of AIH can be recognized: type 1 AIH is associated with antinuclear antibodies and/or smooth muscle antibodies and affects adult patients much more commonly, while type 2 AIH, characterized by antibodies to liver-kidney microsome type 1, is usually

Table 2 Studies reporting the prevalence of positive celiac serology or biopsy-proven celiac disease in children and adolescents with autoimmune liver diseases

Ref.	Study design	Study population with AILD	Number of patients with CD	Effect of GFD
Caprai *et al*[39], 2008	Retrospective	140 patients aged 7-125 mo with AILD	23 (16.4%) (19 with AIH; 2 with AIC; and 2 with overlap syndrome) had CD on the basis of serology (EMA IgA and/or tTGA IgA) Diagnosis of CD preceded the diagnosis of liver disease in 18 of the 23 patients	All patients achieved remission on GFD and immunosuppressive therapy, but 14 relapsed because of discontinuation of therapy or during spontaneous gluten challenge
Diamanti *et al*[40], 2008	Retrospective	40 patients aged 3-13.2 yr with AIH	5 (12.5%) had CD on the basis of serology and histological findings In four patients CD was diagnosed after AIH onset	On GFD four patients showed a mild decrease in transaminases, but never a complete normalization
Tosun *et al*[41], 2010	Retrospective	15 patients aged 4-15 yr with AIH	7 (46.0%) had CD on the basis of serology and histological findings CD and AIH were diagnosed concomitantly	Not available
El-Shabrawi *et al*[42], 2011	Prospective	26 patients aged 3.5-21 yr with AIH	CD serology (tTGA IgA and/or EMA IgA) was positive in 4 (15.4%). Three out of these four AIH (11.5%) showed histological findings of CD	Not available
Nastasio *et al*[43], 2013	Retrospective and Prospective	79 children and adolescents with AIH	15 (19.0%) had CD on the basis of serology and histological findings Diagnosis of CD preceded the diagnosis of liver disease in 8 of the 15 patients	All 15 patients on GFD achieved sustained remission when treated with immunosuppressive therapy

AILD: Autoimmune liver diseases; CD: Celiac disease; GFD: Gluten-free diet; AIH: Autoimmune hepatitis; EMA: Anti-endomysial antibodies; tTGA: Anti-tissue transglutaminase antibodies.

confined to childhood CD[18,31].

In the late 1970s, CD was occasionally reported in patients with AIH[18,32-34]. Then several studies established a relationship between CD and AIH of both types 1 and 2[26]. The first of these studies included the largest cohort of AIH patients (*e.g.*, 181, of whom 157 with type 1 and 24 with type 2) who were screened for CD by serology[18]. Among these patients, eight [4.4% (3.8% with type 1 and 8.3% with type 2 AIH)] were found to have raised levels of EMA IgA. Of these 8 antibody-positive patients, five underwent jejunal biopsy which revealed a subtotal villous atrophy typical of CD. In a recent systematic review[26] performed in adults, the prevalence of CD in AIH ranged between 2% and 20% but was approximately 4% in most studies.

In children, at first the association between CD and AIH was only reported in isolated cases[35-37]. Subsequently pediatric surveys have reported a wide prevalence of CD in AIH ranging from 3.6% to 12% (Table 2)[38-43]. In an Italian retrospective (1990-2005) multicenter study, Caprai *et al*[39] found that among 140 children with AILD, 23 (16%) had CD [19 with AIH (12 with type 1; 4 with type 2; 3 seronegative), 2 with autoimmune cholangitis and 2 with overlap syndrome]. CD was diagnosed before liver disease in 18 of them, though raised aminotransferases were found in 16 at CD diagnosis. Conversely, five of the 23 patients had a diagnosis of AILD before the identification of CD. Nineteen patients had liver-related non-organ-specific autoantibodies. Hepatic biopsy showed inflammatory lesions with features of autoimmune damage and different degrees of fibrosis in all 19 subjects and cirrhosis in 4 of them. All patients on GFD achieved remission on immunosuppressive therapy, but 14 relapsed either because treatment ceased or because the GFD was not respected. Diamanti *et al*[40] retrospectively (1990-2006) evaluated the CD prevalence in 40 AIH children. There were five cases of CD in the 40 AIH patients (12.5%); all five CD patients had type 1 AIH. In four patients (80%), AIH preceded the diagnosis of CD. On GFD the level of transaminases mildly decreased, and never reached normal concentrations. Tosun *et al*[41] who retrospectively evaluated the presence of CD in 15 AIH patients, found a prevalence of 46% (95%CI: 21%-67%), being the highest ever reported in pediatric literature, although the sample size is small. In a prospective study involving 26 Egyptian patients (aged 3.5-21 years) with AIH, El-Shabrawi *et al*[42] reported an 11.5% prevalence of CD. Very recently, in a retrospective and prospective evaluation (1995-2000), Nastasio *et al*[43] reported that among 79 patients with AIH, CD was present in 15 (19%) of them (9 had type 1, 3 type 2, and 3 were seronegative). All these patients achieved sustained remission on a GFD when treated with immunosuppressive therapy.

There are two studies providing prospective data on AIH in children with CD (Table 3)[15,44]. Di Biase *et al*[15] showed that isolated hypertransaminasemia was present in 40% of CD subjects on a gluten-containing diet, and that 2% had AIH, while there were no other AILD. Liver tests became normal after GFD only in CD patients with isolated hypertransaminasemia, but not in AIH cases who required GFD plus immunosuppressant therapy. Ventura *et al*[44] showed that AILD were more frequent in adolescents and young adults with CD than in the general population. In particular, out of 374 CD patients 10 (1.1%) had a diagnosis of AIH. They also

Table 3 Studies reporting the prevalence of autoimmune hepatitis in children and adolescents with celiac disease

Ref.	Study design	Study population with CD	Diagnosis of CD	Number of patients with AIH	Effect of GFD
Ventura *et al*[44], 1999	Prospective	909 children and adolescents with CD (group 1, < 2 yr of age; group 2, 2-10 yr; group 3, > 10 yr)	Serology and intestinal biopsy according to the ESPGHAN criteria	10 (1.1%) had AIH, of whom 2.9% in group 2 and 0.8% in group 3	Not available
Di Biase *et al*[15], 2010	Prospective	350 untreated children with suspected CD aged 1 to 16 yr	Serology and intestinal biopsy according to the ESPGHAN criteria	7 (2.0%) had AIH, of whom 5 type I AIH	During treatment with GFD, steroids and azathioprine for 5 yr, all AIH persistently normalized clinical and biochemical parameters. After withdrawal, 6 patients maintained a sustained remission (12-63 mo)

CD: Celiac disease; AIH: Autoimmune hepatitis; GFD: Gluten-free diet; ESPGHAN: European Society for Paediatric Gastroenterology, Hepatology and Nutrition.

reported that in patients with CD, AILD rates increased as age at diagnosis increased, suggesting a possible relationship with duration of exposure to gluten[44].

PSC is a cholestatic disorder characterized by inflammation and periductal fibrosis of the intrahepatic and/or extrahepatic bile ducts[45-47]. No characteristic autoantibody has been identified in PSC patients. The diagnosis depends on evidencing the characteristic biliary lesions in biopsy tissue or the intra and extrahepatic biliary tree abnormalities by cholangiography[47]. Many patients, especially children, have PSC-AIH overlap with features of both diseases, and this is termed autoimmune sclerosing cholangitis (ASC)[46,48]. ASC refers to cases with PSC who have positive autoantibodies and may have histological features overlapping with those seen in AIH[47]. In adults, PBC may also be found. This additional form of AILD is characterized by the presence of anti-mitochondrial antibodies. It progresses slowly and is more common in females. Histologically, PBC is characterized by portal inflammation and immune-mediated destruction of the intrahepatic bile ducts. Autoimmune cholangitis (AIC) is a cholestatic liver disorder with biochemical signs of cholestasis, histological features of inflammatory bile duct damage, and negativity for anti-mitochondrial antibodies. PSC, PBC, and AIC have been mainly described in adults with CD[21,49-53]. In children, the association between CD and PSC or AIH/ASC overlap syndrome or AIC has been only reported in two studies[39,54].

NONALCOHOLIC FATTY LIVER DISEASE/ NONALCOHOLIC STEATOHEPATITIS

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver conditions ranging from simple, uncomplicated steatosis, to nonalcoholic steatohepatitis (NASH), with inflammation and liver cell injury progressive to cryptogenic cirrhosis. NAFLD has become the most common cause of chronic liver disease in children and adolescents. Case reports and cross-sectional studies describe the association of various forms of fatty liver with CD[55-60]. Wigg *et al*[55] found that 3 of 22 adult patients with NASH had positive AGA IgA and IgG, and one of them had a histological diagnosis of CD. Grieco *et al*[56] reported histologically-diagnosed CD in 4 (13.3%) of 30 patients with laboratory diagnosis of NASH. After one year on GFD, the transaminase levels were normalized, and duodenal histology was improved. Nehra *et al*[57] investigating the relationship between NASH and CD, found that only one (2.1%) of the 47 study obese patients with NASH was positive for EMA IgA. In a study of 59 overweight patients undergoing liver biopsy for persistent hypertransaminasemia, NASH was detected in 38 (64%) whereas simple steatosis was found in 21 (36%)[58]. Six (10%) of the 59 patients showed positivity for tTGA and two (3.4%) of them also positivity for EMA IgA. Histology confirmed CD in the two patients positive for both markers. In both cases, liver enzymes went back to normal after a 6-mo GFD. In a study involving 121 patients with biopsy-proven NAFLD, Lo Iacono *et al*[59] reported that the prevalence of histologically-confirmed CD was 3.3%. In an Iranian population of 116 patients with NAFLD (as diagnosed on the basis of elevated transaminase levels, liver ultrasound and/or liver biopsy), Rahimi *et al*[60] found the prevalence of histologically-confirmed CD to be 2.2%. Interestingly, CD was more commonly diagnosed among NAFLD patients having body mass index (BMI) < 27 kg/m² compared to those with BMI > 27 kg/m² (5.83% *vs* 0%, $P = 0.001$). Very recently, in a nationwide study of more than 26000 children and adults with CD, Reilly *et al*[61] found an increased risk of NAFLD compared to the general population. Excess risks were highest in the first year after CD diagnosis, but persisted through 15 years beyond diagnosis with CD.

On the basis of the above findings, we conclude that there is an association between CD and fatty liver. However, since fatty liver is not an unusual finding in the general population of developed countries, the association of hepatic steatosis with CD may be a coincidental finding rather than a true association.

To complicate matters further, fatty infiltration of the liver may be secondary to rapid weight loss or malabsorption, both etiologically linked to fatty liver. Future investigations should be undertaken to resolve this issue and should include pediatric populations for whom there are very few data at present.

SEVERE LIVER DAMAGE

Although rarely, severe liver disease has been described in adults with CD[62-64]. In a Finnish study, 4 patients with severe liver failure awaiting liver transplantation were discovered to have CD (one had congenital liver fibrosis; one, a massive hepatic steatosis; and two patients had progressive hepatitis with no apparent cause)[62]. Their liver disease improved after GFD. The Authors then screened 185 patients undergoing liver transplantation and found that 8 (4.3%) of them had CD, which is 4-10 times the population prevalence of CD in Finland. Most of these patients had AILD. Only 1 patient was on GFD. This suggests that in some cases of CD, GFD help to avoid end-stage liver disease. Subsequently, in a study from United States involving an ample cohort of individuals with end-stage AILD (n = 310) and non-AILD (n = 178) who underwent liver transplantation[64], the prevalence of tTGA and EMA was significantly greater in HLA-DQ2- or HLA-DQ8-positive patients with end-stage AILD compared with those with end-stage non-AILD (14.2% *vs* 5.4%, P = 0.0001 and 4.3% *vs* 0.78%, P = 0.01, respectively), while the co-occurrence of tTGA and EMA was increased five-fold in end-stage AILD (3% *vs* 0.6%). However, the study was retrospective, and apart from two patients, intestinal tissues were not available for re-review. Thus, a definite diagnosis of CD was not possible for most of the patients positive for CD-related autoantibodies. When serum samples were tested 6-12 or ⩾ 24 mo post-transplantation, tTGA and EMA became normal in 94% and 100% of patients, respectively. This occurred without excluding gluten from the diet which implies no relationship between gluten and autoantibody kinetics. The suppression of tTGA and EMA after the transplant suggests that the lack of autoantibody positivity of post-transplant sera cannot exclude a diagnosis of CD, therefore supporting the pre-transplantation screening of patients with end-stage AILD[64].

In children, severe liver disease has been described in association with CD[65-68]. Demir *et al*[65] reported five celiac children with cryptogenic cirrhosis. In three patients with chronic diarrhea and hepatosplenomegaly, the diagnoses of CD and cirrhosis were concomitant, whereas in two patients, CD was diagnosed following that of cirrhosis. One to five years later, three patients on strict GDF had normal values of serum aminotransferases, and clinical improvement. The other two patients with poor dietary compliance had no improvement in liver function. Al-Hussaini *et al*[66] reported an 11-year-old girl with liver failure due to sclerosing cholangitis associated with CD. Treatment with ursodeoxycholic acid and GFD, and steroid tapered over three months, normalized the liver function tests. A few cases of CD with severe liver involvement requiring liver transplant have been also reported[67,68]. In a case-report, Pavone *et al*[67] described a 14-year-old girl with CD and mild gastrointestinal symptoms developing, after a long exposure to gluten, severe hepatic dysfunction requiring liver transplantation. Casswall *et al*[68] reported six 13- to 36-mo-old girls who within 1-24 mo of the diagnosis of CD developed severe liver damage. Four of these girls had acute liver failure and two needed a liver transplant.

PATHOGENESIS OF LIVER DYSFUNCTION IN CD

The pathogenesis of the hypertransaminasemia and liver damage in CD remains poorly understood. Probably they involve increased intestinal permeability and alterations in gut microbiota, chronic intestinal inflammation, and genetic predisposition (Figure 1).

Since the liver receives three quarters of its blood supply from the intestine, it is one of the organs most exposed to gut-derived toxic factors[69-72]. Cross-talk between the gut and the liver is an intriguing hypothesis that may explain the hepatobiliary changes associated with many intestinal inflammatory diseases including CD. The suggestion that increased intestinal permeability and altered gut microbiota may contribute to the development of several diseases was made since 1890 (Llewellyn Jones: "Theory of auto-intoxication from gut bacteria")[72]. Gut epithelial cells are linked to one another with tight junctions (TJs), which play an essential role in maintaining the integrity of the intestinal barrier and in demarcating microbes in the gut from the host immune system. Zonulin, a human protein known to reversibly regulate intestinal permeability by modulating intercellular TJs[73], is augmented in autoimmune conditions associated with TJ dysfunction including CD[74].

Patients with CD and hypertransaminasemia have an important increase in intestinal permeability compared with those whose liver enzymes are normal[11]. The increased intestinal permeability may ease the entry of toxins, antigens, and inflammatory substances (cytokines and/or autoantibodies) to the portal circulation and these mediators may play a part in the pathogenesis of hepatic involvement in CD. Interestingly, increased intestinal permeability caused by disruption of intercellular TJs in the intestine as well as increased prevalence of small intestinal overgrowth has been reported in adult patients with NAFLD[75]. Moreover, it has been found that serum zonulin concentration is increased in children and adolescents with NAFLD and correlates with the severity of steatosis[76]. This may also explain hepatic fat deposition in CD. Autoantibodies directed against

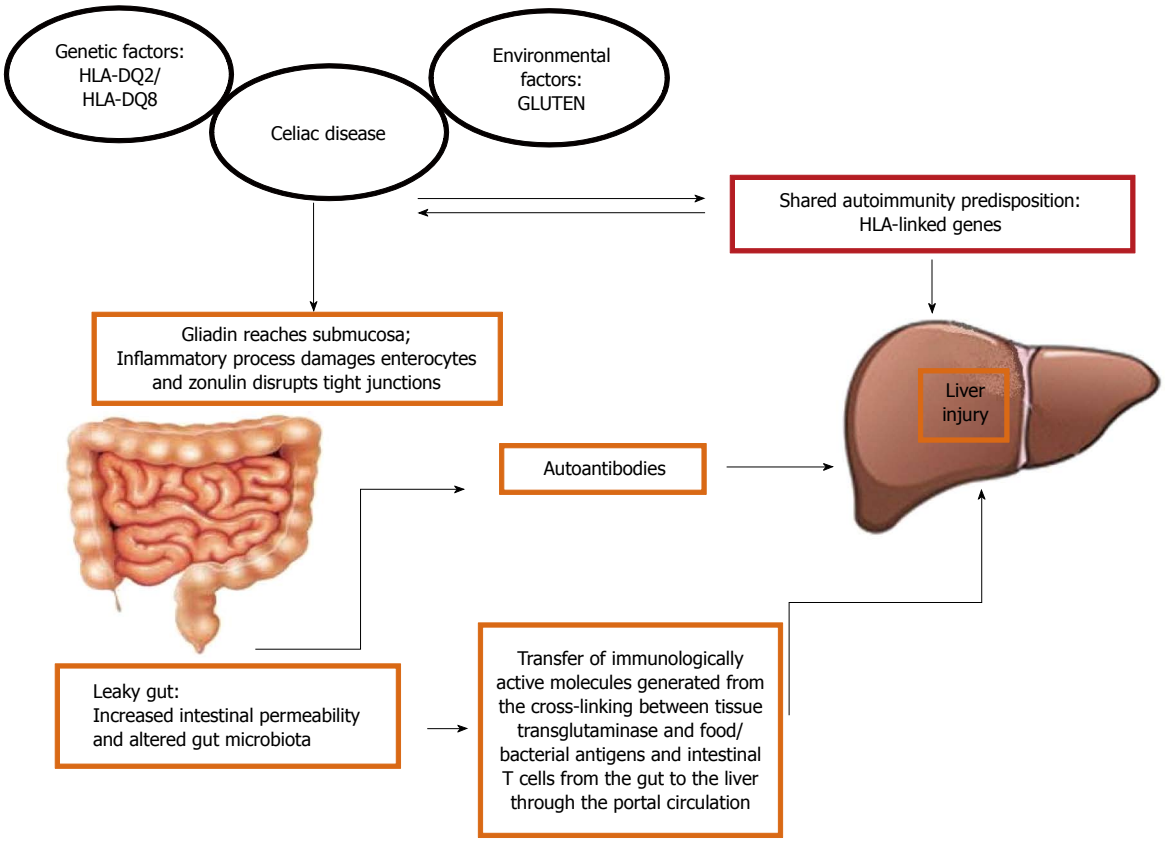

Figure 1 Possible pathogenetic mechanisms between celiac disease and liver abnormalities.

tTG are present in the liver and other extraintestinal tissues in CD. This raises the possibility of a pathogenic role for the humoral-mediated immune responses in liver injury observed in CD. It has also been suggested that an aberrant T lymphocyte homing to the liver may contribute to trigger immune hepatic damage. As matter of fact, an increased number of lymphocytes expressing molecules of intestinal origin have been discovered in hepatic sinusoidal endothelial cells in individuals with liver abnormalities[77]. Moreover, liver-primed T cells have been demonstrated to migrate into the intestine and into the gut-associated lymphoid tissue, suggesting an enterohepatic lymphocyte circulation[78]. The ability of T cells of homing both to the liver and the intestine may explain the link between CD and liver diseases.

Considerable progress has been made toward understanding the role of genetics in autoimmune liver damage. It is well known that CD and some autoimmune liver disorders share HLA class Ⅱ molecules and haplotypes. The main genetic marker of CD is HLA-DQ2, which is present in about 95% of CD patients. HLA-DQ2 is in strong linkage disequilibrium with HLA-DR3, which is the major HLA risk factor for AIH[79].

CONCLUSION

CD is increasingly reported in children who are symptomless or present atypical symptoms and signs. Liver abnormalities are common extraintestinal manifestations in patients with CD and range from mild hepatic injury to severe liver disease. The so-called celiac hepatitis is a frequent, benign, clinically silent condition which resolves on a GFD. Autoimmune liver diseases are less common and are associated in the majority of cases with clinical signs and symptoms of chronic liver disease, which need specific immunosuppressive therapy, rather than just GFD. Although rarely, CD may be also associated with severe liver involvement requiring liver transplant. In light of this background early diagnosis and treatment of CD-associated chronic and severe liver diseases may play an important role in the prognosis of this clinical entities. To this end, screening for liver involvement in celiac children and for CD by means of tTGA and EMA in children with liver diseases should become routine practice.

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Contribution of the IL-17/IL-23 axis to the pathogenesis of inflammatory bowel disease

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Abstract

Inflammatory bowel diseases (IBDs) are chronic disorders of modern society, requiring management strategies aimed at prolonging an active life and establishing the exact etiology and pathogenesis. These idiopathic diseases have environmental, genetic, immunologic, inflammatory, and oxidative stress components. On the one hand, recent advances have shown that abnormal immune reactions against the microorganisms of the intestinal flora are responsible for the inflammation in genetically susceptible individuals. On the other hand, in addition to T helper cell-type (Th) 1 and Th2 immune responses, other subsets of T cells, namely regulatory T cells and Th17 maintained by IL-23 are likely to develop IBD. IL-23 acts on innate immune system members and also facilitates the expansion and maintenance of Th17 cells. The IL-17/IL-23 axis is relevant in IBD pathogenesis both in human and experimental studies. Novel biomarkers of IBD could be calprotectin, microRNAs, and serum proinflammatory cytokines. An efficient strategy for IBD therapy is represented by the combination of IL-17A and IL-17F in acute IL-17A knockout TNBS-induced colitis, and also definite decrease of the inflammatory process in IL-17F knockout, DSS-induced colitis have been observed. Studying the correlation between innate and adaptive immune systems, we hope to obtain a focused review

in order to facilitate future approaches aimed at elucidating the immunological mechanisms that control gut inflammation.

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

Core tip: The pathogenesis of inflammatory bowel diseases (Crohn's disease and ulcerative colitis) is multifactorial and still not completely understood. There is a need to identify new diagnostic biomarkers as well as an efficient therapy for these diseases. A better understanding of the immunological mechanisms that control gut inflammation, is of high clinical importance because it offers the possibility to develop new drugs, which attack the key pro-inflammatory pathways in chronic intestinal inflammation.

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INTRODUCTION

Inflammatory bowel diseases (IBD) including ulcerative colitis (UC) and Crohn's disease (CD) require a better knowledge of the exact autoimmune mechanisms involved in their pathogenesis[1]. A better understanding of the pathogenesis of IBD will allow the better control of the inappropriate and continuing inflammatory response to commensal microbes in a genetically susceptible host[2-5] . In addition, in CD pathogenesis the "dialogue" between the host and intestinal microbiota includes the defensins and dysbiosis as well as the hemostatic system[6]. The pathways for intestinal homeostasis include: epithelial restitution, barrier function, antimicrobial defense, innate and adaptive immunity, "reactive oxygen species" (ROS) generation and the metabolic pathway[4].

IMMUNOLOGICAL MECHANISMS IN IBD

Immune tolerance, inflammation, mucosal pathways and/or epithelial restitution are promoted by intra- and intercellular networks using plastic cellular programs responsible for intestinal homeostasis[7].

UC is characterized by inflammation limited to the colon as a result of a disturbance of the normal immune control of the gut symbiotic bacteria. By contrast, CD involves any part of the gastrointestinal tract. Chronic inflammation involves the submucosal and mucosal layers leading to bleeding, abdominal pain, diarrhea and malnourishment. The gut wall permeability is also increased. The Peyer's patches of the small intestine could be the site of the immune tolerance breakdown to the microorganisms of the intestinal flora[4].

The immune system is made of two compartments: innate and acquired. The function of the non-specific system is to recognize all external agents and also act against them without specificity and memory. The mucosa is the first place where pathogens/allergens encounter polymorphonuclear neutrophils, which directly recognize the PAMP (pathogen associated molecular pattern) through pattern recognition receptors such as Toll-like receptors (TLRs). The key factor between TLRs and neutrophils interactions are ROS. There are at least 10 variants of TLRs expressed on the surface of neutrophils, macrophages, dendritic cells and, to a lesser extent, lymphocytes. Through these types of receptors, the innate immune response has a certain specificity compared to the high degree of variability present on the surface molecules of the pathogenic agents[8].

First, abnormalities of innate immunity in association with epithelial barrier dysfunction could be the "key" point to the initiation of the mucosal inflammation[9,10]. Besides the recognition role, the innate immune response mediated by neutrophils, natural killer (NK) cells, monocytes/macrophages, dendritic cells and the complement system has other important functions such as phagocytosis and direct cell cytotoxicity or Antibody-Dependent Cell Cytotoxicity (ADCC). Moreover, NK cells and NKT cells exhibit cytotoxic activity in cancerous cells. Although the innate immune system is extremely effective as a first line of defense against many aggressors, it also causes multiple collateral effects by producing antibacterial free radicals. This is the point where the specific adaptive immune system intervenes[8].

Second, the adaptive immunity has classically been considered to play a crucial role in the pathogenesis of IBD. The acquired immune response is ensured by T and B lymphocytes. The adaptive immune response eliminates specific pathogens through humoral and cellular response[8]. However, recent research in immunology and genetics has shown that the innate immune response is equally important in inducing inflammation in IBD patients. It has been highlighted that an altered epithelial barrier function contributes to the intestinal inflammation in UC, while aberrant innate immune responses, such as autophagy, innate microbial sensing and antimicrobial peptide production are associated with CD pathogenesis[3].

The immunology of IBD represents an imbalance between two types of T cells populations: regulatory T cells (Treg) and pro- inflammatory T cells (Figure 1).

Besides Th1 and Th2, two other subsets have

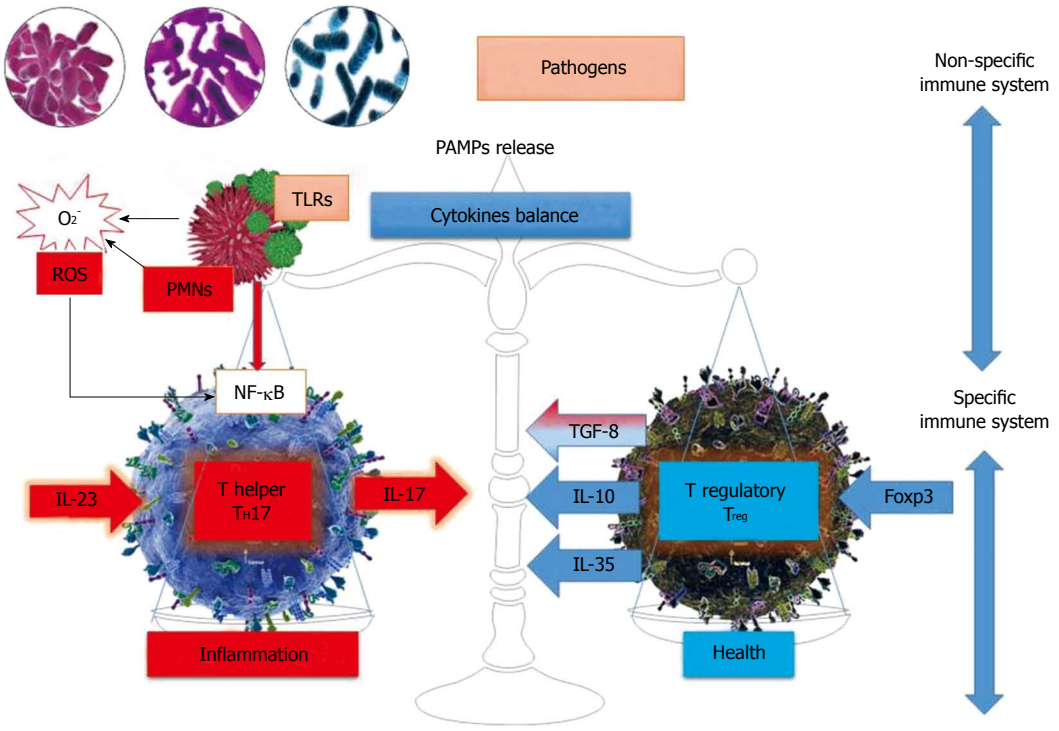

Figure 1 Immunological Balance in inflammatory bowel diseases. IL: Interleukin; PAMPs: Pathogen-associated molecular pattern molecules; Treg: regulatory T cells; Th17: T helper 17 cell; Foxp3: Forkhead box P3; TLRs: Toll-like receptors; ROS: Reactive oxygen species; PMNs: Polymorphonuclear leukocytes.

recently been described: $CD4^+CD25^+$T regulatory cells and Th17 cells (Table 1). Th17 cells expressing RORγ1 favor the occurrence of allergic reactions and autoimmune phenomena by producing IL-17 cytokines. Therefore, the Th17/Treg balance prevents or promotes inflammation and autoimmune diseases. After activation, naïve CD4-T cells differentiate into three subsets of Th effector cells, each subset having a unique cytotoxic profile and specific biological functions. Treg cells that differentiate in the presence of the Foxp3 transcription factor (90-100 aminoacids that form a DNA binding motif) produce anti-inflammatory mediators such as IL-10 and TGF-β, whose function is to preserve immune tolerance and homeostasis[8]. In addition to natural thymic Treg cells, Treg could be induced in the periphery under specific conditions. Th17 and iTregs are closely related so that they can generate one another under the control of TGF-β maintaining their balance in the context of IBD[10,11].

The most commonly described effector T cells are Th1, Th17 in CD and Th2 in UC, Th17 being responsible for the IL-17 production, a key pro-inflammatory cytokine that is increased in IBD[12]. Conversely, given the redundancy of effector and regulatory pathways leading to IBD lesions and the Th17 cells protective functions, the neutralization of IL-17A did not diminish the inflammatory process in CD[3]. In addition, effector T-cells and cytokines have been targets of many recently developed treatment agents for CD[13].

The first interaction between the innate and the acquired systems occurs when antigen-presenting cells (APCs), which are part of the innate system, present antigens to the T cells belonging to the innate system[8]. "Polarization" of adaptive immune responses was firstly presented in 1986[14]. This hypothesis was that on encountering an antigen presented by APC, naïve $CD4^+$T-cells acquired a unique effector immunophenotype, representing the final stage of non-reversible differentiation and being defined by specific effector cytokines[15,16].

The Th cell polarization model is promoted by cytokines which derive from the innate immune system recognizing microbe-associated molecular patterns (MAMPs) and establishing proinflammatory antimicrobial responses[11].

The Th1 *vs* Th2 "polarization model" was presented twenty-five years ago in the context of responses against infectious agents. It is generally accepted that a deregulation in the immune response towards the gut flora is responsible for the intestinal chronic inflammation in genetically predisposed individuals. Caspase recruitment domain 9 (CARD9) is a nonredundant adapter protein that conveys signal information downstream of pattern recognition receptors. CARD9 has also been associated with autoinflammatory disorders members and with

Table 1 Main characteristics of CD4 T-cell subsets

CD4 T-cell subsets	Surface expression	Polarizing Cytokines	Master regulator transcription factor	STAT regulators	Effector profile/signature cytokines
Th1	IL-12RB2 IFN-γR Tim3	IL-12, IFN-γ, IL-27	T-bet	STAT1, STAT4	IFN-γ, IL-2, TNF-α, IL-10
Th2	IL-17RB Tim1	IL-4, IL-25, IL-6	GATA-3 MAF	STAT6	IL-4, IL-5, IL-13, IL-25, IL-2, IL-10
Th17	IL-1R1 IL-12RB IL-23R CCR6 CD161 IL-13Ra1	TGF-β, IL-6, IL-1β, IL-21, IL-23	RORγt, RORα,	STAT3	IL-17A, IL-17F, Il-21, IL-22
TREG	CD25 CD39 CD73 CD101 CD127	TGF-β	Foxp3	STAT5	TGF-β, IL-10
TFh	CD84 CXCR5 IL-6R IL-21R gp130	IL-6, IL-1β, TNFα IL-21 CXCL13	Bcl6 IRF4, c-Maf, Batf,	STAT3/5	IL-6, IL-2, IL-10, IL-21

TFh: Follicular B helper T cells found in the B cell follicles; STAT3: Signal transducer and activator of transcription 3/5; Foxp3- forkhead box P3, a key transcription factor controlling T regulatory cell (Treg); t-bet: T-box transcription factor TBX21 which initiates Th1 lineage development from naïve Th precursor cells; GATA-3: GATA binding protein 3, an important regulator of T cell development; MAF: Proto-oncogene c-Maf or V-maf musculoaponeurotic fibrosarcoma oncogene homolog; Batf: B-cell-activating transcription factor required for the differentiation of IL-17-producing Th17 cells and T_{FH} cells; Bcl6- B-cell-activating transcription factor; Tim-3: T-cell immunoglobulin domain and mucin domain 3, a negative regulator of Th1-cell responses, CXC13- C-X-C motif chemokine 13.

the activation of NF-κB family, enhancing the Th17 cytokines production[17]. Chronic inflammation is coordinated by the oxidative stress manifested by the increase in pro-inflammatory cytokines encoded by NF-κB genes[8].

The "polarization model" was also used to explain the immunophenotype of non-infectious inflammatory conditions. Expression studies on the signature cytokines demonstrated the crucial role of IL-23, Il-17 and IL-6 in the development of experimental colitis, Th17 cells being involved in disease propagation. Moreover, IL-23 promotes the expansion and maintenance of Th17 cells, which secrete the proinflammatory cytokine IL-17 involved in the pathogenesis of many chronic inflammatory disorders. Other studies have shown that IL-23 acts on the cells of the innate immune system and contributes to the inflammatory cytokine production and tissue inflammation. Therefore, the role of the IL-23/ IL-17 axis in the pathogenesis of IBD chronic intestinal inflammation has been highlighted both in animal and human studies, this pathway being relevant especially in the pathogenesis of Crohn's disease[17].

The combinations of cytokines, which initiate the Th17 polarization process as well as the functional stability of Th17 lineage, have been extensively studied[18]. In humans, the initial development of Th17 cells is induced by TGF-β and IL-6 synergism as well as the presence of low levels of TGF-β and IL-1β[19]. The second stage is the expansion of the Th17 population under the activation of IL-21. This stage is followed by the stabilization phase driven by the pro-inflammatory cytokine IL-23, generating the cytokine profile for the CD4 Th17cell subset which has a unique property named plasticity[20]. In other words, the functions of Th17 cells depend on the immunological environment in which they developed.

The main pro-inflammatory function of the Th17cells is ensured *via* IL-17 secretion. From this point of view and according to the "polarization model", effector lineage is generally exclusive. To be more precise, an inflammatory state can be either Th-17 or Th-1-driven. However, it has recently been shown that Th17 cells acquire the ability to upregulate t-bet (transcription factor) and produce IFN-γ, generating IL-17/ IFN-γ double positive cells, Th1/ Th17[21].

Such a population with the ability to produce both IL-17 and IFN-γ is also present in the inflamed gut mucosa[22]. Moreover, Th1- and Th17-mediated signaling may act in synergy and have deleterious effects on the gut mucosa, Th1-effects being more prominent during the chronic phase of CD as compared to the Th17 signaling pathway[23].

IL-23 is also upregulated in the active disease. However, all the necessary constituent elements for the IL-23/Th17 polarization process are to be found only in the chronic inflammatory lesions of CD[24].

In addition, this unique population may differentiate into specific Th1 IFN-γ producing cells, thus excluding the Th17 population[25-27]. The extraordinary T17 cells plasticity, confirmed in experimental and clinical studies, could be explained in at least three different ways: firstly, by inducing Th1 cells- IFN-γ secretion, secondly by expressing T-bet and producing IFN-γ in addition to IL-17, and thirdly completely trans-differentiating into Th1-subtype, IFN-γ producing cells expressing CD161, the surface marker of Th17 cells progenitors. Moreover, Th17-derived Th17/Th1 and Th1 cells, rather than Th17 cells alone, have an important function in IBD[11,20].

Th17 cells also possess a regulatory function recently described as human IL-17-producing Foxp3$^+$/RORγt double positive T-cells[19]. In conclusion, the effector lymphocytes are not terminally differentiated. They may differentiate into regulatory pathways and alternative effectors under the local immunological pressure[28]. In patients with IBD the prevalence of circulating Foxp3 DE CD4 (+) and IL-17 T cells is increased and the coexpression of Foxp3 and RORγt in these cells requires conversion from Treg cells to Th17 cells associated with a decreased suppressive function of Foxp3 CD4(+) T lymphocytes[29].

GENETIC CORRELATIONS

Only three genetic polymorphisms have a pathogenetic role in IBD. The first one is related to NOD2 and mainly occurs in Caucasians[4,30]. GWAS (genome-wide association studies) for CD show the genes associated with CD risk such as ATG16L1 (autophagy 16-like 1), IRGM (immunity- related guanosine triphosphatase M) and LRRK2 (leucine-rich repeat kinase 2)[31].

GWAS have identified certain polymorphisms relevant to CD which are detected in genes encoding for IL-23/Th17 pathway proteins. The most important of IL-23r gene is the Arg381Gln polymorphism which confers protection against developing IBD. Conversely, the polymorphism associated with significantly elevated IL-17A mRNA transcripts is IL-17a variant IVS1+ 18 G>C[32]. The strategy of treatment is also affected by the gene polymorphisms, the IL-23R genotype status determining the early response to anti-TNF[33].

BIOMARKERS FOR INTESTINAL INFLAMMATION

Inflammation is the "key" player in IBD, elevated levels of serum tumor necrosis factor alpha, TNF-α and CRP (C-reactive protein) being associated with CD[34]. Mucosal biomarkers in IBD as predictors of response to therapy and disease severity are: cytokines, adhesion molecules, intracellular markers of activation, immune and non immune cells and other factors (TLRs, mucin, MUC, G6PD, Glucose-6-phosphate dehydrogenase)[7]. Serum and colonic omentin-1, a newly discovered adipokine, acts as an anti-inflammatory agent as well as a new biomarker for the CD evolution, its correlation with the disease activity being superior to that of CRP[35].

Calprotectin is a more recently established marker for intestinal inflammation. This paper revealed the present knowledge on fecal calprotectin testing as predictor of intestinal inflammation. In inflammatory bowel disease, the calprotectin fecal test shows higher intensity values[36].

Thirty prospective studies confirmed that fecal calprotectin from granulocytes and macrophages released by activated innate immunity had been a real value in the diagnosis and disease activity evaluation, reaching a sensitivity and specificity up to 95% and 91%, respectively. Fecal calprotectin is more efficient than CRP, ESR (erythrocyte sedimentation rate), ASCAs (anti-Saccharomyces cerevisiae antibodies), ANCAs (antineutrophil cytoplasmic antibodies) and Omp C (outer membrane porin C)[37]. Fecal lactoferrin reflects the inflammation status of the intestine, the diagnostic rate being similar to that of fecal calprotectin and better than that of CRP. Another fecal marker correlated with endoscopic scores is neopterin, an index of disease activity in UC and CD[38]. A non-invasive biomarker of inflammation in IBD, which has high sensitivity and specificity and good compliance is S100A12; it stimulates the proinflammation NF-κB pathway. In addition, fecal S100A12 reflects the drug treatment response[34].

Serum microRNAs, which are small non-coded single-stranded RNAs, could be upregulated or downregulated in IBD[37]. It is very important to confirm the existence of specific expression patterns of miRNAs associated with IBD in different stages and demonstrate the utility of miRNA as ideal biomarkers[39]. Thus, it was demonstrated that miR-31, miR-206, miR-424 and miR-146a were novel biomarkers of inflammatory bowel disease[40].

THERAPEUTIC IMPLICATIONS

The crucial role of the IL-23/Th17 axis in intestinal inflammation was demonstrated once again by eliminating certain components of this pathway and thus ameliorating the severity of the disease. Corticosteroids are classical immunosuppressive drugs of pro-inflammatory cytokine production used for the induction of clinical remission in IBD, but they may cause severe side effects. Consequently, it is of high clinical importance to define other drugs that

attack the key pro-inflammatory pathways in chronic intestinal inflammation[41].

Small molecules that neutralize specific elements of the IL-23/Th17 pathway were used[42]. Drugs that target IL-17 include the anti IL-17 monoclonal antibody named secukinumab and the small molecule vidofludimus that blocks IL-17 release[43]. Apilimod mesylate inhibits IL-12 and IL-23 transcription, the anti-IL-12 ABT-874/J695 monoclonal antibodies ustekinumab and briakinumab target the p40 unit which is common to both IL-23 and IL-12; SCH-900222 targets the p19 subunit specific to IL-23[44]. Ustekinumab is efficient in moderate-to-severe CD, especially in patients in whom anti-TNF treatment had previously failed. An anti-IL-21 antibody neutralizes IL-21 and reduces the IL-17 secretion by lamina propria lymphocytes isolated from IBD patients[45]. The importance of ustekinumab is due to its simultaneous inhibition of Th17 and Th1 cells, both of them being involved in IBD etiopathology[46].

A potential strategy for IBD therapy is represented by the IL-17A and IL-17F combination, an important protection being observed against intestinal inflammation in an acute IL-17A knockout TNBS- induced colitis and also a clear improvement in the inflammatory process in IL-17F knockout DSS-induced colitis. Only IL-17A KO in the DSS model worsened the colonic inflammation[47].

A novel efficient and safe oral immunomodulatory drug, vidofludimus inhibits DHODH (dihydroorotate dehydrogenase), which is the key enzyme involved in pyrimidine biosynthesis in activated lymphocytes by reducing the IL-17A and IL-17F expression through the NF-κB pathway[48,49] .

The data presented in this review are mainly based on studies carried out on humans. Animal models of IBD are not totally relevant for this human specific condition; differences between human disease and animal models exist[50]. Animal models of intestinal inflammation may help understanding the action of inflammatory cytokines[51]. This topic was not the focus of the present review, where we looked for human data, for better accuracy. Data from clinical studies are highly relevant. A recent review shows the importance of immunological changes in the gut inflammation and these offer the background for emerging therapies[52].

This review presents the contribution of the IL-17/IL-23 axis in the pathogenesis of inflammatory bowel disease. Better understanding of the pathogenesis of this condition represent the background for the progress in therapy.

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ORIGINAL ARTICLE

Basic Study

Magnetic resonance imaging of the pancreas in streptozotocin-induced diabetic rats: Gadofluorine P and Gd-DOTA

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Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at verocay@snuh.org or moonwk@snu.ac.kr.

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Abstract

AIM: To investigate the performance of Gadofluorine P-enhanced magnetic resonance imaging (MRI) on the diagnosis of diabetes in a streptozotocin (STZ) -induced diabetic rat model.

METHODS: Fischer 344 rats were treated with STZ. Rats not treated with STZ served as controls. T1-

weighted MRI was performed using a 3T scanner before and after the injection of Gd-DOTA or Gadofluorine P (6 diabetic rats, 5 controls). The normalized signal intensity (SI) and the enhancement ratio (ER) of the pancreas were measured at each time point, and the values were compared between the normal and diabetic rats using the Mann-Whitney test. In addition, the values were correlated with the mean islet number. Optimal cut-off values were calculated using a positive test based on receiver operating characteristics. Intrapancreatic Gd concentration after the injection of each contrast media was measured using laser ablation-inductively coupled plasma-mass spectrometry in a separate set of rats (4 diabetic rats, 4 controls for Gadofluorine P; 2, 2 for Gd-DOTA).

RESULTS: The normalized SI and ER of the pancreas using Gd-DOTA were not significantly different between diabetic rats and controls. With Gadofluorine P, the values were significantly higher in the diabetic rats than in the control rats 30 min after injection ($P < 0.05$). The area under the receiver operating characteristic curve that differentiated diabetic rats from the control group was greater for Gadofluorine P than for Gd-DOTA (0.967 *vs* 0.667, $P = 0.085$). An increase in normalized SI 30 min after Gadofluorine P was correlated with a decrease in the mean number of islets ($r^2 = 0.510$, $P = 0.014$). Intra-pancreatic Gd was higher in rats with Gadofluorine P injection than Gd-DOTA injection (Gadofluorine P *vs* Gd-DOTA, 7.37 *vs* 0.00, $P < 0.01$). A significant difference in the concentration of intrapancreatic Gd was observed between the control and diabetic animals that were sacrificed 30 min after Gadofluorine P injection (control *vs* diabetic, 3.25 ng/g *vs* 10.55 ng/g, $P < 0.05$)

CONCLUSION: In this STZ-induced diabetes rat model, Gadofluorine P-enhanced MRI of the pancreas showed high accuracy in the diagnosis of diabetes.

Key words: Gadofluorine P; Gd-DOTA; Magnetic resonance contrast media; Type 1 diabetes; Pancreas

Core tip: Early changes in type 1 diabetes involve islet microvasculature such that vascular permeability increases. We used noninvasive magnetic resonance imaging to image, *in vivo*, the vasculature of the pancreas in streptozotocin-induced diabetic rats using Gadofluorine P. We anticipate that with further development, this technique could diagnose type 1 diabetes early, as well as monitor vascular and islet changes noninvasively and quantitatively.

Cho HR, Lee Y, Doble P, Bishop D, Hare D, Kim YJ, Kim KG, Jung HS, Park KS, Choi SH, Moon WK. Magnetic resonance imaging of the pancreas in streptozotocin-induced diabetic rats: Gadofluorine P and Gd-DOTA. *World J Gastroenterol* 2015; 21(19): 5831-5842 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5831.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5831

INTRODUCTION

Type 1 diabetes is an autoimmune disease characterized by the specific destruction of insulin-producing β-cells, which are located in the islets of Langerhans in the pancreas[1]. Pancreatic islets receive 10% to 20% of the blood flow of the pancreas despite accounting for only 1% to 2% of the pancreas by weight[2]. Accumulating evidence suggests that the vascular endothelium is of crucial importance for the development of type 1 diabetes. Increases in the vascular permeability of islet blood vessels in the pancreas during the development of type 1 diabetes have been described in animal models of spontaneous autoimmune diabetes[3,4]; alloxan-induced diabetes in mice[5]; and streptozotocin (STZ)-induced diabetes in rats[6] and mice[7,8]. Moreover, vascular swelling and increased vascular permeability precede insulitis in nonobese diabetic mice and in STZ-induced diabetes models[6-12]. Accordingly, pancreatic islet vascular dysfunction can represent an early and potentially predictive biomarker for the loss of β-cell mass.

We used Gadofluorine P (invivoContrast GmbH, Berlin, Germany)-enhanced magnetic resonance imaging (MRI) in a rat model of STZ-induced diabetes. Gadofluorine P is a gadolinium (Gd)-based T1 contrast agent; it is commercially available but not approved for clinical use[13]. We hypothesized that increased vascular permeability of the islets could be detected using long-circulating Gadofluorine P, which extravasates from leaky vessels into the surrounding tissue and binds to extracellular proteins. Thus, our purpose was to determine the diagnostic performance of Gadofluorine P-enhanced MRI of the pancreas in the diagnosis of diabetes in a STZ-induced diabetic rat model and to compare the results with an extracellular agent, Gd-DOTA (Dotarem®, Guerbet, Paris, France).

MATERIALS AND METHODS

The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (Number 11-0019). The animal protocol was designed to minimize pain or discomfort to the animals. Briefly, diabetic rats were given STZ for three consecutive days (day 0, 1, 2). MR was performed from day 4 to day 6. Blood glucose and weight were measured from day 0 to day 3. Eleven rats (6 diabetic rats, 5 control rats) that underwent MRI were euthanized on day 6 for histological analysis. Twelve rats (8 diabetic rats, 4 control rats) that were given each contrast media were euthanized on day 4 for measurement of

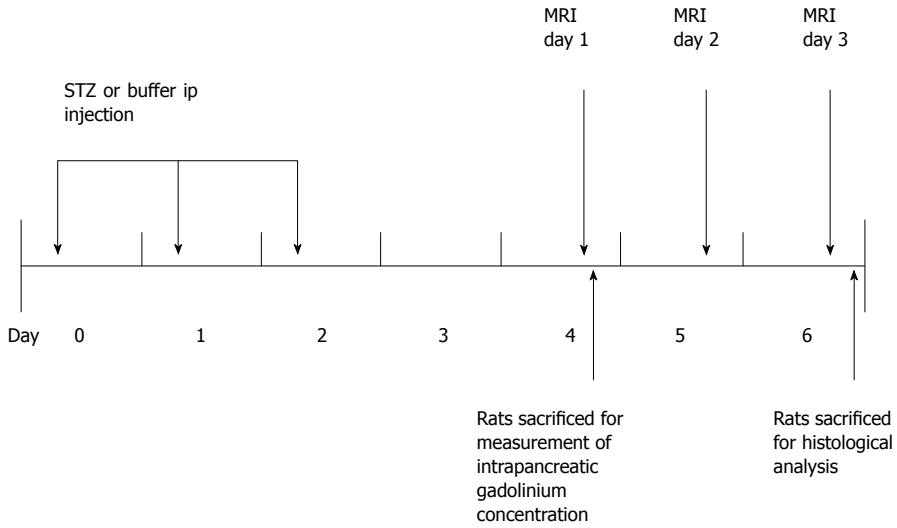

Figure 1 Experimental design. MRI: Magnetic resonance imaging; STZ: Streptozotocin.

intrapancreatic Gd concentrations using laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). These rats did not undergo MRI (Figure 1).

Animal preparation

The experiment was undertaken in female Fischer 344 rats from Charles River (Orient-Bio Inc., Seongnam, South Korea) weighing 140-210 g at 6-7 wk of age. For the insulin-dependent diabetic rat model, we used the multiple low-dose STZ model[14-16]. For three consecutive days, the diabetic rats received daily intraperitoneal injections of 15 mg/kg STZ (S0130-1G, Sigma-Aldrich Korea, Seoul, South Korea) dissolved in a citrate buffer (0.09 mol/L). For the control group, the rats received an equal volume of citrate buffer intraperitoneally.

Blood glucose concentrations were measured once a day using a commercial kit (BAROZEN®, Handok, South Korea) and blood obtained from the tail vein. On day 0, blood glucose levels were measured after 6 h of fasting, and after day 0, non-fasting blood glucose levels were measured. Hyperglycemia was defined as a blood glucose level ⩾ 2 g/L in two consecutive measurements.

Contrast media

Gadofluorine P (molecular weight 1322 g/mol) is a daughter product of Gadofluorine M (Bayer Schering Pharma, Berlin, Germany) (molecular weight 1528 g/mol) with improved tolerability. Gadofluorine agents are amphiphilic gadolinium complexes, synthesized by adding a perfluoroctyl chain to a gadolinium-containing macrocycle (Figure 2)[13]; therefore, they form micelles in water. In blood or extracellular tissue, Gadofluorine agents strongly interact with hydrophobic proteins (*e.g.*, albumin, extracellular matrix proteins), leading to a breakdown of the micelles[17,18]. After intravenous injection, Gadofluorine P also binds reversibly to plasma proteins and forms semi-stable, macromolecular Gadofluorine-protein complexes[19]. In 37 ℃ plasma, the R1 relaxation rate of Gadofluorine P at 1.41 T was about 17.5 $mmol^{-1}s^{-1}$[20]. The median lethal dose of Gadofluorine P is twice of that of Gadofluorine M and similar to that of Gd-DOTA (5 mmol/kg for Gadofluorine M; 12.5 for Gadofluorine P; and 11 for Gd-DOTA). Blood retention is shorter for Gadofluorine P than for Gadofluorine M. In rabbits, the plasma elimination half-life of Gadofluorine P is approximately 2 h, and Gadofluorine P is almost completely eliminated from blood/plasma within 24 h after intravenous injection[19]. In contrast, the plasma half-life of Gadfluorine M in rabbits is approximately 10 h[21,22]. In mice, contrast enhancement in the blood almost disappeared 6 h after the injection of Gadofluorine P, whereas it was 24 h for Gadofluorine M[23]. Gd-DOTA is a macrocyclic gadolinium paramagnetic complex with a molecular weight of approximately 558 g/mol (Figure 2)[24]. In 37 ℃ plasma, the R1 relaxation rates of Gd-DOTA at 1.5 T were approximately 3.5 $mmol^{-1}s^{-1}$[25]. Gd-DOTA is a first generation extracellular fluid space contrast MR agents. After intravenous injection, Gd-DOTA randomly distributes in intravascular and interstitial extracellular fluid spaces, and is eliminated rapidly through glomerular filtration in the kidney[26]. In rats, the biodistribution of Gd-DOTA has a distribution half-life of 3 min and an elimination half-life of 18 min[25,27].

MRI

Before MRI acquisition, animals were sedated with an intramuscular injection of tiletamine hydrochloride with the sedative zolazepam hydrochloride at 30 mg/kg body weight (Zoletil®; Virbac, Carros, France) and xylazine hydrochloride (Rompun®; Bayer, Seoul, Korea) at 10 mg/kg body weight. We used a 3.0 T MRI scanner (Trio; Siemens, Erlangen, Germany) with a 4-channel wrist coil. On day 4, we obtained T1-weighted image (T1WI) before, immediately

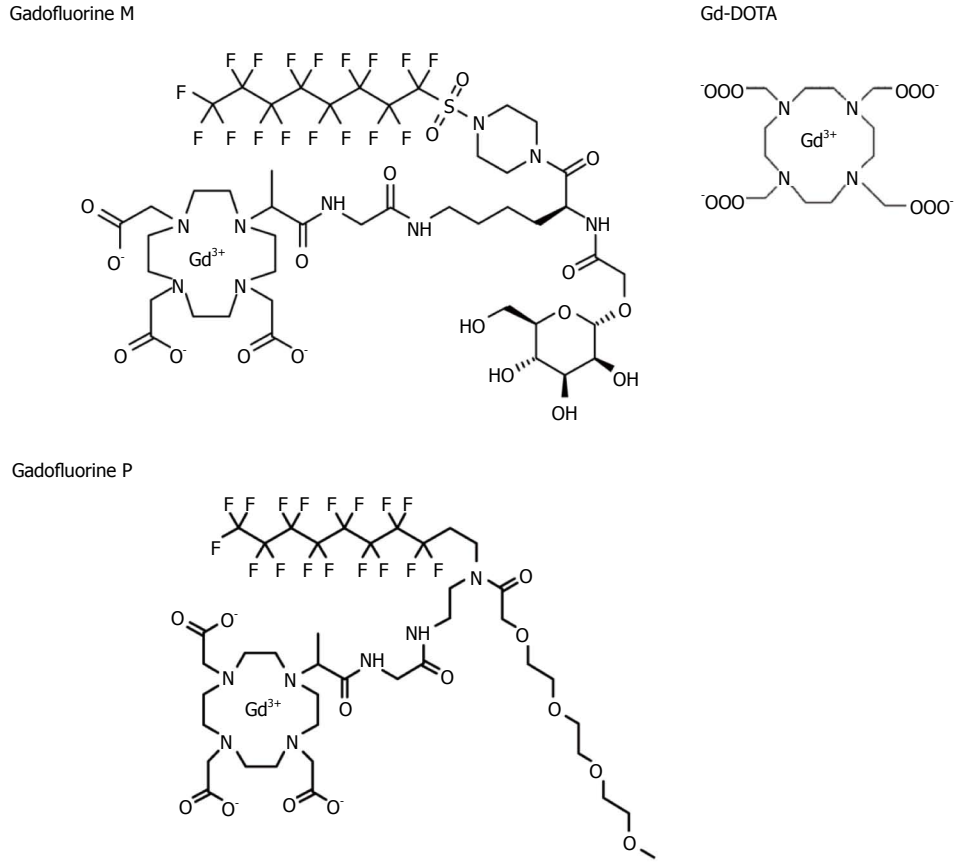

Figure 2 Structures of Gadofluorine M, Gadofluorine P, and Gd-DOTA.

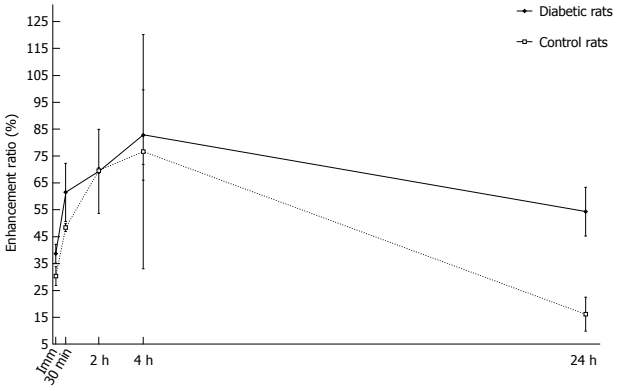

Figure 3 Time-enhancement ratio curve pattern of Gadofluorine P.

and 3 min after injection of Gd-DOTA (0.1 mmol/kg body weight). On day 5, we obtained T1WI data immediately and 30 min after Gadofluorine P injection (0.1 mmol/kg body weight). On day 6, we obtained T1WI data at 24 h after the injection of Gadofluorine P. Both contrast media injections were in the tail vein using an injection cap followed by flushing with 1 mL of normal saline. The 6 diabetic rats and the 5 controls underwent MRI for 3 d. We performed MRI first with Gd-DOTA and 24 h later with Gadofluorine P because the elimination half-life of Gd-DOTA is much shorter than that of Gadofluorine P[19,25]. The imaging time point for Gd-DOTA and Gadofluorine P was based on our time-intensity curve data (Figure 3) and data from other studies[13,23].

The rats were scanned in the supine position with no respiratory gating. A water phantom of normal saline was positioned alongside the body during the imaging of each rat. Transverse T1WI with a 3D gradient-echo sequence was obtained as follows: repetition time, 25 ms; echo time, 5.1 ms; flip angle, 25°; number of excitations, 2; slice thickness, 1 mm; FOV 100 mm × 50 mm; matrix 256 × 123; and spatial resolution, 0.39 mm × 0.41 mm × 1 mm. Transverse images obtained from the level of liver dome to the mid pole of the kidney to cover the entire pancreas.

Time-intensity curve

For the time-intensity curve, four rats were used to generate a time-enhancement ratio curve pattern for Gadofluorine P (Separate rats from rats for MRI imaging, and measurement of intrapancreatic Gd concentrations using laser ablation-inductively coupled plasma-mass spectrometry). Two diabetic rats and two control rats underwent this MRI. The blood glucose level of both diabetic rats was greater than 2 g/L from day 1. In MR image analysis, the pancreas signal intensity was normalized to the paravertebral muscle. With Gadofluorine P, the mean enhancement ratio of

the pancreas increased for 240 min, with a maximal difference in enhancement ratio between the groups at 30 min after Gadofluorine P injection (Figure 3). However, no significant difference was found at any time point for both contrast media.

MRI analysis

The MR images were analyzed by a radiologist who was blinded to the group (control or diabetic) and the histological results. The MR data from each animal were processed with ImageJ (http://rsb.info.nih.gov/ij/) and a software program developed in-house (Y-J. K. and K.G.K.) using Visual C++ (Microsoft, Redmond, Wash). In each rat, regions of interest (ROIs) were drawn within the entire pancreas through consecutive transverse planes while avoiding surrounding structures including the stomach lumen, the caudate lobe of the liver and the spleen. The signal intensities (SIs) of the pancreata were normalized using the SIs of a water phantom (normalized SI of the pancreas = SI of the pancreas/SI of a water phantom). The mean value of the summation of consecutive ROIs was used for interpretation of the normalized SIs of the pancreas. The contrast enhancement ratio (ER) was calculated as follows: ER (%) = 100 × (normalized $SI_{enhanced}$ - normalized $SI_{unenhanced}$)/normalized $SI_{unenhanced}$, and the ER of the pancreas was calculated at each time point (*e.g.*, immediately and 3 min after Gd-DOTA injection; and immediately, 30 min and 24 h after the Gadofluorine P injection).

Histological analysis

After the acquisition of the MRIs (day 6), the pancreata (n = 11) were immediately removed and fixed in 10% buffered formalin. Paraffin-embedded pancreata were cut into 4-μm-thick sections and stained with hematoxylin and eosin (HE). The islet size and number were measured for statistical analysis. Slides were observed under a light microscope at magnification × 100 by a pathologist. Ten non-consecutive slides were chosen from each rat, and the islet size was measured using an Olympus DP70 digital microscope camera and its associated software (Olympus Corporation, Tokyo, Japan). The largest diameter of each islet was measured using a digital scale bar. The number of islets was determined as the mean number of islets in a field of 2.2 mm diameter at 100 × magnification. Three separate fields were viewed in each slide.

Measurement of intrapancreatic Gd concentrations using laser ablation-inductively coupled plasma-mass spectrometry

We measured Gd concentrations in 10 μm sections of the pancreas using laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS). The pancreas was sectioned on a coronal plane to cover the entire pancreas (head, body, tail); the representative section of the whole pancreas was used. On day 4, four diabetic rats and four control rats were euthanized 30 min after Gadofluorine P injection, and two diabetic rats and two control rats were sacrificed 3 min after Gd-DOTA injection. They did not undergo MRI.

We measured the intrapancreatic Gd levels of the rat in the diabetic and normal groups using LA-ICP-MS for pancreas sections. The analysis was performed using a New Wave Research NWR193 laser ablation instrument (Kennelec Technologies) fitted with a Large Format Cell. Argon was used as the carrier gas. The laser unit was hyphenated to an Agilent Technologies 7700s ICP-MS instrument fitted with a "cs" lens system, a platinum sampler, and skimmer cones. Prior to analysis, the system was tuned for sensitivity using a NIST 612 Trace element in glass and in-house-produced tissue standards. Oxide formation was controlled by limiting the amount of $^{248}Th16O+/^{232}Th+$ to < 0.3% for the ablation of NIST 612.

Data were acquired by ablating adjacent lines down each specimen (10 μm thick) with a beam diameter of 50 m and a scan speed of 200 m/s. Mass spectrometer integration times were chosen in order to maintain the true image dimensions[28] when processed, such that a single pixel represented 50 (or is that 2500) m^2. Variations in laser power output and instrument drift were compensated for through normalization to the ^{13}C signal[29].

Quantitative data were produced through representative ablation of tissue standards, using a previously described method[30,31]. Briefly, chicken breast tissue was purchased from a local market and stripped of all fatty and connective tissue. Five-gram aliquots of dissected tissue were partially homogenized using an OmniTech TH tissue homogenizer (Kelly Scientific) fitted with a polycarbonate probe. Then, 5 to 50 L aliquots of high purity standard solution (Choice Analytical) were added to each standard preparation, which were then further homogenized. Next, six approximately 250 mg aliquots of each standard were digested in HNO_3:H_2O_2 (3:1 ratio) in a Milestone MLS 1200 microwave digester (John Morris Scientific), and each standard was analyzed by solution nebulization ICP-MS to accurately determine the trace metal concentration and homogeneity of the standards.

Data were reduced into multispectral images using the Interactive Data Imaging Spectral Data Analysis Software (ISIDAS) developed by the University of Technology, Sydney Computational Research Support Unit. ISIDAS is a specialized data reduction package written in the Python programming language. Images were exported from ISIDAS in a Visualization Toolkit (.vtk) format into Enthought MayaVi2 for color rendering. Quantitative data were extracted by freehand outlining of the ROIs using ISIDAS.

Statistical analysis

Statistical analyses were performed using MedCalc

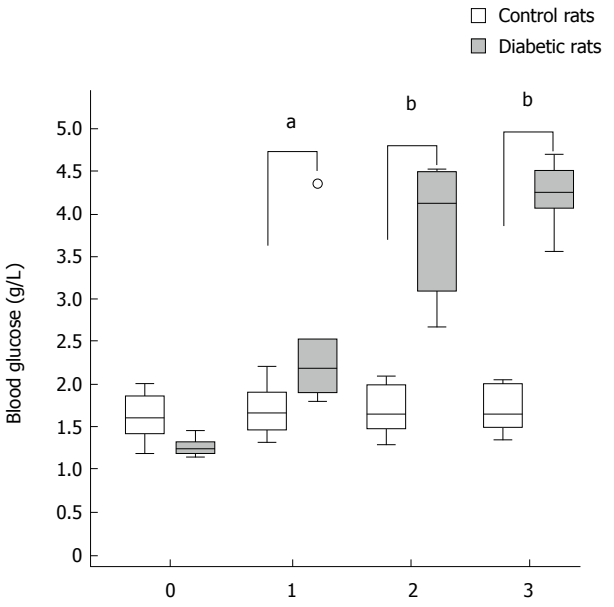

Figure 4 Blood glucose level of the streptozotocin-injected rat model of type 1 diabetes. Blood glucose level of the control (n = 5) and diabetic (n = 6) rat groups over 4 d. The blood glucose level increased more than 2 g/L from day 2 in the diabetic rat group. [a]P < 0.05 control rats *vs* diabetic rats; [b]P < 0.01 control rats *vs* diabetic rats.

version 14.8.1 for Windows (MedCalc Software, Mariakerke, Belgium). Descriptive statistical measures, including the mean, median, standard deviation (SD), and range were calculated for quantitative measurement.

Data are expressed as the mean ± SD or median (lowest value, highest value). Differences in the blood glucose levels, intrapancreatic gadolinium concentration using LA-ICP-MS and the normalized SI and ER of the pancreata between the diabetic and control group at each time point were compared using the Mann-Whitney test. We obtained the area under the receiver operating characteristics (ROC)-curve (AUC), and the sensitivity, specificity, and accuracy of the ER to determine the diabetic rat group with an optimal cut-off value which was calculated using a ROC-based positive test with the categorical variables of diabetes or control.

We used linear regression analysis to determine the association between the histological results and the normalized SI and ER of the pancreata.

A P value < 0.05 was considered statistically significant.

The statistical methods of this study were reviewed by Yunhee Choi, PhD, from the Medial Research Collaborating Center at Seoul National University Hospital.

RESULTS

Comparison of the change in the normalized SI and ER of the pancreas between Gd-DOTA and Gadofluorine P

The blood glucose levels for STZ-injected rats were elevated compared with control rats from day 1 after the injection of STZ (day 1, P < 0.05; day 2, P < 0.01; day 3, P < 0.01). The blood glucose level of rats that underwent MRI are shown in Figure 4 (from day 0 to day 3).

Table 1 summarizes the normalized SI and ER of the pancreata after the injection of Gd-DOTA and Gadofluorine P in control and diabetic rats. After the injection of Gd-DOTA, we did not observe a significant difference in either normalized SI or ER of the pancreata between control and diabetic rats at each time point. In terms of Gadofluorine P-enhanced MRI, both the normalized SI and ER of the pancreata of the diabetic rats were significantly higher than those of the control rats 30 min after injection (309.8 ± 21.5 *vs* 251.7 ± 27.6; 111.8 ± 22.3 *vs* 78.0 ± 17.5, respectively), whereas we did not observe a significant difference at any other time point. Figure 5 shows the change in the normalized SI and ER of the pancreata after the injection of Gd-DOTA and Gadofluorine P in the control and diabetic rats; representative images (Figure 6) are included for each group.

To differentiate diabetic rats from the control group, ROC analysis showed that ER 30 min after Gadofluorine P injection had a greater AUC than did ER 3 min after Gd-DOTA injection (Table 2), which was not statistically significant (P = 0.085). The optimal ER value 30 min after Gadofluorine P injection was > 101.6%; the sensitivity, specificity and accuracy were 83.3% (5 of 6 rats), 100% (5 of 5 rats) and 90.9% (10 of 11 rats). The optimal ER value 3 min after Gd-DOTA injection was > 9.4%; the sensitivity, specificity and accuracy were 83.3% (5 of 6 rats), 60% (3 of 5 rats) and 72.8% (8 of 11 rats).

Histological results

The mean islet diameters were 315.89 ± 101.44 and 481.34 ± 69.32 μm in the diabetic and control rat groups, respectively, which were significantly different (P = 0.013). The mean numbers of islets per 2.2-mm diameter field were 3.7 ± 1.5 and 6.8 ± 2.3 in the diabetic and control rat groups, respectively, which were also statistically significant (P = 0.023) (Figure 7).

The pathologist did not report any specific HE findings other than decreased islet numbers and sizes.

Correlation of normalized SI and ER with histological results

Simple linear regression analysis revealed that the increase in normalized SI 30 min after Gadofluorine P injection was associated with a decrease in the mean number of islets per field (r^2 = 0.510, P = 0.014) but that normalized SI 3 min after Gd-DOTA was not associated (r^2 = 0.013, P = 0.743) (Figure 8).

Intrapancreatic Gd concentration using LA-ICP-MS

A significant difference in the concentration of intrapancreatic Gd was observed between the control

Table 1 Normalized signal intensity and mean enhancement ratio of the pancreas in the diabetic rat group (n = 6) and the control rat (n = 5) group

	Pre	Imm after Gd-DOTA	3 min after Gd-DOTA	Imm after GdP	30 min after GdP	24 h after GdP
Normalized signal intensity of the pancreas						
Control rats	137.9 (132.0, 152.4)	165.6 (160.0, 177.8)	158.2 (141.2, 161.3)	238.4 (201.8, 311.6)	264.7 (212.5, 280.7)[b]	171.0 (147.3, 178.9)
Diabetic rats	146.6 (135.2, 161.7)	184.4 (164.0, 218.7)	173.3 (153.2, 185.9)	277.1 (217.0, 317.4)	302.6 (295.6, 353.3)[b]	177.9 (145.9, 189.7)
Enhancement ratio of the pancreas (%)						
Control rats		20.5 (8.7, 27.9)	9.3 (-7.3, 21.6)	80.8 (46.3, 111.2)	79.5 (54.1, 101.6)[a]	17.4 (11.6, 24.0)
Diabetic rats		29.3 (11.8, 45.5)	14.8 (4.9, 34.4)	79.3 (47.5, 129.3)	103.3 (87.9, 151.1)[a]	16.5 (-0.1, 34.8)

Data are shown as the median (lowest value, highest value). P values were calculated using the Mann-Whitney test. Imm: immediate; GdP: Gadofluorine P. [a]P < 0.05 control rats *vs* diabetic rats; [b]P < 0.01 control rats *vs* diabetic rats.

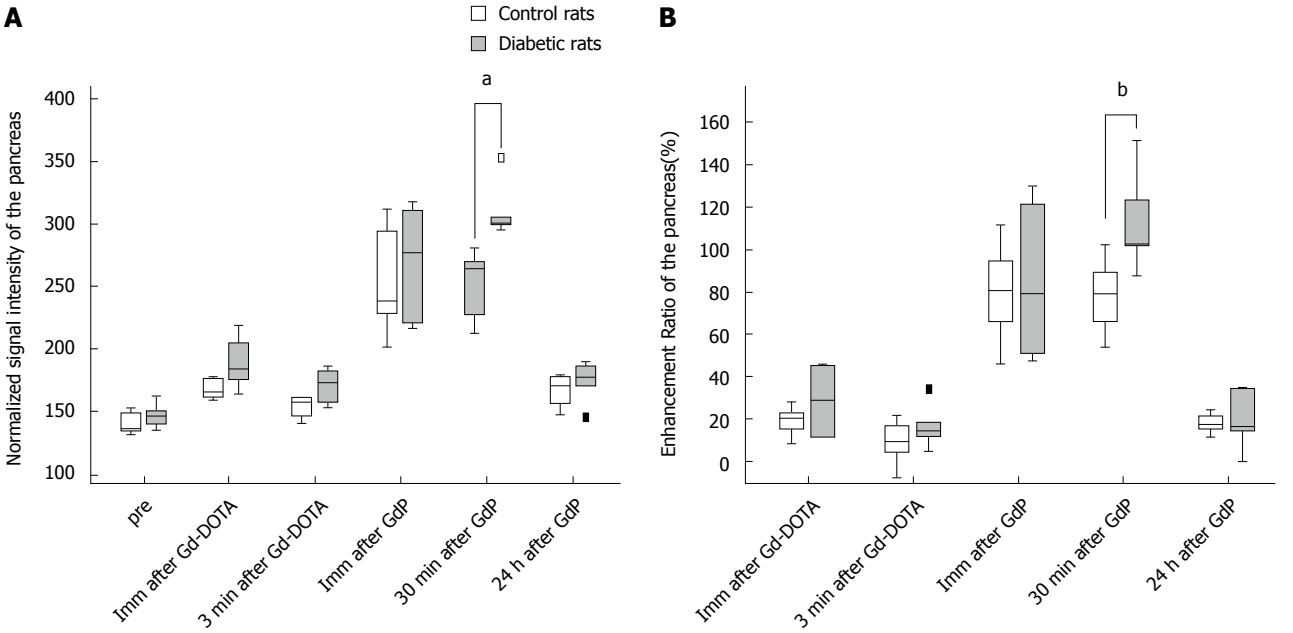

Figure 5 Enhancement of the pancreata of the control and diabetic rats. The normalized signal intensity (SI) (A) and enhancement ratio (ER) (B) of the pancreata at each time point after the injection of Gd-DOTA and Gadofluorine P in the control (n = 5) and diabetic (n = 6) rat groups ([b]P < 0.01). Imm, immediate. [a]P < 0.05 control rats *vs* diabetic rats; [b]P < 0.01 control rats *vs* diabetic rats. GdP: Gadofluorine P.

Table 2 Area under the receiver operating characteristics curve for Gadofluorine P and Gd-DOTA for differentiating diabetic rats from the control group

	AUC
Enhancement ratio 3 min after Gd-DOTA	0.667 (0.334-0.908)
Enhancement ratio 30 min after GdP	0.967 (0.664-1.000)

Numbers in parentheses indicates the 95%CI. AUC: Area under the receiver operating characteristics curve; GdP: Gadofluorine P.

and diabetic animals that were euthanized 30 min after Gadofluorine P injection (control (n = 4) *vs* diabetic (n = 4), 3.25 (2.48, 5.35) ng/g *vs* 10.55 (9.39, 14.92) ng/g, P < 0.05; Figure 9A, B). No significant difference was noted between the control and diabetic rats that were euthanized 3 min after Gd-DOTA injection [control (n = 2) *vs* diabetic (n = 2), 0.00 (0.00, 0.00) *vs* 0.78 (0.00, 1.55) ng/g; Figure 9C, D]. The concentration of intrapancreatic Gd was higher in rats treated with Gadofluorine P than the rats treated with Gd-DOTA injection [Gd-DOTA (n = 4) *vs* Gadofluorine P (n = 8), 0.00 (0.00, 1.55) *vs* 7.37 (2.48, 14.92), P < 0.01].

DISCUSSION

This study demonstrates that Gadofluorine P-enhanced MRI can be used to assess the changes in the pancreas in the STZ-induced diabetes model. This is attributed to the increased vascular permeability of the pancreas and the physicochemical properties of Gadofluorine P.

We suggest several working mechanisms of Gadofluorine P enhancement of the pancreas in STZ-induced diabetes. First, Gadofluorine P has a strong blood pool effect[23]. Unlike Gd-DOTA, which is also T1 contrast media but is rapidly cleared from the intravascular space and eliminated[32], Gadofluorine P can re-circulate in the blood by binding to albumin via its nonspecific protein binding properties[19]. The elimination half-life of Gadofluorine P is approximately six times greater than that of Gd-DOTA[19,25]. In addition, the R1 relaxivity of Gadofluorine P is

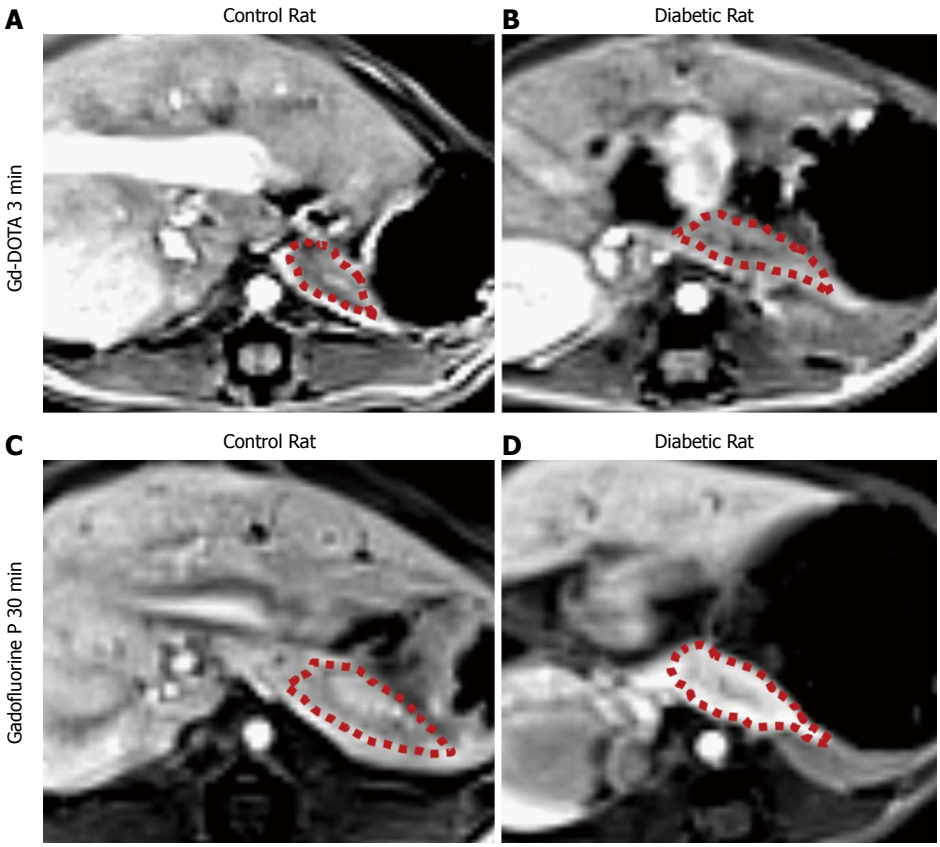

Figure 6 T1-weighted image magnetic resonance imaging obtained 3 min after Gd-DOTA injection and 30 min after Gadofluorine P injection and a diabetic rat (D) revealed the pancreas of a control rat (A), a diabetic rat (B), a control rat (C) and a diabetic rat (D), respectively. The normalized SI and ER were as follows: a control *vs* a diabetic rat, 158.2 *vs* 153.2 and 14.7 *vs* 4.9, respectively. The normalized SI and ER were as follows: a control versus a diabetic rat, 212.5 *vs* 295.6 and 54.1 *vs* 102.3, respectively. The area of the pancreas is outlined with a red dotted line. MRI: Magnetic resonance imaging; SI: Signal intensity; ER: Enhancement ratio.

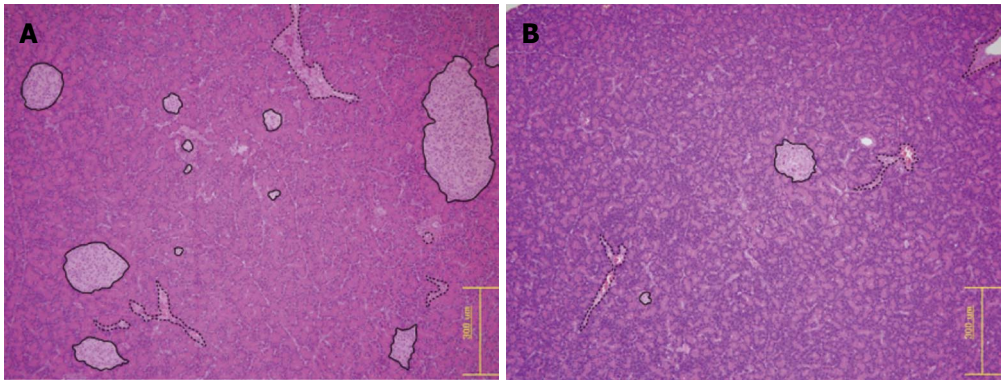

Figure 7 Hematoxylin and eosin staining of the pancreas of a control rat and a diabetic rat. A light microscope at magnification × 100. All islets are outlined with solid lines, and the structures outlined with dotted line are the vessels. Both the islet size and the islet number were decreased in the diabetic rats (B) compared with the control rats (A).

approximately four times greater than that of Gd-DOTA. Second, Gadofluorine P can extravasate in regions of increased vascular permeability and bind to extracellular protein in the interstitial space. In our results obtained from LA-ICP-MS, the concentration of intrapancreatic Gd was significantly higher in diabetic rats than the control rats with Gadofluorine P injection. With Gd-DOTA injection, intrapancreatic Gd was significantly lower than with Gadofluroine P injection in the pancreas of diabetic or control rats. These results revealed the extravasation and accumulation of Gadofluorine P in the pancreas of the diabetic rat. Gadofluorine M accumulation in the extracellular matrix in regions with disturbed epithelial membranes has been confirmed in other disease models including atherosclerosis, and inflammatory bowel disease[18,22]. In atherosclerotic plaques, the Gadofluorine M-albumin complex leaks into the extravascular space and then

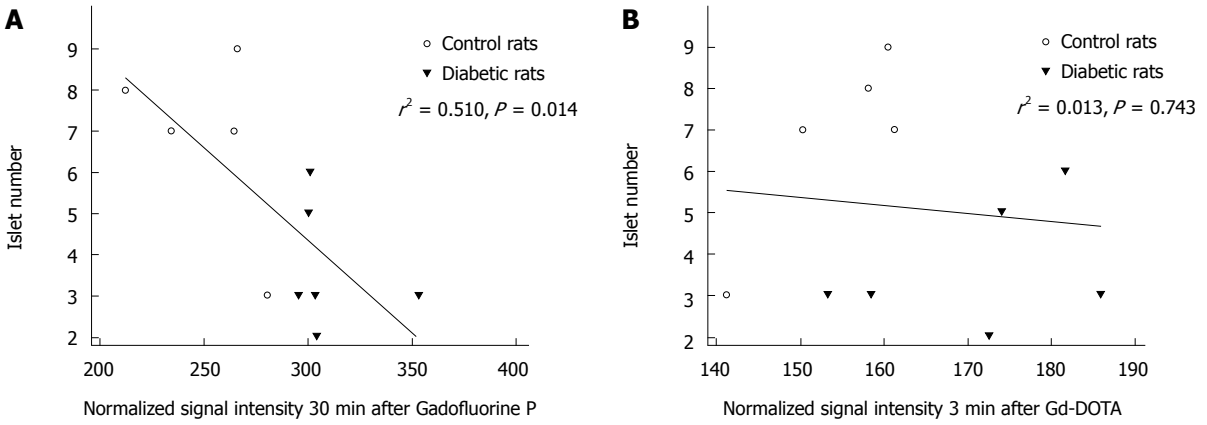

Figure 8 Correlation of magnetic resonance parameters of the pancreas after Gadofluorine P injection with islet number. A: Normalized signal intensity of the pancreas at 30 min after Gadofluorine P injection correlated well with the mean number of islet per field (r^2 = 0.510, P = 0.014); B: Normalized signal intensity of the pancreas at 3 min after Gd-DOTA injection did not correlated with the mean number of islets per field (r^2 = 0.013, P = 0.743).

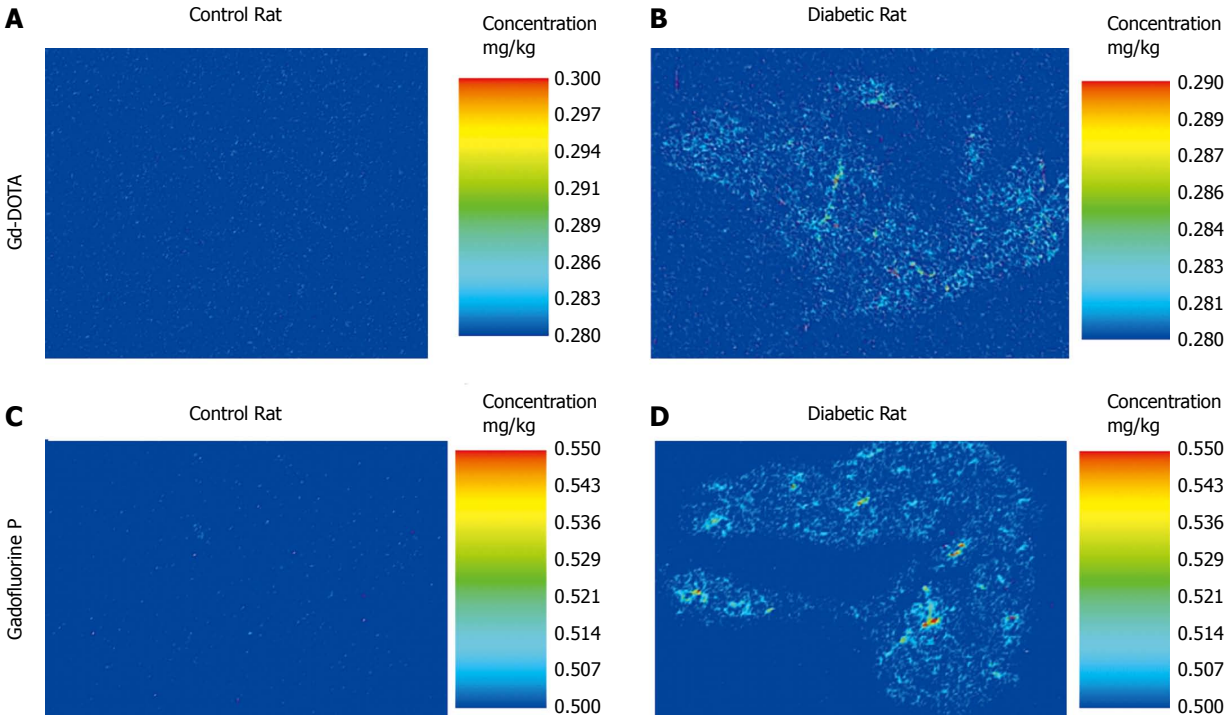

Figure 9 Determination of intra-pancreatic Gadolinium concentration using laser ablation-inductively coupled plasma-mass spectrometry. Higher average Gadolinium concentration (10.49 ng/g) in the pancreas section of a diabetic rat (A) than a control rat (3.89 ng/g) (B) 30 min after Gadofluoine P injection. The average Gadolinium concentration was 0 ng/g in a control rat (C) and 1.55 ng/g in a diabetic rat (D).

accumulates within the extracellular compartment and the fibrous parts of the plaque by binding to collagens, proteoglycans and tenascin[18]. In inflammatory bowel disease, Gadofluorine M accumulates in the lamina propria mucosae of the bowel beyond the intravascular space[22]. We also demonstrated the accumulation of Gadofluorine P in the pancreas using LA-ICP-MS.

In our results, the normalized SI of the pancreas 30 min after Gadofluorine P injection negatively correlated with pancreatic islet number of the pancreas. These results correspond with the results of an earlier study by Medarova *et al*[33] which demonstrated that T1 relaxation time was significantly lower compared to controls after the injection of T1 contrast medium (protected graft copolymer covalently linked to Gd-diethylenetriaminepentaacetic acid residues labeled with fluorescein isothiocyanate) in the pancreata of STZ-induced diabetic animals. However, they did not quantitatively correlate the MR parameters with islet mass or β-cell function.

There have been other studies evaluating the pancreas of type 1 diabetes using MRI: Denis *et al*[34] used long-circulating magnetofluorescent nanoparticles (iron oxide cored particle attached fluorochrome) in nonobese diabetic mice and Gaglia *et al*[35] used iron-based magnetic nanoparticles in

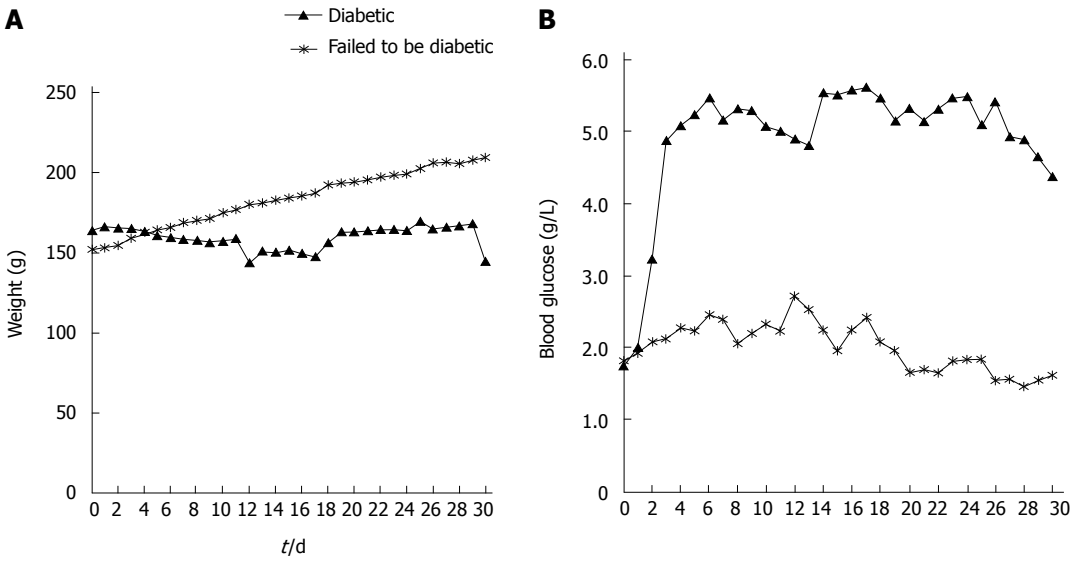

Figure 10 Thirty days' monitoring of body weight (A) and blood glucose level (B) of rats with streptozotocin intraperitoneal injection. Nine separate rats were treated with streptozotocin in the same way as the material and method. They are monitored for blood glucose level and weight for 30 d. Among them, two rats failed to develop hyperglycemia. In diabetic rats, blood glucose level was elevated and plateaued above 4.5 g/dL from day 3.

recent onset type 1 diabetes patients. In contrast to our study, these studies used iron-based negative T2 contrast agents. They postulated that their iron-based contrast agents extravasate from leaky vessels into the surrounding tissue and are engulfed by activated macrophages in the tissue. Denis *et al*[34] demonstrated that accumulation of the nanoparticles in mice correlates with insulitis intensity as revealed by HE histological analysis. However, in our study, we could not find evidence of insulitis in the histological analysis of the pancreas: there was no inflammatory cell accumulation. Similarly, Medarova *et al*[33] reported that in an STZ-induced model of type 1 diabetes, the pancreatic islets were less abundant and islet morphology was abnormal, but there was no inflammatory cell infiltrates.

Antkowiak *et al*[36] reported that measurements of pancreatic R1 in manganese-enhanced MRI accurately reflected changes of functional β cell mass in their mouse model of type 1 diabetes. Manganese ion, which is a T1 contrast medium, enters β cells through voltage-gated calcium channels and specific β cell uptake occurs after glucose stimulation[37]. However, that study, which emphasizes the detection of early changes before overt diabetes, does not account for the increase of vascular permeability that can influence manganese ion uptake by β cell.

Dhyani *et al*[38] reported that the kinetic parameters of dynamic manganese-enhanced MRI could be used to assess β-cell functionality; however, this method is limited by motion artifacts and low signal-to-noise ratios during pancreatic imaging. In our study, we used noninvasive MRI to image *in vivo* the vasculature of the pancreas in STZ-induced diabetic rats using the blood-pool agent Gadofluorine P. The strengths of the present method are as follows: (1) possible application for pancreatic MRI using conventional sequences; (2) relatively short circulation time as a blood pool agent; and (3) strong T1 contrast between the normal and diabetic pancreas.

In addition, MRI can evaluate pancreatic morphology, allowing the assessment of pancreatic volume and other pancreatic diseases (chronic pancreatitis, pancreatic cancer) that can cause diabetes.

The next logical step of our study is to test whether this method can detect early changes before overt diabetes, as blood glucose was significantly elevated one day after STZ injection in our model. We anticipate that with further development, this technique could diagnose type 1 diabetes early, as well as monitor vascular and islet changes noninvasively and quantitatively.

There are several limitations to this study. First, we used sequential imaging in the same rats for both contrast media. If we use a longer time interval, contrast media are more easily eliminated from the body, so the potential interaction between the contrast media might be avoided. However, the elimination half-life of Gd-DOTA is 18 min; therefore, the 24-h interval is considered sufficient for rats with healthy kidneys. In addition, the changes in the pancreas may progress during the delay between MRI. However, we did not perform histological analyses of the pancreas on day 4, which is a limitation of our study. However, we had a separate set of rats for blood glucose level monitoring for 30 d. In Figure 10, blood glucose level was elevated and plateaued above 4.5 g/L from day 3. MRI, which was selected for comparison of Gd-DOTA and Gadolinium P in our study, was obtained on day 4 and day 5. Moreover, we attempted to minimize the number of animals following the policy of our

institutional animal care and use committee. Second, there was a 24 h interval between the times of rat euthanasia for HE staining for islet number count (day 6) and the time when normalized SI and ER of control and diabetic rats showed a significant difference (day 5). Therefore, it is possible that islet number may be greater at 30 min after Gadofluorine P injection than at 24 h. Third, we demonstrated that Gadofluorine P accumulated in the pancreata of diabetic rats using LA-ICP-MS but Gd-DOTA did not; however, we did not demonstrate the location of Gadofluorine P in the pancreas. However, in the study of Medarova *et al*[33] of an STZ-induced mouse model of type 1 diabetes, they observed their fluorescence-conjugated T1 contrast medium in islet interstitium and islet-feeding blood vessels in diabetic rats but not in control rats using fluorescence microscopy.

In conclusion, in this STZ-induced diabetic rat model, Gadofluorine P-enhanced MRI of the pancreas showed high accuracy in the diagnosis of diabetes.

ACKNOWLEDGMENTS

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COMMENTS

Background

Diabetes mellitus is a group of metabolic diseases resulting from defects in insulin secretion or insulin action. Type 1 diabetes is characterized by insulin deficiency, which is caused by specific destruction of insulin-producing β-cells, which are located in the islets of Langerhans in the pancreas.

Research frontiers

Magnetic resonance imaging (MRI) is used in diabetes research including imaging of islet vasculature, imaging of islet transplantation, imaging of autoimmune attack in type 1 diabetes, and imaging of β cell mass.

Innovations and breakthroughs

This study compared two MRI T1 contrast media to detect diabetes in rats. This study showed that an increase in normalized signal intensity 30 min after Gadofluorine P injection was correlated with a decrease in the mean number of islets.

Applications

The ability to detect changes in the pancreas through noninvasive methods can have diverse important clinical benefits. For example, it can reduce potentially harmful biopsy during the pancreas transplantation. With further development, MRI and MRI contrast media or probes can detect the early changes of diabetes earlier than blood tests.

Terminology

Gd-DOTA is a first generation nonspecific extracellular MR contrast medium, which distributes into the intravascular and interstitial spaces and does not have a protein interaction. Gadofluorine P is an amphiphilic, water-soluble Gd complex. Gadofluorine P has strong interactions with hydrophobic proteins in blood (albumin) or extracellular tissue (extracellular matrix proteins). Therefore, Gadofluorine P has longer half-life in the blood and a higher tissue protein binding than that provided by standard extracellular MR contrast media. However, the protein binding of Gadofluorine P has no direct specific molecular targeting.

Peer-review

The authors utilized MRI and contrast media in the diagnosis of type 1 diabetes in rats and compare two MRI contrast media. Imaging methods can also evaluate other pancreatic disease (chronic pancreatitis and pancreatic cancer) with known association with diabetes. The manuscript is very well written.

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ORIGINAL ARTICLE

Basic Study

Expression profile of microRNAs in gastrointestinal stromal tumors revealed by high throughput quantitative RT-PCR microarray

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Abstract

AIM: To investigate the microRNA (miRNA) expression profile in gastrointestinal stromal tumor (GIST) tissues that could serve as a novel diagnostic biomarker for GIST detection.

METHODS: We performed a quantitative real-time quantitative reverse transcriptase polymerase chain reaction assay to analyze the expression of 1888 miRNAs in a sample set that included 54 GIST tissue samples.

RESULTS: We found that dysregulation of several miRNAs may be related to the malignant potential of GISTs. Six of these miRNAs, hsa-let-7c, miR-218, miR-488#, miR-4683, miR-34c-5p and miR-4773, were selected as the final list of biomarkers to separate the malignant GISTs (M group) from the benign GISTs (B group). In addition, MiR-29b-2#, hsa-let-7c, miR-891b, miR-218, miR-204, miR-204-3p, miR-628-5p, miR-744, miR-29c#, miR-625 and miR-196a were used to distinguish between the borderline (BO group) and M groups. There were 11 common miRNAs selected to separate the benign and borderline (BB) group from the M group, including hsa-let-7c, miR-218, miR-628-5p, miR-204-3p, miR-204, miR-891b, miR-488#, miR-145, miR-891a, miR-34c-5p and miR-196a.

CONCLUSION: The identified miRNAs appear to

be novel biomarkers to distinguish malignant from benign GISTs, which may be helpful to understand the mechanisms of GIST oncogenesis and progression, and to further elucidate the characteristics of GIST subtypes.

Key words: Gastrointestinal stromal tumors; MicroRNAs; Microarray analysis; Real-time polymerase chain reaction; Diagnosis

Core tip: Using high throughput quantitative reverse transcription-polymerase chain reaction microarray, we obtained a panel of miRNAs including hsa-let-7c, miR-218, miR-488#, miR-4683, miR-34c-5p and miR-4773, which can distinguish malignant from benign gastrointestinal stromal tumors (GISTs). Understanding the mechanisms of GIST tumorigenesis and development, may help to further elucidate the characteristics of GIST subtypes.

Tong HX, Zhou YH, Hou YY, Zhang Y, Huang Y, Xie B, Wang JY, Jiang Q, He JY, Shao YB, Han WM, Tan RY, Zhu J, Lu WQ. Expression profile of microRNAs in gastrointestinal stromal tumors revealed by high throughput quantitative RT-PCR microarray. *World J Gastroenterol* 2015; 21(19): 5843-5855 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5843.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5843

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal neoplasms of the gastrointestinal tract. GISTs were commonly misdiagnosed as leiomyomas or leiomyosarcomas until the 1990s when Miettinen *et al*[1] discovered that the expression of CD34 in the tumor can distinguish GISTs from leiomyomas and leiomyosarcomas. Currently, the diagnoses of GISTs have become more precise through immunohistochemical staining with the discovery of surface markers such as CD117 (positive rate, 95%) and DOG1 (95%).

GISTs are most commonly found in the stomach (60%) and small intestine (25%), while approximately 5% are found in the colon or rectum, and only 2% in the esophagus or other organs[2]. The clinical symptoms of GISTs include abdominal pain, nausea, dysphagia, and chronic gastrointestinal bleeding.

GISTs are a type of oncogenic mutation driven tumors, with common mutations arising in the KIT receptor tyrosine kinase gene (80%-90%) and the platelet-derived growth factor receptor alpha (PDGFRA) gene (5%)[3,4]. Recent research has revealed links to BRAF and SDH mutations[5,6]. GISTs are thought to originate from interstitial cells of Cajal (ICC), therefore, GISTs are found to express KIT and CD34 which are the characteristic features of ICC[7].

GISTs may recur in nearly 50% of patients after complete resection. In fact, half of high-risk GISTs may recur within 2 years of surgery[8]. Most GISTs recur in the liver or peritoneum[9]. The risk of recurrence is based on the following aspects: large tumor size (> 5 cm), high mitotic rate (> 5 mitoses per high power field), tumor location, margins of resection, and rupture of tumor[10]. Some retrospective studies indicated that GISTs located in the stomach have better prognosis than those arising from the small intestinal and rectum.

In the past decade, the search for oncogenes and tumor suppressor genes has focused on microRNAs (miRNAs), which negatively regulate target mRNAs[11]. Dysregulation of miRNAs can influence tumor differentiation, invasion, metastasis and recurrence[12]. Consequently, the development of new therapeutic strategies to target dysregulated tumor-driving miRNAs is vital. Recent investigations have focused on the relationship between miRNAs and GISTs, including studies of miR-494[13], miR-17, miR-20a, and miR-222[14].

The objective of this study was to analyze miRNA expression in different types of GISTs using a quantitative polymerase chain reaction (qPCR) array platform. Identifying and isolating dysregulated miRNAs may contribute to understanding of the mechanisms involved in GIST malignant progression.

MATERIALS AND METHODS

Study design, patients and samples

This study enrolled 54 GIST patients who were seen at Zhongshan Hospital, Fudan University during the period of October 2011 to July 2012. According to our previous study[15,16], the patients were classified into a benign GIST group (B group, n = 9), a borderline GIST group (BO group, n = 14) or a malignant GIST group (M group, n = 31). All samples were collected from consenting individuals according to the protocols approved by Zhongshan Hospital Ethics Committee. Patients were eligible for the study if they were 18 years of age or older and had a pathological diagnosis of GIST following surgical resection that met histological or cytological criteria. Histological typing of the tumors was performed according to the 2012 National Comprehensive Cancer Network (NCCN) Soft Tissue Sarcoma Guideline.

A total of 54 samples were analyzed and classified into 1 of 3 risk levels. Of these, 15 were characterized as low risk, 9 as intermediate risk, and 30 as high. The demographic and clinical features of the patients are summarized in Table 1.

RNA isolation from fresh tissues

Total RNA was isolated from 20 to 30 mg of frozen tissue with the miRNeasy Mini Kit (Qiagen, 217004)

Table 1 Characteristics of tissue samples

Characteristic	Benign GISTs (*n* = 9)	Borderline GISTs (*n* = 14)	Malignant GISTs (*n* = 31)
Age (yr)			
Median	68	58	58
Gender			
Male	4	6	19
Female	5	8	11
Risk			
Low	6	6	3
Intermediate	3	4	2
High	0	4	26

GISTs: Gastrointestinal stromal tumors.

according to the manufacturer's instructions. The quality of the isolated RNA was detected by agarose gel electrophoresis and the quantity was analyzed by an ultraviolet spectrophotometric method using Biomate3 (Thermo).

Total RNA pool making

The total RNA concentration of each sample was diluted to 62.5 ng/μL, and 10 μL of the total RNA from each sample was combined to make the total RNA pool.

First-strand cDNA synthesis

First-strand cDNA was generated from the total RNA sample by reverse transcription (RT) using the Sharpvue™ miRNA First Strand Kit (Biovue, 9000004) following the manufacturer's protocol. A poly (A) tail was added to the 3' end of miRNAs, and RT of total RNA to the first-strand cDNA was performed using a universal RT primer. The RT reaction was carried out using a GeneAmp PCR 9700 Thermocycler (Applied Biosystems). The reactions were incubated at 37 ℃ for 60 min and were inactivated by incubation at 95 ℃ for 10 min.

Real-time quantitative PCR

Single-tube miRNA assays were used to detect and quantify mature miRNAs by Sharpvue™ 2 × Universal qPCR Master Mix High Rox (Biovue, 9000008) and Sharpvue™ Human miRNA Primer assay (Biovue) under conditions defined by the supplier. The RNA pool was detected using 1920 miRNA primers including 35 controls in five 384-well plates. Each plate contained 3 endogenous controls (hsa-7SL-scRNA, hsa-RNU6B, and hsa-RNU48) in duplicate and one no template control. MiRNA expression levels were quantified using the 7900HT Fast Real-Time PCR System (Applied Biosystems). The reactions were incubated in a 384-well optical plate at 95 ℃ for 2 min, followed by 3 cycles of 96 ℃ for 5 s and 60 ℃ for 1 min, then 37 cycles of 96 ℃ for 5 s and 60 ℃ for 30 s.

Statistical analysis

Data analyses were performed using R and Bioconductor packages. For the data obtained by qRT-PCR, raw cycle threshold (Ct) measurements were used for the comparison among the 3 risk levels. To remove differences in the RNA input used to profile the 54 samples analyzed in the study, we used the Quantile-Median method to process the raw Ct measurements. Samples that showed significantly different profiles (mean absolute difference) from all other samples (Bioconductor package "arrayQualityMetrics") were considered to be outlier samples and were removed from downstream analysis. No sample was removed. Differential expression analysis was performed on the samples using *t*-test (R package "limma"). MiRNAs producing false discovery rate (FDR) adjusted *P*-values below 0.01 and fold-changes above 2 were judged as differentially expressed. The predicted probability of being diagnosed with GIST was used as a surrogate marker to construct receiver operating characteristic (ROC) curves. The area-under-the-ROC curve (AUC) was used as an accuracy index for evaluating the diagnostic performance of the selected miRNA panel.

RESULTS

Clinicopathological characteristics

The 54 GIST patients ranged in age from 32 to 78 years (median: 60.2 years). Of these, 30 were cases located in the stomach (55.6%), 8 in the small intestine (14.8%), 4 in the pelvic cavity (7.4%), 4 in the abdominal cavity (7.4%), 2 in the duodenum (3.7%), and 1 case each (1.9% each) in the rectum, colon, and esophagus, respectively. Among these 54 cases, 3 developed hepatic metastases (5.6%). All samples were diagnosed by pathology, and the macroscopic description was available.

Among these GIST samples, 5 were wild-type GISTs (9.3%), 19 had the C-kit exon 11 mutation (35.2%), and 4 had the C-kit exon 17 mutation (7.4%). For the remaining cases, gene mutation status was unknown.

Fifteen cases were defined as low-risk GISTs (27.8%), 9 as intermediate risk (16.7%), and the remaining 30 as high risk (64.8%).

Pathological analyses unveiled 31 cases as malignant GISTs (57.4%), 14 as borderline GISTs (25.9%), and 9 as benign GISTs (16.7%).

MiRNA data testing and analysis

For the data obtained by qRT-PCR, the GIST miRNA functional panel (3 cards including 849 human miRNAs and 2 endogenous controls) was developed by Biovue Technology Ltd based on the miRNA expression results from the GIST tissue pool. The 851 miRNAs had Ct values below the detection threshold of 35 in 54 samples.

Data analysis was performed using the "arrayQualityMetrics" Bioconductor package. This procedure removed 1 tumor tissue sample (GIST-018, malignant) that showed significantly different profiles from the

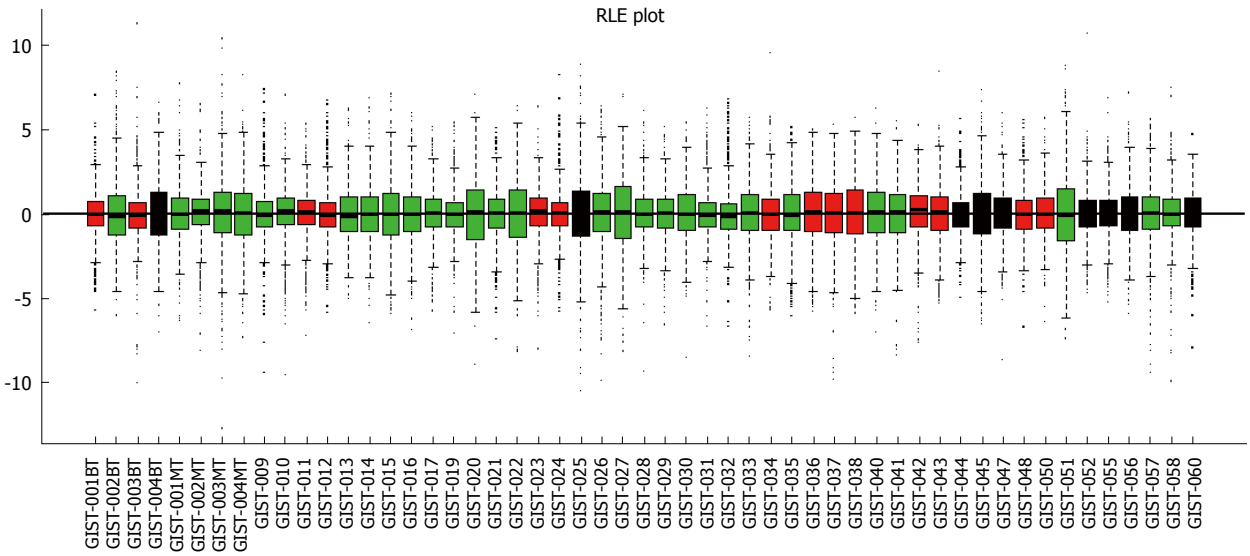

Figure 1 Relative log expression plot (quantile) of normalized data. There were no significantly different profiles between samples. GIST: Gastrointestinal stromal tumor; RLE: Relative log expression.

other samples (Figure 1). Thus, 53 tissue samples were analyzed for miRNA expression, including 9 benign GIST tissue samples, 30 malignant GIST tissue samples, and 14 borderline GIST tissue samples.

Differentially expressed miRNAs between different GIST types

The miRNA expression in the 53 tissue samples were compared in 3 group pairs: the B group (9 benign GIST tissue samples) compared with the M group (30 malignant GIST tissue samples); the BO group (14 borderline GIST tissue samples) compared with the M group (30 malignant GIST tissue samples); and the benign and borderline (BB) group (9 benign GIST tissue samples and 14 borderline GIST tissue samples) compared with the M group (30 malignant GIST tissue samples). The hierarchical clustering of the miRNAs was shown by adjusted *P*-values (FDR) below 0.1 and fold-changes above 2 (red point). Figure 2 shows the comparisons for 4 miRNAs that were differentially expressed in the B group and M groups, while Figure 3 shows the comparisons for 82 miRNAs that were differentially expressed in the BO group and M groups. Figure 4 shows the comparisons for 54 miRNAs that were differentially expressed in the BB group and M groups.

Biomarker selection

To develop a prediction algorithm for malignant GIST diagnosis from a population of samples containing malignant GIST tissue samples (n = 30), borderline GIST tissue samples (n = 14), and benign GIST tissue samples (n = 9). Three classification methods were used: support vector machine (SVM, Bioconductor package "e1071"), K-nearest neighbors (KNN, Bioconductor package "class"), and diagonal linear discriminate analysis (Bioconductor package "sfsmisc"). Algorithm performance was initially evaluated using a leave-one-out cross-validation procedure for different numbers of predictor markers. For each set of training samples, miRNAs were ranked based on their *t*-test *P*-values generated when comparing malignant *vs* normal tissues. The top "*n*" miRNAs (where *n* was allowed to range between 2 and 35) were used to build a prediction model based on the training samples, and applied to the remaining test samples. Prediction class and probability were recorded for each sample and classification algorithm. Figure 5A shows that the resulting error rates were relatively stable when 4 predictor markers were used. Due to the limited number of samples available for this study, we chose the common miRNAs from these lists as the final list of predictors (selected markers).

Differential miRNAs between the B and M groups

Six common miRNAs were selected as the final list of biomarkers for malignant GIST diagnosis (Table 2). The characteristics of these miRNAs, including fold-changes between malignant and normal tissues, together with *t*-test *P*-values and FDR-adjusted *P*-values, are presented in Table 2. The 4 miRNAs included hsa-let-7c, miR-218, miR-488#, and miR-4683, which had lower expression levels in the M group than in the B group. On the contrary, other miRNAs such as miR-34c-5p and miR-4773 had significantly higher expression levels in the M group.

The sensitivity of the selected markers in the detection of malignant GISTs was 97% and the specificity was 67%, with an AUC of 0.874 (Figure 5B). The classification performance of the selected 6 miRNAs for the SVM algorithm, when the leave-one-out cross-validation procedure was used, is presented in Figure 1).

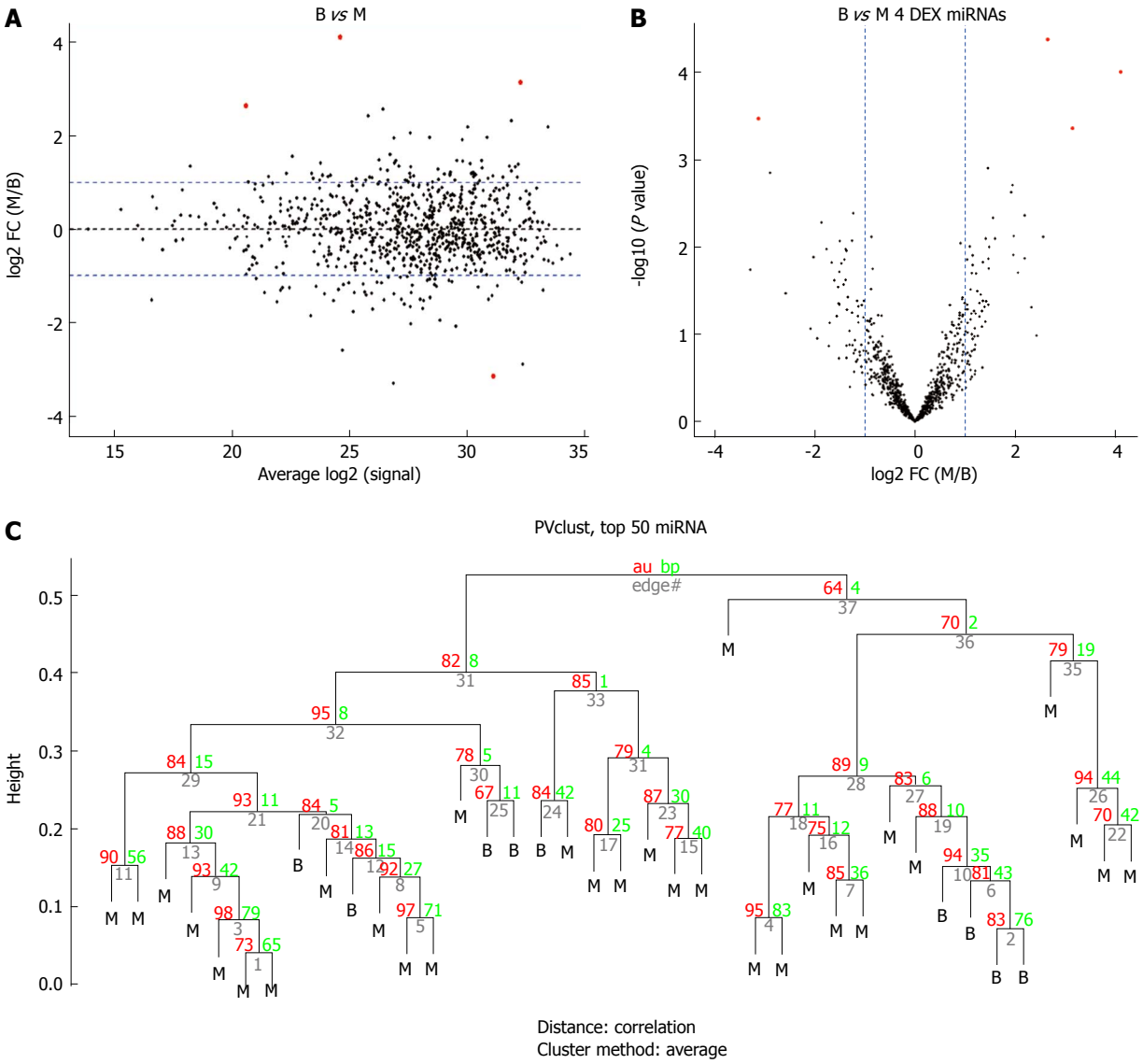

Figure 2 Comparison of the benign gastrointestinal stromal tumors (B group) *vs* the malignant gastrointestinal stromal tumors (M group). A: MA plot of assays used to profile the compared samples: fold-change (Y-axis) *vs* normalized Ct measurements; B: Volcano plot of the resulting *P* values of the *t*-test between the B and M groups. Four miRNAs show adjusted *P* values (FDR) below 0.1 and fold-changes above 2 (shown in red); C: Hierarchical clustering of the 9 benign GIST tissues and 30 malignant GIST tissues based on the 50 most variable (top 50) miRNA assays. GIST: Gastrointestinal stromal tumor; FDR: False discovery rate.

Differential miRNAs between the BO and M groups

Eleven common miRNAs were also selected to separate the BO group from the M group (Table 3), including 29b-2#, hsa-let-7c, miR-891b, miR-218, miR-204, miR-204-3p, miR-628-5p, miR-744, miR-29c# and miR-625, which were overexpressed in the BO group. In contrast, miR-196a had lower expression in the BO group. The predicted probability was used to construct an ROC curve. The AUC was 0.96, with an 87% sensitivity and a 93% specificity (Figure 6).

Differential miRNAs between the BB and M groups

There were 11 common miRNAs selected to separate the BB group from the M group, including hsa-let-7c, miR-218, miR-628-5p, miR-204-3p, miR-204, miR-891b, miR-488#, miR-145 and miR-891a, which were overexpressed in the BB group (Table 4). In contrast, miR-34c-5p and miR-196a had lower expression in the BB group. The predicted probability was used to construct an ROC curve. The AUC was 0.906, with an 87% sensitivity and an 82% specificity (Figure 7).

DISCUSSION

By comparing benign, borderline, and malignant GISTs, we found that the dysregulation of several miRNAs was related to the malignant potential of GISTs. An expression signature of 6 miRNAs was selected to separate malignant GISTs from benign GISTs. Of these, 4 miRNAs (hsa-let-7c, miR-218, miR-488#, and miR-4683) were down-regulated in malignant GISTs, and 2 miRNAs (miR-34c-5p and miR-4773) were up-regulated in malignant GISTs. An expression signature of 11 miRNAs (miR-29b-2#, hsa-let-7c,

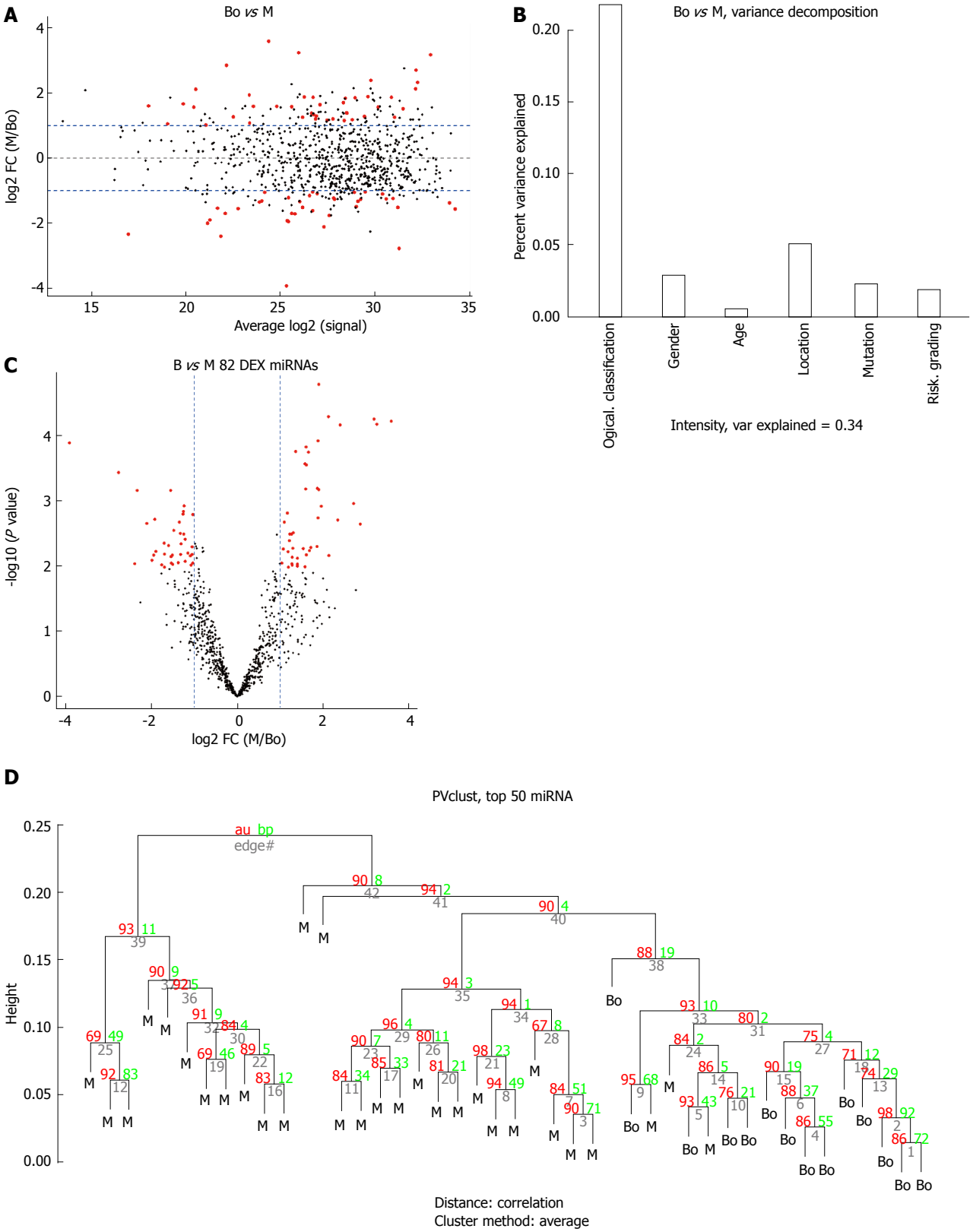

Figure 3 Comparison of the borderline gastrointestinal stromal tumors (borderline group) *vs* the malignant gastrointestinal stromal tumors (M group). A: MA plot of assays used to profile the compared samples: fold-change (Y-axis) *vs* normalized Ct measurements; B: 45% of the variance observed in the Ct measurements of the 50 most variable (top 50) miRNA assays across all samples can be attributed to the sample description (BO or M). The remaining covariates considered here ("gender", "tumor grade", or "stage") account for less than 5%; C: Volcano plot of the resulting *P*-values of the *t*-test between the Bo and M groups. Eighty-two miRNAs show adjusted *P*-values (FDR) below 0.1 and fold-changes above 2 (shown in red); D: Hierarchical clustering of the 14 borderline GIST tissues and 30 malignant GIST tissues based on the 50 most variable (top 50) miRNA assays. GIST: Gastrointestinal stromal tumor; FDR: False discovery rate; M: Malignant GIST tissues.

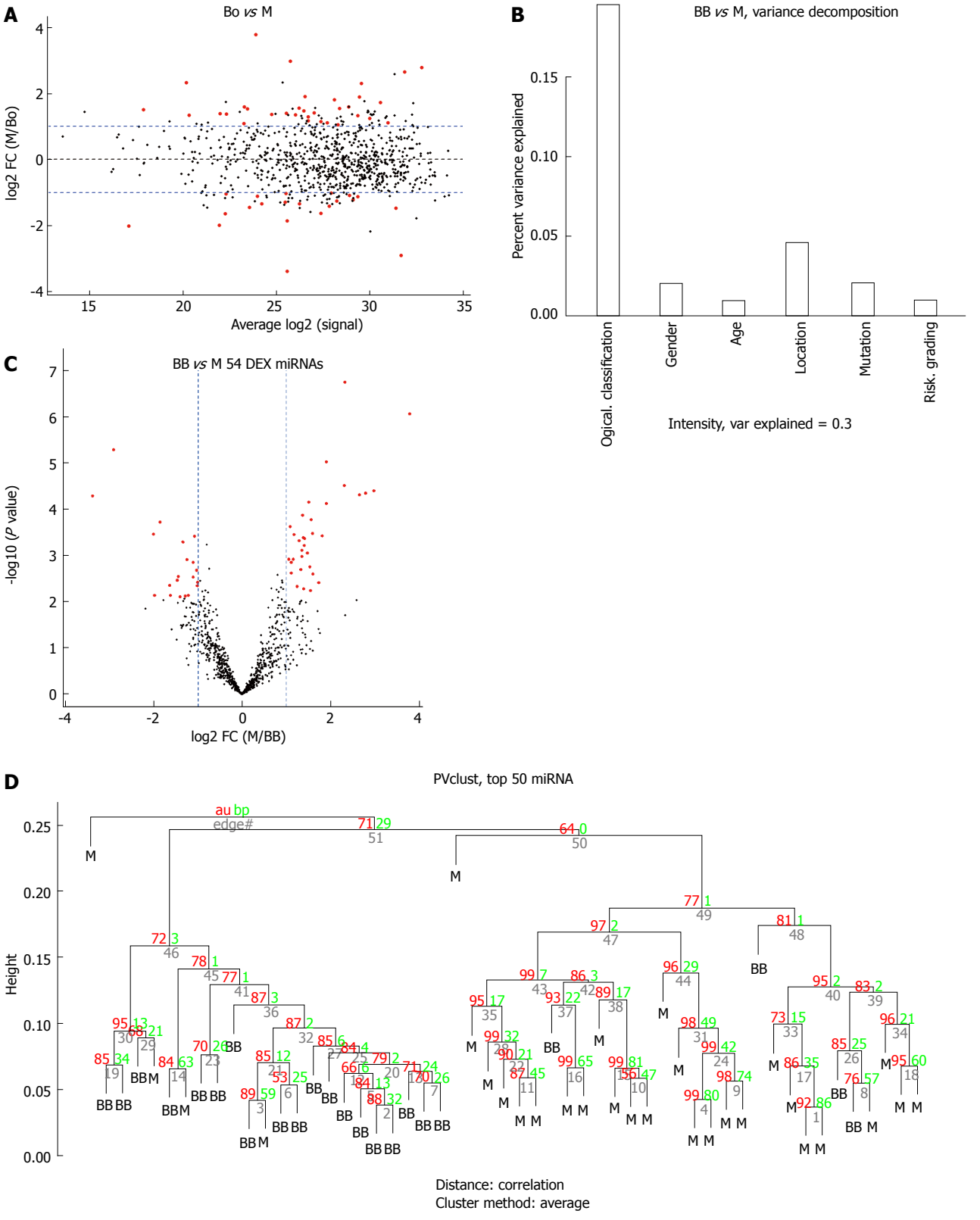

Figure 4 Comparison of the benign or borderline gastrointestinal stromal tumors (benign and borderline group) *vs* the malignant gastrointestinal stromal tumors (M group). A: MA plot of assays used to profile the compared samples: fold-change (Y-axis) *vs* normalized Ct measurements; B: Forty-five percent of the variance observed in the Ct measurements of the 50 most variable (top 50) miRNA assays across all samples can be attributed to the sample description (BB or M). The remaining covariates considered here ("gender", "tumor grade" or "stage") account for less than 5%; C: Volcano plot of the resulting *P*-values of the *t*-test between the Bo and M groups. Fifty-four miRNAs show adjusted *P*-values (FDR) below 0.1 and fold-changes above 2 (shown in red); D: Hierarchical clustering of the 23 benign or borderline GIST tissues and 30 malignant GIST tissues based on the 50 most variable (top 50) miRNA assays. GIST: Gastrointestinal stromal tumor; FDR: False discovery rate; M: Malignant GIST tissues.

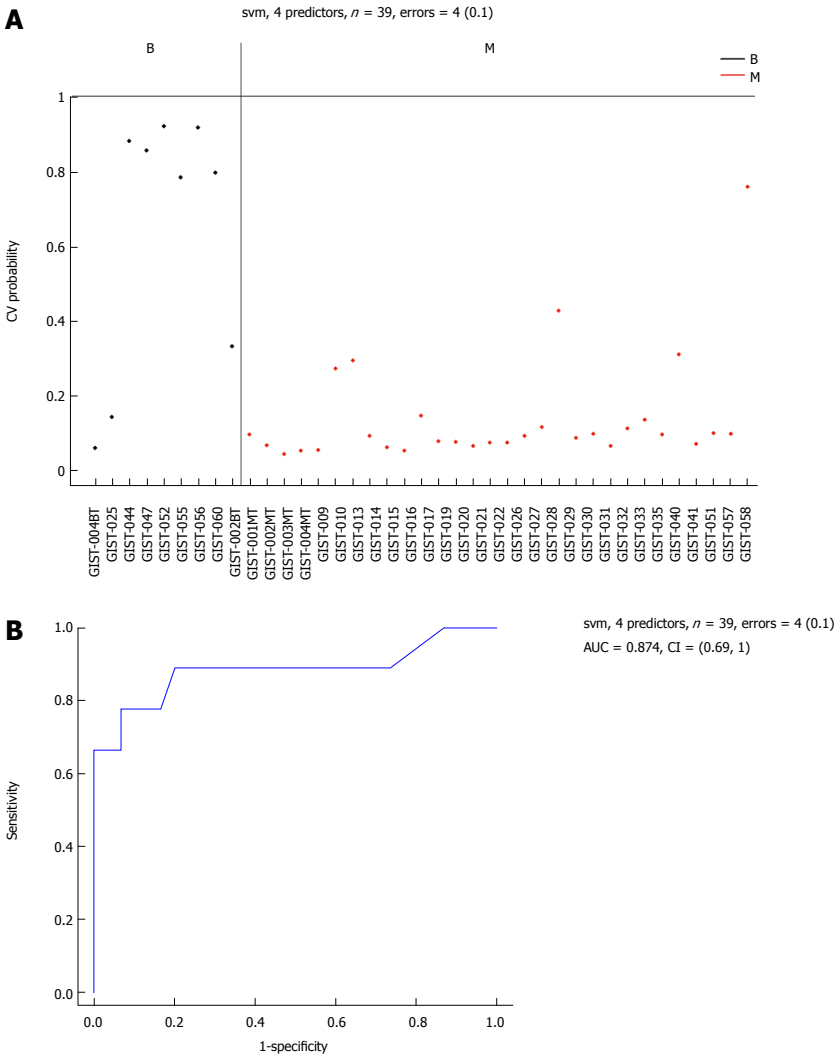

Figure 5 Area-under-the-curve (0.874) estimation for the miRNA panel in the B and M groups. Performance of the 6 selected miRNAs in Table 2 for classification of benign gastrointestinal stromal tumor (GIST) tissues compared with malignant GIST tissues, using a support vector machine (SVM) algorithm and leave-one-out cross-validation procedure. A: Benign GIST tissue prediction probabilities for each sample used in this study: 39 samples with an error = 4 (0.1); B: Receiver operating characteristic (ROC) curve [area-under-the-curve (AUC) = 0.874]. M: Malignant GIST tissues.

Table 2 MicroRNA signatures between the benign gastrointestinal stromal tumors (B group) and the malignant gastrointestinal stromal tumors (M group)

ID	logFC	AveExpr	*t*	*P* value	Adjusted *P* value
hsa-let-7c	2.645556	20.57949	4.561671	4.20E-05	0.035449
MIR-218	4.103333	24.58974	4.293033	9.85E-05	0.041616
MIR-34C-5P	-3.13333	31.12308	-3.89399	0.000339	0.091870
MIR-488#	3.141111	32.27179	3.811858	0.000435	0.091870
MIR-4683	1.455556	29.83077	3.452312	0.00126	0.199198
MIR-4773	-2.89333	32.37436	-3.41224	0.001414	0.199198

Negative numbers indicate that the fold change is lower in the B group than in the M group. LogFC: Logarithm fold change; Aveexpr: Average expression.

miR-891b, miR-218, miR-204, miR-204-3p, miR-628-5p, miR-744, miR-29c#, miR-625 and miR-196a) was selected to separate borderline GISTs from malignant GISTs. An expression signature of 11 miRNAs (hsa-let-7c, miR-218, miR-628-5p, miR-204-3p, miR-204, miR-891b, miR-488#, miR-145, miR-891a, miR-34c-5p and miR-196a) was selected to separate benign GISTs and borderline GISTs from malignant GISTs.

In these 3 comparisons, let-7c and miR-218 were down-regulated in malignant GISTs. The down-expression of let-7 family genes has been reported in many cancers. For example, a low level of let-7c expression was observed in human non-small cell lung cancer, and down-regulation of let-7c could inhibit

Table 3 MiRNA signatures between the borderline gastrointestinal stromal tumors (borderline group) and malignant gastrointestinal stromal tumors (M group)

ID	logFC	AveExpr	*t*	*P* value	Adjusted *P* value	B
MIR-29B-2#	1.887619	26.27273	4.791486	1.67E-05	0.009785	2.889621
hsa-let-7c	2.118571	20.51591	4.449458	5.20E-05	0.009785	1.850695
MIR-891b	3.170952	32.94773	4.420680	5.71E-05	0.009785	1.764525
MIR-218	3.579524	24.39773	4.399150	6.13E-05	0.009785	1.700197
MIR-204	3.238095	25.98636	4.368453	6.78E-05	0.009785	1.608688
MIR-204-3P	2.390000	29.77955	4.360826	6.95E-05	0.009785	1.585990
MIR-628-5P	1.875238	26.75000	4.185139	0.000123	0.013800	1.067600
MIR-196a	-3.906670	25.33636	-4.165320	0.000131	0.013800	1.009689
MIR-744	1.590476	24.82727	4.120092	0.000151	0.013983	0.877951
MIR-29C#	1.350476	26.76364	4.070312	0.000177	0.013983	0.733696
MIR-625	1.648095	27.10227	4.061304	0.000182	0.013983	0.707679

Negative numbers indicate that the fold change is lower in the borderline group than in the M group. LogFC: Logarithm fold change; AveExpr: Average expression.

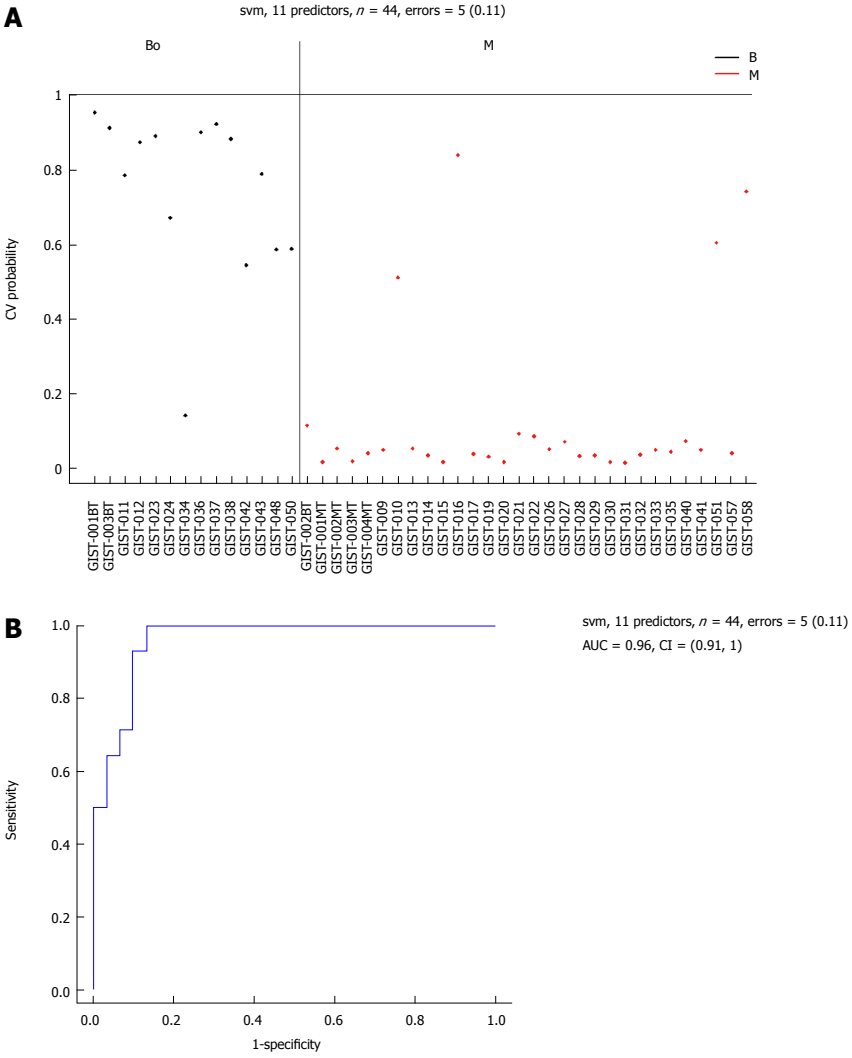

Figure 6 Area-under-the-curve (0.96) estimation for the miRNA panel in the borderline and M groups. Performance of the 11 selected miRNAs for classification of borderline gastrointestinal stromal tumor tissues compared with malignant gastrointestinal stromal tumor tissues. A: Support vector machine (SVM) prediction probability for 44 samples with an error = 5 (0.11); B: Area-under-the-curve (AUC = 0.96) estimation for the miRNA panel in the borderline gastrointestinal stromal tumor (GIST) tissues and the malignant GIST tissues. M: Malignant GIST tissues.

the tumor migration and investigation by targeting ITGB3 and MAP4K3[17]. TRIB2 was a target and was negatively regulated by let-7. As the expression of let-7 increased, the downstream effectors of TRIB2

Table 4 miRNA signatures between the benign gastrointestinal stromal tumors or borderline gastrointestinal stromal tumors (benign and borderline group) and the malignant gastrointestinal stromal tumors (M group)

ID	logFC	AveExpr	*t*	*P* value	Adjusted *P* value	B
hsa-let-7c	2.324783	20.18113	5.938844	1.86E-07	0.000157	7.046674
MIR-218	3.784493	23.89434	5.519007	8.86E-07	0.000375	5.587287
MIR-34C-5P	-2.908700	31.66226	-5.032010	5.24E-06	0.001477	3.931173
MIR-628-5P	1.903188	26.52075	4.863745	9.58E-06	0.002023	3.370903
MIR-204-3P	2.309565	29.53774	4.525668	3.14E-05	0.004925	2.268094
MIR-204	2.973188	25.72642	4.455823	4.00E-05	0.004925	2.044513
MIR-891b	2.787101	32.74717	4.417100	4.57E-05	0.004925	1.921237
MIR-488#	2.648841	31.84717	4.392406	4.98E-05	0.004925	1.842883
MIR-196a	-3.389280	25.56415	-4.376970	5.25E-05	0.004925	1.794012
MIR-145	1.505217	17.88679	4.290314	7.05E-05	0.005869	1.521146
MIR-891a	1.900870	29.41509	4.266462	7.64E-05	0.005869	1.446506

Negative numbers indicate that the fold change is lower in the BB group than in the M group. LogFC: Logarithm fold change; AveExpr: Average expression.

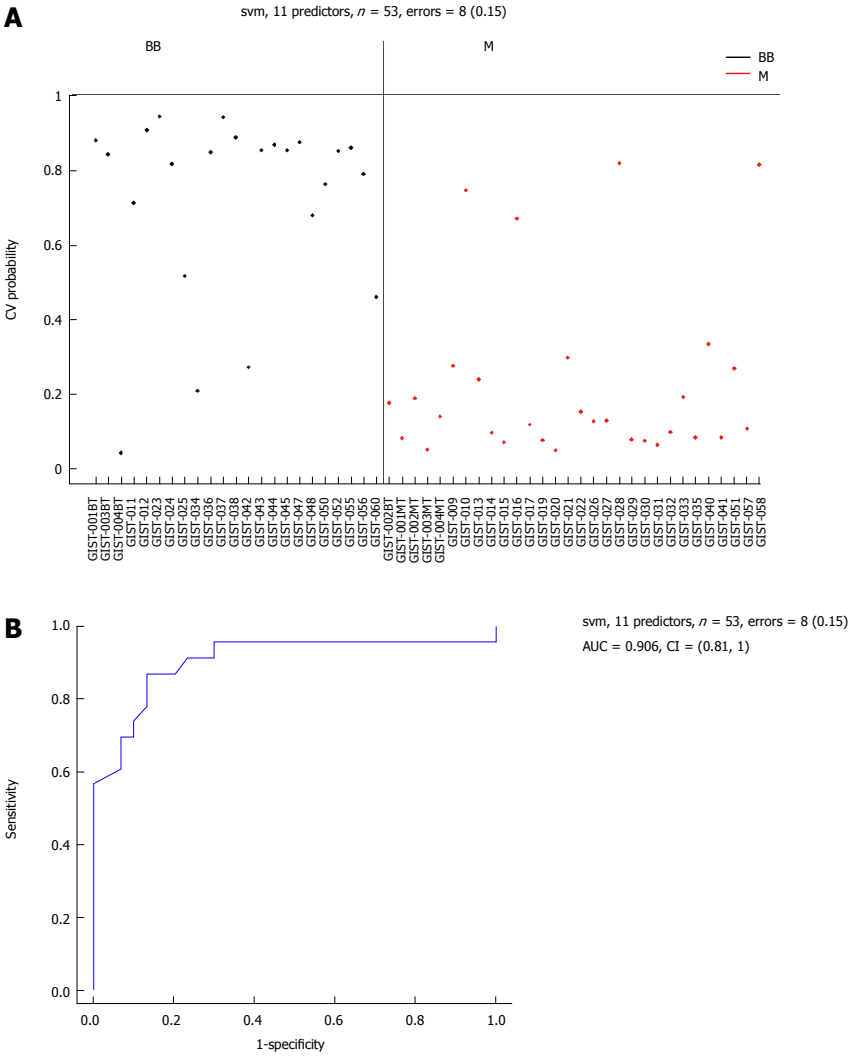

Figure 7 Area-under-the-curve (0.906) estimation for the miRNA panel in the benign or borderline gastrointestinal stromal tumor tissues and M groups. Eleven selected miRNAs comparing the BB and M groups. A: Support vector machine (SVM) prediction probability for 53 samples with an error = 8 (0.15); B: Area-under-the-curve (AUC = 0.906) estimation for the miRNA panel in the BB and M groups. BB: Benign or borderline GIST tissues; M: Malignant GIST tissues; GIST: Gastrointestinal stromal tumor.

was increased, and the activities of C/EBP-α and phosphorylated p38MAPK were also increased. TRIB2, C/EBP-α and phosphorylated p38MAPK were related to cell proliferation[18]. Other studies showed that let-7c inhibited cell proliferation by targeting other genes such as HOXA1[19]. Furthermore, a study performed by Brennan *et al*[20] concluded that let-7 targets several members of the TGF-β1 signaling pathway.

As a well-known homeodomain protein, PBX2 was identified as a novel let-7c target that may contribute to the AML phenotype[21]. In colorectal cancer, let-7c can suppress metastasis by targeting MMP1 and PBX3[22].

Let-7c overexpression enhanced apoptosis in endothelial cells[23]. In esophageal squamous cell carcinoma, the expression of let-7c is a significant factor of response to chemotherapy by regulation of the IL-6/STAT3 pathway[24]. Taken together, most of these studies suggest that downregulation of let-7c was related to tumor metastasis and cell differentiation, and all of these studies imply that let-7c may be related to GIST immigration and invasion.

The expression of miR-218 was previously found to be reduced in many cancers. For example, the upregulation of miR-218 was found to reduce the migration and invasion of glioma cells, while the suppression of miR-218 is able to increase the invasive ability of the cells[25]. In oral squamous cell carcinoma, miR-218 was found to target the gene Rictor, and through its regulation of the expression of Rictor, miR-218 is able to activate the TOR-Akt pathway[26]. A recent study found that the expression of miR-218 decreases in human GIST tissue and cell lines, and miR-218 can negatively regulate the expression of KIT protein and inhibit the proliferation and invasion of GIST cells[27].

For those miRNAs found to be upregulated in malignant GISTs, miR-34c and miR196a seem to be most important. miR-34c was revealed as a tumor suppressor in prostate cancer by targeting MET, a receptor tyrosine kinase activated by hepatocyte growth factor, which is crucial for metastatic progression[28]. In osteosarcoma, miR-43c controls cell proliferation by influencing the p53-miR-34c-RUNX2 network[29]. Yang *et al*[30] found that miR-34a/c is lowly expressed in metastatic breast cancer cells and human primary breast tumors with lymph node metastases and overexpressing miR-34c can also inhibit cancer cell migration and invasion. In uveal melanoma, miR-34c suppressed cell proliferation *via* the cell cycle proteins CDK4, CDK6, and Rb[31]. A study revealed that miR-34c downregulates the expression of ULBP2, and diminishes tumor cell recognition by NK cells[32]. In our study, miR-34c was overexpressed in malignant GISTs, which may indicate a role in tumorigenesis and tumor progression.

MiR-196a is also an important miRNA that is related to tumor progression. A positive correlation has been found with miR-196a expression and the progression from intestinal metaplasia to adenocarcinoma[33]. Some researchers found that the overexpression of miR-196a was associated with high-risk grade, metastasis and poor survival, and implied that the genes HOXC and HOTAIR may be miR-196a target genes[7]. In that study, the authors revealed that miR-196a upregulated in gastric cancer tissues and cell lines was related to tumor size, poor pT stage, pN stage and survival time. Downregulation of miR-196a can suppress cancer cell proliferation. Dysregulation of miR-196a has also been found in other cancers such as breast cancer and pancreatic adenocarcinoma[34].

Recently, some studies indicated that deregulation of miRNAs had close relation to imatinimb resistance in GISTs. Akçakaya *et al*[35] found that miR-125a-5p is overexpressed in imatinib resistant GISTs, and could down-regulate PTPN18 to induce imatinib resistance in GISTs. A study showed that low expression of miR-320a was correlated with short time to imatinib resistance, and proposed the potential mechanism of miR-320a for imatinib resistance[36]. These studies mean that miRNAs may have potential to be targets for imatinib resistance in GISTs.

Although these specific miRNAs were isolated in our assays, further studies need to be performed to confirm the veracity of our results. For example, validation of these results in a study with a larger sample size would provide a more clear understanding and interpretation of these preliminary data.

In conclusion, through the utilization of real-time quantitative RT-PCR-based miRNA assays to analyze the expression of 1888 miRNAs in GIST samples, 3 expression signatures of miRNAs were selected to diagnose malignant GISTs. The dysregulation of these miRNAs may be related to the malignance of GISTs, and therefore serves as a valuable target to further study the mechanisms of malignant GIST development.

COMMENTS

Background

Gastrointestinal stromal tumor is the most common mesenchymal neoplasia in the gastrointestinal tract and has a broad spectrum of pathological patterns and clinical features ranging from benign to malignant. Advances in high-throughput technologies such as high-throughput real-time quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) microarray for gene expression profiling have reinforced the identification of molecular characteristics of tumors. Screening and testing for the role of miRNAs in distinguishing malignant gastrointestinal stromal tumor (GIST) from benign GISTs is of great clinical value.

Research frontiers

MicroRNAs have an important role in the pathogenesis of various human cancers by regulating the expression of target genes post-transcriptionally. High-throughput RT-qPCR microarray combined with clinical statistical analysis enabled the identification of biomarkers in hundreds of microRNAs.

Innovations and breakthroughs

A panel of miRNAs, including hsa-let-7c, miR-218, miR-488#, miR-4683, miR-34c-5p and miR-4773, as biomarkers, are able to separate the malignant GISTs

from the benign GISTs. These may contribute to a better understanding of the mechanisms involved in GIST oncogenesis and progression, and further elucidation of the characteristics of GIST subtypes.

Applications

The use of high-throughput RT-qPCR microarray to evaluate the expression profile of microRNAs in tumor tissues will contribute to the characterization of cancer heterogeneity in order to potentially develop personalized therapy for patients.

Terminology

A microarray is a multiplex lab-on-a-chip that assays large amounts of biological material using high-throughput screening methods. The types of microarrays include DNA microarray, microRNA microarray, protein microarray, and tissue microarray. High-throughput RT-qPCR is a current technique widely used in studying expression patterns of genes.

Peer-review

This study aims at identifying an miRNA expression profile in GIST tissues to be used as a novel diagnostic biomarker and as the basis for investigation of novel target therapies. Although the cohort is not large, the authors were able to separate the M, B, and BB groups based on their deregulated expression profile. The methods are accurate and the results are convincing.

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ORIGINAL ARTICLE

Basic Study

Hepatocyte nuclear factor 4α induces a tendency of differentiation and activation of rat hepatic stellate cells

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Abstract

AIM: To investigate the effect of hepatocyte nuclear factor 4α (HNF4α) on the differentiation and transformation of hepatic stellate cells (HSCs).

METHODS: By constructing the recombinant adenovirus vector expressing HNF4α and HNF4α shRNA vector, and manipulating HNF4α expression in HSC-T6 cells, we explored the influence of HNF4α and its induction capacity in the differentiation of rat HSCs into hepatocytes.

RESULTS: With increased expression of HNF4α mediated by AdHNF4α, the relative expression of Nanog was downregulated in HSC-T6 cells (98.33 ± 12.33 *vs* 41.33 ± 5.67, $P < 0.001$). Consequently, the expression of G-P-6 and PEPCK was upregulated (G-P-6: 14.34 ± 3.33 *vs* 42.53 ± 5.87, $P < 0.01$; PEPCK: 10.10 ± 4.67 *vs* 56.56 ± 5.25, $P < 0.001$), the expression of AFP and ALB was positive, and the expression of Nanog, Type Ⅰ collagen, α-SMA, and TIMP-1 was significantly decreased. HNF4α also downregulated vimentin expression and enhanced E-cadherin expression. The ultrastructure of HNF4α-induced cells had more mitochondria and ribosomes compared with the parental cells. After silencing HNF4α expression, EPCK, E-cadherin, AFP, and ALB were downregulated and α-SMA and vimentin were upregulated.

CONCLUSION: HNF4α can induce a tendency of differentiation of HSCs into hepatocyte-like cells. These findings may provide an effective way for the treatment

of liver diseases.

Key words: Hepatocyte nuclear factor 4α; Hepatic stellate cells; Adenovirus vector; Differentiation; Rat

Core tip: Hepatocyte nuclear factor 4α (HNF4α) is an important transcription factor in liver differentiation. When enhancing HNF4α expression in hepatic stellate cell line hepatic stellate cells (HSCs)-T6, the expression of G-P-6, PEPCK, and E-cadherin was upregulated, the expression of Type Ⅰ collagen, α-SMA, TIMP-1, and vimentin was downregulated, and the induced cells were positive for AFP and ALB. When silencing HNF4α expression with shRNA vector, EPCK and E-cadherin were downregulated and α-SMA and vimentin were upregulated. The results demonstrated that HNF4α can induce a tendency of differentiation of HSCs into hepatocyte-like cells. These findings may provide an effective method for treating liver diseases.

Liu K, Guo MG, Lou XL, Li XY, Xu Y, Ji WD, Huang XD, Yang JH, Duan JC. Hepatocyte nuclear factor 4α induces a tendency of differentiation and activation of rat hepatic stellate cells. *World J Gastroenterol* 2015; 21(19): 5856-5866 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5856.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5856

INTRODUCTION

Hepatic stellate cells (HSCs) are stem-like cells that have recently been described as a liver-resident mesenchymal stem cell (MSC) population. This is due to their MSC-related expression profile, which expresses a variety of stem cell markers such as nestin, CD24, CD105, CD133, and c-kit, but can also serve as a progenitor cell population with hepatobiliary characteristics[1,2]. HSCs play crucial roles in liver repair and regeneration after liver injury[3-5]. Transplanted HSCs can home to the injured liver and contribute to tissue regeneration by developing into putative progenitor cells, epithelial cells, and mesenchymal tissues[2]. Under incubation of different cytokines in the culture medium, selected CD133+ cells from fresh rat HSCs have been shown to differentiate into stromal cells, endothelial cells, and hepatocyte-like cells *in vitro*[6,7]. In the glial fibrillary acidic protein (GFAP)-Cre/green fluorescent protein (GFP) transgenic mouse liver injury animal model, HSCs displayed the capacity to develop into albumin-expressing hepatocytes[5]. Following liver injury, activated HSCs secret cytokines, such as hepatocyte growth factors (HGF), activate hedgehog receptors to promote liver repair and regeneration[8,9]. In contrast, Foxf1$^{+/-}$ mice exhibited abnormal liver repair, diminished HSC activation, and aggravated liver tissue damage following CCl_4 injury[10]. Therefore, differentiated HSCs can be used as seed cells in hepatocyte transplantation, and can also secrete cytokines to promote liver repair and regeneration. However, the differentiation capacity of HSCs and related molecular mechanisms remain unclear.

Genetic engineering techniques can regulate important genes in stem cell differentiation. How to directionally induce the differentiation of stem cells into hepatic cells and enhance their biological function by genetic techniques has become a central topic in the treatment of end-stage liver disease by cell transplantation[11,12]. The hepatocyte nuclear factor (HNF) family is a group of important transcription factors in the regulation of liver differentiation. Members of the HNF family include HNF1, HNF3, HNF4, HNF6, and CCAAT/enhancer-binding protein (C/EBP). Of these, HNF4 is a vital transcriptional regulator in the differentiation of liver function, and consists of three types: HNF4α, HNF4β, and HNF4γ. HNF4α regulates the differentiation of hepatocytes, preserves their biological function, and is highly expressed in mature hepatic cells, where it plays a vital role in maintaining the epithelial phenotype of hepatocytes.

The expression of HNF4α in HSCs has been reported to significantly decrease in hepatocyte injury and chronic liver disease[13]. Activated HSCs transform into myofibroblasts and secrete extracellular matrix (ECM)[14]. If the HNF4α expression in HSCs is rescued by transfection, the biological character of HSCs can be reversed, indicating that HNF4α is an important regulatory factor in maintaining the epithelial phenotype of hepatocytes. Upregulated expression of HNF4α can inhibit transformation of HSCs into stromal cells, and promote cell differentiation and regeneration into hepatocytes[15]. All of these findings indicate that HNF4α is a vital regulator that maintains the endothelial cell state of HSCs. Because of the importance of HSCs in the progression of liver fibrosis, we intend to clarify the functions of HNF4α and the mechanism by which it regulates the participation of HSCs in liver fibrosis. The results of this investigation will provide a new direction in which the pathogenesis and prevention of liver fibrosis can be studied. Therefore, in this study, we constructed a recombinant adenovirus expression vector (AdHNF4α) capable of carrying the full-length cDNA of HNF4α. By manipulating HNF4α expression using AdHNF4α, we explored the influence of HNF4α and its induction capacity in the differentiation of HSCs into hepatic cells in the rat HSC-T6 cell line.

MATERIALS AND METHODS

Amplification and purification of recombinant adenovirus vectors

The recombinant adenovirus vector AdHNF4α,

containing the human HNF4α gene (GenBank: NM_000457.4) expression cassette, and the control adenovirus vector AdGFP, containing the green fluorescent protein (GFP) gene, were recombined as previously based on the recombinant system of adenovirus vector AdEasy and kept in the Department of Gastroenterology, Shanghai Changzheng Hospital (Shanghai, China)[16,17]. The adenovirus AdHNF4α was demonstrated to efficiently express HNF4α factor with biological functions on both human and rat cells[16]. Human embryonic kidney 293 cells (HEK293, Shanghai Institute of Cell biology, Chinese Academy of Sciences) were used as the virus carrier to amplify the recombinant adenovirus, and the adenovirus vector was purified by cesium chloride density gradient centrifugation. The virus titer was measured by the tissue culture infectious dose (TCID50) method (Q Biogene Inc.). The AdHNF4α and AdGFP titers were 1 × 10^{10} pfu/mL and 3 × 10^{10} pfu/mL, respectively.

Recombinant adenovirus-mediated HNF4α expression in rat HSC-T6 cells

The rat hepatic stellate cell line HSC-T6 was established by Scott L Friedman and William S Blaner's research group (Department of Medicine, College of Physicians and Surgeons of Columbia University, NY, United States)[18]. The cell line was kindly gifted by Scott L Friedman[16,19] and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/mL of streptomycin, and 100 units/mL of penicillin. These cells were cultured at 37 ℃ with 5% CO_2. The medium was changed once every 1-2 d and the cells were passaged every 2-3 d. For trypsinization, the cells were treated with 0.25% trypsin with 1 mmol/L ethylenediaminetetraacetic acid (EDTA) solution and incubated at 37 ℃ for 5 min. The reaction was stopped *via* the addition of Hank' s solution and the cells were collected for subsequent passage. HSC-T6 cells (1 × 10^5) were transferred into a well of a 6-well plate. After 24 h, the cells adhered to the well and the culture medium was replaced by a serum-free medium. The cells were incubated with AdHNF4α containing supernatant at multiplicities of infection (MOIs) of 50, 100, 200, 400, and 600 pfu/mL for 2 h. The control groups were treated with virus-free supernatant and supernatant containing AdGFP. After the medium was replaced by serum-containing medium, the cells were cultured for an additional 72 h and collected from both the test and control groups.

To calculate the efficiency of virus transfection, the GFP-positive cells in the AdGFP group were visualized by microscopy, and fluorescence antibodies were used to detect the expression of HNF4α in the AdHNF4α and virus-free groups. 4′,6′-Diamidino-2-phenylindole (DAPI) was used for nuclear staining. Goat anti-human HNF4α antibody (1:200), mouse anti-rat Nanog antibody (1:500), FITC-labeled goat anti-mouse IgG (1:500), and Cy3-labeled donkey anti-goat IgG (1:500) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, United States).

Total RNA was isolated with TRIzol reagent. HNF4α were quantified by RT-PCR. β-actin was used as the control for equal cDNA inputs. Primer sequences for HNF4α are as follows: forward primer, 5′-AAATGTGCAGGTGTTGACCA-3′ and reverse primer, 5′-CACGCTCCTCCTGAAGAATC-3′. The expression of HNF4α at the protein level was quantified by Western blot analysis. Whole-cell extracts were isolated by incubation with 40 μL cell lysis buffer/well for 10 min. The cell lysate was collected and centrifuged, and the supernatant was transferred to an Eppendorf tube and boiled for 10 min. After measuring the protein concentration, 10 μg of the protein was separated by electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride (PVDF) membrane. Horseradish peroxidase (HRP)-labeled donkey anti-goat secondary antibodies (1:2000) were purchased from Rockland Immunochemicals Inc. (Gilbertsville, PA, United States).

HNF4α induces transformation of phenotype during the differentiation of rat HSC-T6 cells

To evaluate the effect of HNF4α on directional differentiation, immune phenotype, cell function, and epithelial-mesenchymal transition (EMT) index after transfection, RT-PCR was used to detect expression genes, such as stem cell markers, hepatocyte differentiation markers, EMT-specific markers, and ECM synthesized molecules. The primers used in this study are listed in Table 1. Products of RT-PCR were identified by electrophoresis on 1.5% gel. The gels were scanned by a UV transilluminator. The optical densities of the bands were analyzed by Multi-Analyst software. The expression of G-6-P, PEPCK, Collagen Ⅰ, α-SMA, and TIMP-1 were detected by Western blotting. Primary antibodies were purchased from Santa Cruz Biotechnology Inc. The cells were fixed in 4% paraformaldehyde and 1% glutaraldehyde, and the EPON 812-embedded ultra-thin sections were prepared for observing cell ultrastructure under transmission electron microscope.

Interference of HNF4α expression reverses the phenotypic differentiation of rat HSC-T6 cells

Based on the HNF4α sequence (GenBank: NM_000457.4), a specific 19-bp shRNA (5′-CTGTAGCCACACTTTATGA-3′) was designed to bind with exon 3 of HNF4α. The shRNA was carried in the pGensil1.1-shHNF4α vector, which was transfected into HSC-T6 cells, with Western blotting then being used to measure indices that may have been altered by HNF4α interference.

Statistical analysis

Results were expressed as mean ± SD. Significance was established using analysis of variance by SPSS

Table 1 Primer sequences used to identify the transformation of the immune phenotype during hepatocyte nuclear factor 4α-induced differentiation of rat hepatic stellate cells-T6 cells

Classification	Molecules	Sequence (Primer sequences)
Stem cell-related	CD133	F: 5′-TTAATGCAGCACCAGGTACATC-3′
		R: 5′-TCGTTGAGCAGGTAGGGAGTAT-3′
	CD105	F: 5′-ATCCCTCTGACCAGTGATGTCT-3′
		R: 5′-CTTTTTCCGAAGTGGTGGTAAG-3′
	Nestin	F: 5′-GAGTGTCGCTTAGAGGTGCAA-3′
		R: 5′-TGTCACAGGAGTCTCAAGGGTA-3′
Hepatocyte differentiation-related	ALB	F: 5′-TGCAGGCTTGCTGTGATAAG-3′
		R: 5′-AGTAATCGGGGTGCCTTCTT-3′
	AFP	F: 5′-TACGTCCCTCCACCATTCTC-3′
		R: 5′-ATCCTGGTCTTTGCAGCACT-3′
	G-6-P	F: 5′-AAGAGGGCATAGCCCAGACT-3′
		R: 5′-TTGGAAGCTTCGTTGGTCTT-3′
	PEPCK	F: 5′-CAGGTTCCCAAAGGTCTGAA-3′
		R: 5′-TTCACTAGGGCCTGCTTGAT-3′
Fibroblast cell-related	Collagen Ⅰ	F: 5′-CCGTGACCTCAAGATGTGCC-3′
		R: 5′-GCTCATACCTTCGCTTCCAA-3′
	α-SMA	F: 5′-CCGAGATCTCACCGACTACC-3′
		R: 5′-TCCAGAGCGACATAGCACAG-3′
	TIMP-1	F: 5′-TCCCCAGAAATCATCGAGAC-3′
		R: 5′-TCAGATTATGCCAGGGAACC-3′
EMT index	Snail	F: 5′-GAGGACAGTGGCAAAAGCTC-3′
		R: 5′-TCGGATGTGCATCTTCAGAG-3′
	Vimentin	F: 5′-AGATCGATGTGGACGTTTCC-3′
		R: 5′-CACCTGTCTCCGGTATTCGT-3′
	E-cadherin	F: 5′-GGGTTGTCTCAGCCAATGTT-3′
		R: 5′-CACCAACACACCCAGCATAG-3′
Target	HNF4α	F: 5′-AAATGTGCAGGTGTTGACCA-3′
		R: 5′-CACGCTCCTCCTGAAGAATC-3′
Control	β-actin	F: 5′-ACCCACACTGTGCCCATCTATG-3′
		R: 5′-AGAGTACTTGCGCTCAGGAGGA-3′

F and R stand for forward and reverse primers, respectively. HNF4α: Hepatocyte nuclear factor 4α.

11.0 software. Differences were considered significant when P value < 0.05 and very significant when P < 0.01.

RESULTS

HNF4α expression mediated by recombinant adenovirus vector in HSC-T6 cells

To optimize the transfection efficiency of recombinant adenovirus vector, HSC-T6 cells were transfected with the recombinant adenovirus vector AdGFP at MOIs of 50, 100, 200, 400, and 600 pfu/mL. After 72 h, the transfection efficiency was considered proportional to the ratio of GFP-positive cells to the total number of cells. The transfection efficiencies for the different MOIs used were 20%, 42%, 59%, 78%, and 90%, respectively (Figure 1A). Based on these results, 600 pfu/mL was the MOI used for further experiments.

After HSC-T6 cells were transfected with adenovirus AdHNF4α, RT-PCR and Western blot analysis were used to measure the expression of HNF4α. The results revealed that AdHNF4α can mediate highly efficient expression of HNF4α in HSC-T6 (Figures 1B and C). In order to determine the specificity of the adenovirus vector, HSC-T6 cells were transfected with two vectors: AdHNF4α and AdGFP. Immunostaining results showed that HNF4α was only expressed in the nuclei of cells in the AdHNF4α group, and not in the AdGFP group (Figure 1D). Under electron microscope, HNF4α-induced cells had more mitochondria and ribosomes when compared with the parental cells (Figure 1E).

Identification of stem cell properties in rat HSC cells

In order to measure the stemness of HSCs, the expression levels of stem cell-related genes such as CD133, CD105, and nestin were measured using RT-PCR. The results revealed that the three molecules were positively expressed (Figure 2A), indicating that the rat HSCs were progenitor cells in the liver.

After transfection of adenovirus AdHNF4α, the expression of HNF4α and traditional stem cell marker Nanog was observed by co-focal immunofluorescent staining. With the increased expression of HNF4α, the relative expression of Nanog was downregulated from 98.33 ± 12.33 to 41.33 ± 5.67 (P < 0.001; Figures 2B and C).

HNF4α induced a tendency of cell differentiation of HSC cells

To investigate the role of HNF4α in HSC cell differentiation, we detected molecular markers related to the differentiation of HSCs into hepatocytes by

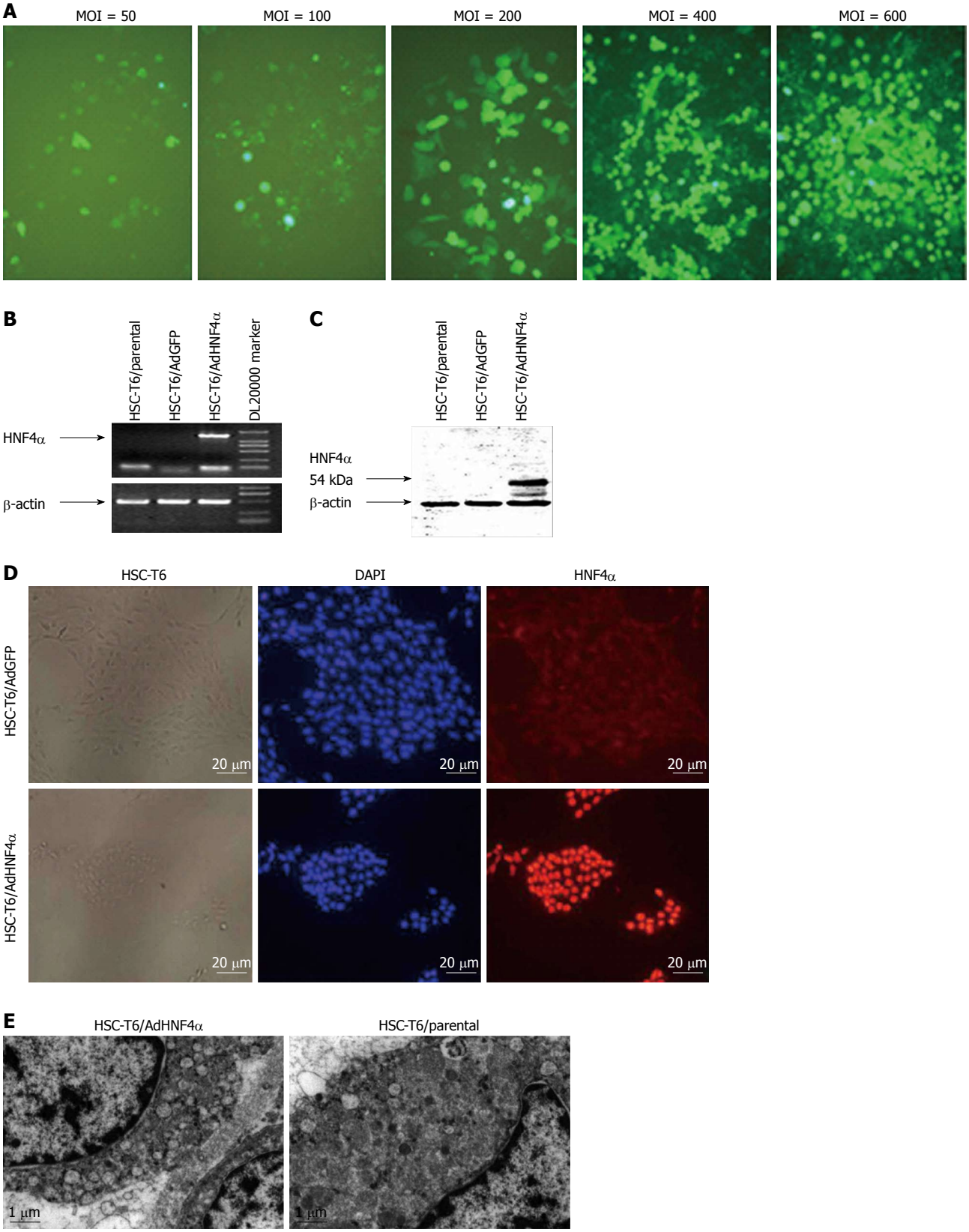

Figure 1 Ad-hepatocyte nuclear factor 4α-mediated hepatocyte nuclear factor 4α expression in rat hepatic stellate cells-T6 cells. A: AdGFP was transfected to HSC-T6 at multiplicities of infection (MOIs) of 50, 100, 200, 400, and 600 pfu/mL. After 72 h, the GFP-positive cells were counted under a microscope. The transfection efficiency was proportional to the MOIs; original magnification × 200 ×; B-D: 72 h after transfection of AdHNF4α, HNF4α expression in HSC-T6 cells was detected by RT-PCR (B), Western blotting (C), and immunofluorescence (D); original magnification × 100; The cells were harvested and fixed in 4% paraformaldehyde and 1% glutaraldehyde, and the EPON 812-embedded ultra-thin sections were observed under transmission electron microscope (E). HNF4α: Hepatocyte nuclear factor 4α; HSCs: Hepatic stellate cells.

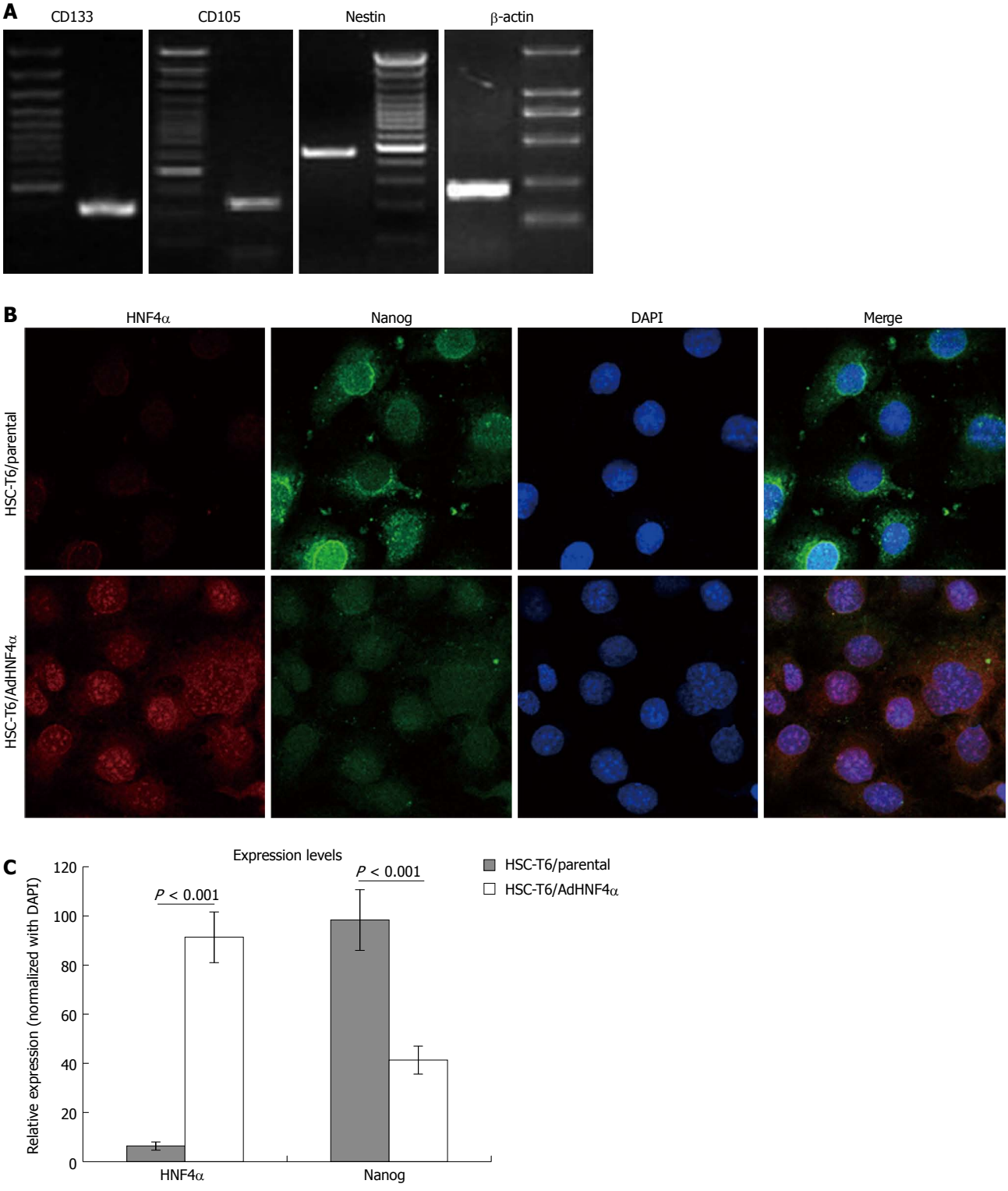

Figure 2 Identification of the stemness of rat hepatic stellate cells. A: The expression of all the stem cell markers (CD133, CD105, and nestin) was positive in HSC-T6 cells; B: After transfection of adenovirus AdHNF4α, the expression of HNF4α and Nanog was observed by co-focal immunofluorescent staining; original magnification × 400; C: The relative expression levels of HNF4α and Nanog were calculated by image density analysis with the Image-Pro Plus V6.0 (Media Cybernetics, Inc., Rockville, MD, United States) normalized with DAPI staining. HNF4α: Hepatocyte nuclear factor 4α; HSCs: Hepatic stellate cells.

RT-PCR and Western blot analysis. These molecules included the functional genes involved in the differentiation of HSCs to hepatocytes and fibroblasts. The functional genes of hepatocytes (G-P-6 and PEPCK) were expressed at much higher levels in the AdHNF4α group than in the control group. The expression of AFP and ALB was detected in the AdHNF4α group (Figures 3A and B). Furthermore, the expression levels of Collagen Ⅰ, α-SMA, and TIMP-1 were significantly decreased in the AdHNF4α group

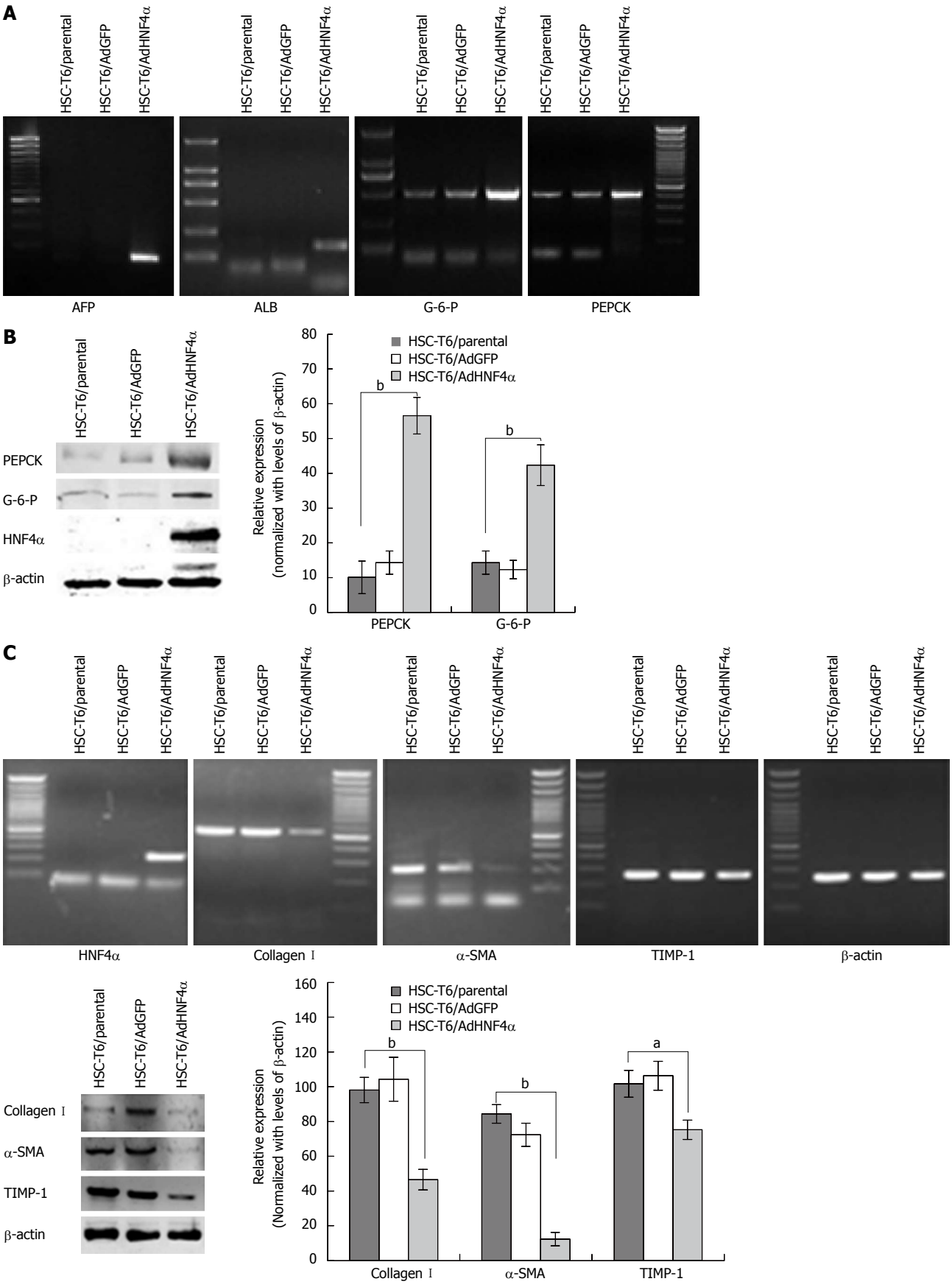

Figure 3 Identification of hepatic stellate cells differentiation mediated by hepatocyte nuclear factor 4α expression. By reverse transcription-polymerase chain reaction and Western blotting, the expression of differentiation functional genes of hepatocytes (A and B) and genes related to fibroblast cells (C) was detected in the AdHNF4α- and AdGFP-infected groups. The relative expression levels of the indicated factors were calculated by image density analysis normalized with β-actin. $^{a}P < 0.05$, $^{b}P < 0.01$, HSC-T6/parental *vs* HSC-T6/AdHNF4α. HNF4α: Hepatocyte nuclear factor 4α; HSCs: Hepatic stellate cells.

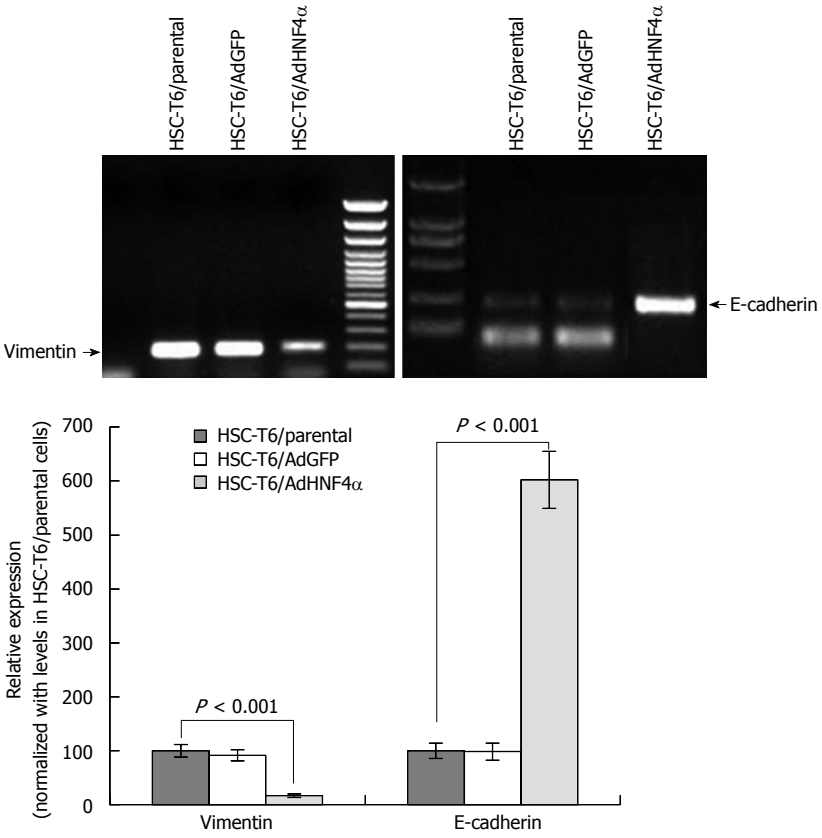

Figure 4 Hepatocyte nuclear factor 4α mediated change of epithelial-mesenchymal transition phenotypic markers in hepatic stellate cells. After transfection of adenoviruses AdHNF4α and AdGFP, the expression of vimentin and E-cadherin was detected by RT-PCR, and relative expression was calculated by image density analysis normalized with the expression levels in HSC-T6 parental cells. HNF4α: Hepatocyte nuclear factor 4α; HSCs: Hepatic stellate cells.

compared with the control group (Figure 3C).

HNF4α-mediated changes in the EMT phenotypic markers in HSC cells

To investigate the phenotypic character of HSCs after HNF4α transfection, we tested the EMT indicators by RT-PCR. As compared with the AdGFP control group, HNF4α obviously downregulated the expression of the mesenchymal phenotypic gene vimentin and significantly enhanced the expression of the epithelial phenotypic gene E-cadherin (Figure 4).

HNF4α interference affects phenotypic differentiation of HSCs

To investigate the biological characteristics of HSCs in HNF4α knockdown, the pGensil1.1-shHNF4α vector was transfected into HNF4α-positive HSC-T6 cells. Western blotting analysis revealed that silencing of HNF4α expression resulted in obvious changes to many genes. With the decrease of HNF4α expression, AFP, ALB, PEPCK, and E-cadherin were downregulated, while α-SMA and vimentin were upregulated (Figure 5).

DISCUSSION

HSCs play an important role in the regulation of liver injury repair and in the development of liver fibrosis. Despite intensive research into the biological and pathophysiological role of HSCs in fibrogenesis, the states of HSCs in the different stages of fibrogenesis are still a matter of debate. In the quiescent state, HSCs exhibit properties of stem cells in the liver. Following liver injury, HSCs become activated. Their potential to differentiate into epithelial or hepatocyte lineages demonstrates their important functions during liver regeneration. HSC activation may be stimulated by most causes of liver injury, with injured hepatocytes and activated Kupffer cells being considered as the leading cause of HSC activation. Injured hepatocytes release a wide array of soluble mediators, including lipid peroxide, hepatotoxin, and reactive oxygen species (ROS). These mediators can strongly activate HSCs and stimulate the potential of these cells in fibrogenesis[20]. Meanwhile, the homeostatic states between the activation and quiescence of HSCs can be regulated by HNF4α.

HNF4α is a nuclear transcription factor that binds to DNA as a homodimer. It can activate the expression of target genes by adjusting the structure of chromosomes and depolymerizing them. The results of chromatin immunoprecipitation (ChIP) showed that HNF4α could combine with the promoter regions of up to 12% of intracellular genes, 80% of which are combined with RNA polymerase Ⅱ. Thus, we can infer that HNF4α controls a large proportion of active transcriptional genes in the liver[21,22]. The gain

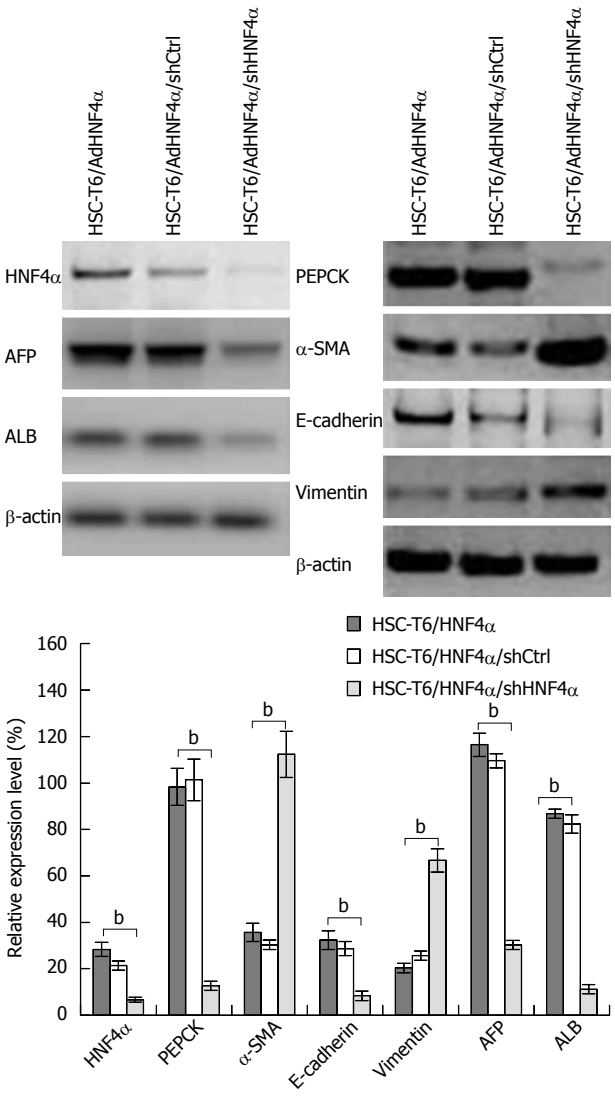

Figure 5 Effect of hepatocyte nuclear factor 4α knockout on phenotypic differentiation of hepatic stellate cells. After HNF4α was knocked out by shHNF4α, the expression of AFP, ALB, PEPCK, E-cadherin, α-SMA, and vimentin was detected by Western blotting. β-actin was used as the control. $^{b}P < 0.01$, *vs* control. HNF4α: Hepatocyte nuclear factor 4α.

or loss of HNF4α function can lead to the inhibition of many genes at different stages of liver development. By comparing the different gene expression profiles between HNF4α-knocked out mice and normal mice with a gene chip array, it was found that silencing HNF4α expression results in a decrease of liver function. Possible mechanisms for this may be through causing liver developmental disorders by destroying cellular close connections, adhesion connections, and gap junctions, as well as affecting the adhesion molecules between desmosomes and the cell matrix and affecting the polarity of epithelial cells and cytoskeleton proteins[23]. Additionally, loss of HNF4α function can result in cell phenotypic abnormalities, thereby affecting liver cell phenotypes and important liver functions such as liver cell metabolism, albumin synthesis, and drug detoxification[22-24]. Inducible expression of HNF4α by oncostatin M (OSM) can promote differentiation of hepatocytes and enhance the functions of hepatocytes[25]. Moreover, upregulated HNF4α can induce hepatoma stem cells to differentiate into mature hepatocytes, inhibit the proliferation of cancer cells, and reverse the differentiation of cancer cells into a differentiated state. These results demonstrate that upregulation of HNF4α is a promising candidate for the treatment of liver cancer[26].

In the mature liver, HNF4α expression is induced when oval cells differentiate into hepatocytes, suggesting its pivotal role in the differentiation and proliferation of hepatocytes from oval cells. However, very few studies have investigated the regulation and function of HNF4α in HSCs. Previous studies have shown that the expression of HNF4α is significantly decreased in liver injury and chronic liver diseases of different causes (*e.g.*, viral hepatitis)[27]. Decreased HNF4α expression can induce EMT in hepatocytes and HSCs[28,29]. EMT is a phenotypic change of epithelial cells induced by various cytokines, such as transforming growth factor TGF-β, following which the epithelial cells exhibit properties of mesenchymal cells. Following EMT, HSCs proliferate rapidly, transform to myofibroblast cells, generate ECM, eliminate lipid droplets, and positively induce the expression of α-SMA and Snail[18]. When HNF4α is rescued by exogenous gene transduction, EMT can be reversed to mesenchymal-epithelial transition (MET)[14,30,31]. This observation tells us that HNF4α is an important regulator for maintaining the epithelial phenotype of HSCs. HNF4α not only inhibits the mesenchymal phenotype of HSCs, but also promotes the differentiation of liver stem cells and the regeneration of hepatocytes[15]. Because of the importance of HSCs in liver fibrosis, understanding the function of HNF4α in regulating HSCs to participate in liver fibrosis will provide a new approach to studying the pathogenesis and prevention of liver fibrosis.

In this study, we sorted and cultured the HSC-T6 cell line. In a quiescent state, the HSCs showed stem cell characteristics, as evidenced by the expression of stem cell markers (CD133, Nanog, nestin, and CD105). To investigate the regulatory role of HNF4α in hepatocyte differentiation, we transfected HNF4α gene in HSCs and upregulated its expression. After transfection with AdHNF4α, the expression levels of HNF4α and E-cadherin was increased while vimentin expression levels decreased. Moreover, the HNF4α-induced HSC-T6 cells showed morphological changes that led to more mitochondria and ribosomes. These results suggested that HNF4α is an important transcriptional factor in maintaining the epithelial phenotype and facilitating the EMT of HSCs. In addition, HNF4α obviously upregulated the expression of genes related to hepatocyte function, such as ALB, AFP, G-6-P, and PEPCK, illustrating that HNF4α can induce a tendency of differentiation of HSCs to hepatocyte-like cells. Meanwhile, the transduction of HNF4α downregulated the expression of α-SMA,

type Ⅰ collagen, and TIMP-1, demonstrating that HNF4α inhibits the differentiation of HSCs to fibroblast cells.

In conclusion, HSCs have a high capacity of proliferation and a low level of differentiation. HNF4α can induce the expression of important epithelial cell genes in HSCs, promote HSC differentiation to hepatocyte-like cells, and inhibit HSC differentiation to the mesenchymal phenotype. All these observations suggest that HNF4α can induce a tendency of differentiation of HSCs into hepatocyte-like cells. The findings of this research may provide an effective method for treating liver diseases.

COMMENTS

Background

Hepatic stellate cells (HSCs) are stem-like cells that play a crucial role in liver repair and regeneration. However, the differentiation capacity of HSCs and the related molecular mechanisms remain unclear. Hepatocyte nuclear factor 4α (HNF4α) is highly expressed in mature hepatic cells, lowly expressed in HSCs, and the upregulated expression of HNF4α can maintain the endothelial cell state of HSCs, thereby demonstrating that HNF4α may play a vital role in promoting the differentiation and regeneration of HSCs into hepatocytes.

Research frontiers

The HNF family is a group of important transcription factors in the regulation of liver differentiation, in which HNF4α can regulate the differentiation of hepatocytes and preserve their biological function. Due to the importance of HSCs in liver fibrosis, this study has clarified the functions of HNF4α and the mechanism by which it regulates the participation of HSCs in liver repair. By constructing an HNF4α-expressing adenovirus vector and manipulating HNF4α expression in rat HSC-T6 cells, the influence of HNF4α and its induction capacity in the differentiation of HSCs into hepatic cells was explored.

Innovations and breakthroughs

HNF4α is a nuclear transcription factor that controls a large proportion of active transcriptional genes in the liver. HNF4α not only inhibits the mesenchymal phenotype of HSCs, but also promotes the differentiation of liver stem cells and the regeneration of hepatocytes. To investigate the regulatory role of HNF4α in hepatocyte differentiation, the authors upregulated HNF4α expression in HSCs by transfection of HNF4α gene. The results showed that HNF4α can promote differentiation of HSCs to hepatocyte-like cells and inhibit differentiation of HSCs to the mesenchymal phenotype by regulating some target genes involved in HSC differentiation, such as Nanog, α-SMA, collagen Ⅰ, TIMP-1, E-cadherin, and vimentin.

Applications

HNF4α can induce a tendency of differentiation of HSCs into mature hepatocytes, which may provide an effective method for treating liver diseases.

Terminology

HSCs were positive for the stem cell-related markers (CD133, CD105, Nanog, and nestin), indicating that the HSCs were progenitor cells in the liver. The increased expression of G-P-6, PEPCK, AFP, ALB, and E-cadherin indicated that the HNF4α-induced cells had a tendency of differentiation into hepatocytes, and the decreased expression of collagen Ⅰ, α-SMA, TIMP-1, and vimentin demonstrated that the HNF4α-induced cells lost the capacity to differentiate towards mesenchymal cells.

Peer-review

The authors presented interesting results suggesting that a mesenchymal to epithelial transition occurs in hepatic stellate cells following forced expression of HNF4α after infection with an adenovirus vector. Their conclusion was that novel donor cells should be provided for cell transplantation.

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ORIGINAL ARTICLE

Basic Study

MiR-451 inhibits proliferation of esophageal carcinoma cell line EC9706 by targeting CDKN2D and MAP3K1

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Author contributions: Zhao GQ and Zang WQ designed and guided the study; Zang WQ, Yang X, Wang YY, Wang T, Li M and Du YW performed and participated in analysis of laboratory experiment data; Zang WQ and Chen XN acquired and preserved the clinical samples; Zhao GQ and Zang WQ provided administrative support and funded experiments; all authors have contributed and approved the final manuscript.

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Abstract

AIM: To investigate the underlying molecular mechanisms of miR-451 to inhibit proliferation of esophageal carcinoma cell line EC9706.

METHODS: Assays for cell growth, apoptosis and invasion were used to evaluate the effects of miR-451 expression on EC cells. Luciferase reporter and Western blot assays were used to test whether cyclin-dependent kinase inhibitor 2D (CDKN2D) and MAP3K1 act as major targets of miR-451.

RESULTS: The results showed that CDKN2D and MAP3K1 are direct targets of miR-451. CDKN2D and MAP3K1 overexpression reversed the effect of miR-451. MiR-451 inhibited the proliferation of EC9706 by targeting CDKN2D and MAP3K1.

CONCLUSION: These findings suggest that miR-451 might be a novel prognostic biomarker and a potential target for the treatment of esophageal squamous cell carcinoma in the future.

Key words: Esophageal squamous cell carcinoma; MiR-451; Cyclin-dependent kinase inhibitor 2D; MAP3K1; Proliferation

Core tip: Recently miR-451 has been reported to be tumor suppressor in human cancer cells. In the previous studies we have reported that miR-451

expression in esophageal squamous cell carcinoma (ESCC) tissues was significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in esophageal carcinoma. However, the underlying molecular mechanisms remain unclear. In this study, we supposed and showed that cyclin-dependent kinase inhibitor 2D (CDKN2D) and MAP3K1 are the targets of miR-451 by the bioinformatics algorithms (TargetScan and miRBase). Moreover, we found that CDKN2D and MAP3K1 contributed to ESCC malignancy.

Zang WQ, Yang X, Wang T, Wang YY, Du YW, Chen XN, Li M, Zhao GQ. MiR-451 inhibits proliferation of esophageal carcinoma cell line EC9706 by targeting CDKN2D and MAP3K1. *World J Gastroenterol* 2015; 21(19): 5867-5876 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5867.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5867

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies worldwide[1,2]. ESCC is the 8th most common cancer and the 6th leading cause of cancer-related death. The traditional treatment for ESCC includes chemotherapy and radiation therapy[3,4]. However, many patients who are treated with such traditional therapy still experience disease progression, which suggests that ESCC is resistant to traditional therapy. New treatment choices are critically required and the mechanism of tumorigenesis is to be further clarified.

MicroRNAs (miRNAs) are small, endogenous noncoding RNAs that have been identified as post-transcriptional regulators of gene expression. MiRNAs exert their functions through imperfect base-pairing with the 3'-untranslated region (3'-UTR) of target mRNAs[5-8]. In human cancer, miRNAs can act as oncogenes or tumour suppressor genes during tumourigenesis. Recently, miR-451 has been reported to be induced during zebrafish, mouse, and human erythroid maturation as a key factor involved in regulating erythrocyte differentiation[9-11]. It was also reported that miR-451 might function as a tumor suppressor and modulate MDR1/P-glycoprotein expression in human cancer cells[12]. In previous studies we have reported that miR-451 expression in ESCC tissues was significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in esophageal carcinoma[13,14]. However, the underlying molecular mechanisms remain unclear. In this study, we supposed and showed that cyclin-dependent kinase inhibitor 2D (CDKN2D) and MAP3K1 are the targets of miR-451 by the bioinformatics algorithms (TargetScan and miRBase). Moreover, we found that CDKN2D and MAP3K1 contributed to ESCC malignancy. Our data demonstrate that miR-451 has potential values as a prognostic marker and a therapeutic target for ESCC.

MATERIALS AND METHODS

Cell culture

EC9706 and KYSE150 cell lines were purchased from the Chinese Academy of Sciences Cell Bank. All cells were cultured in RPMI-1640 (Gibco, United States) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, United States) and grown in humidified 5% CO_2 at 37 ℃.

Oligonucleotides and cell transfection

The miR-451 mimics used in this study was synthesized by Shanghai GenePharma Co. Ltd. For transfection, 2×10^5 cells were seeded into each well of six well plates and grown overnight until they were 50%-80% confluent. Cells were washed, placed in serum-free medium, and transfected using Lipofectamine™2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, United States). After 6 h, the medium was changed to complete medium, and cells were cultured at 37 ℃ in 5% CO_2.

Cell growth assay

The different experimental groups of EC9706 and KYSE150 cells were plated in 96-well plates at 1×10^4 cells per well and incubated for 48 h after transfection. The viability of cells was determined using Cell Counting Kit-8 (CCK-8; Dojindo, Japan) according to the manufacturer's instructions. Viable cell numbers were estimated by measurement of optical density (OD) at 450 nm. All experiments were performed in triplicate.

Colony formation assay

Cells were suspended in RPMI-1640 containing 0.35% low melting agarose, and plated onto solidified 0.6% agarose containing RPMI-1640 in six-well culture plates at a density of 1×10^5 cells per dish. The plates were incubated for 2 wk at 37 ℃ in a 5% CO_2 incubator, and the number of colonies was counted after staining with 0.1% crystal violet solution. All experiments were performed in triplicate.

Cell invasion assay

The experimental groups of EC9706 cells were adjusted at 2×10^5/mL in each group 48 h after transfection. The upper chamber of 24-well Transwell Permeable Supports with 8 μm pores (Corning Cat. No. 3422) was loaded with 200 μL of cell suspension, and the lower chamber was loaded with 500 μL of medium containing 10% serum for incubation in an atmosphere of 5% CO_2 at 37 ℃ for 48 h. Five wells were set for each group. The number of cells invading the matrigel

was counted from 5 randomly selected visual fields using an inverted microscope. All experiments were performed in triplicate.

Apoptosis assay

EC9706 cells were harvested 48 h after transfection and cell concentration was adjusted to 1 × 10^6 cells. Annexin V-FITC/PI Apoptosis Detetion Kit I (BestBio, Shanghai, China) was used to detect Annexin V. Results were obtained using FACScan Flow Cytometer (BD Biosciences, San Jose, CA, United States). Tests were repeated in triplicate. Data were analyzed with Cell Quest software. All experiments were performed in triplicate.

Cell cycle analysis

For cell cycle analysis by flow cytometry, cells in the logarithmic phase of growth were harvested by trypsinization, washed with PBS, fixed with 75% ethanol overnight at 4 ℃ and incubated with RNase at 37 ℃ for 30 min. Nuclei were stained with propidium iodide for 30 min. A total of 10^4 nuclei were examined in a FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, United States). All experiments were performed in triplicate.

Western blot

The experimental groups of EC9706 and KYSE150 cells in each group were lysed in lysis buffer for total protein extration. Protein concentrations were measured using the BCA method (KeyGEN, China), and 30 μg of protein was separated by 12% SDS-PAGE and electroblotted onto a nitrocellulose membrane (Whatman, United States). The membrane was blotted overnight at 4 ℃ with primary antibodies (mouse anti- CDKN2D and anti-MAP3K1, 1:1000) in Tris-buffered saline with 5% non-fat milk. A secondary antibody (HRP-conjugated goat anti-mouse IgG) was incubated with the membrane for 1 h after three washes with TBST. The protein band density was determined with Kodak Digital ID Image Analysis Software and was normalized with the density of β-actin. All experiments were performed in triplicate.

Dual luciferase assay

The human CDKN2D and MAP3K1 fragments containing putative binding sites for miR-451 were amplified by PCR from human genomic DNA. The mutant CDKN2D and MAP3K1 3′-UTRs were obtained by overlap extension PCR. The fragments were cloned into a pmirGLO reporter vector (Promega), downstream of the luciferase gene, to generate the recombinant vectors pmirGLO-CDKN2D-wt, pmirGLO-CDKN2D-mut, pmirGLO-MAP3K1-wt and pmirGLO-MAP3K1-mut. For the luciferase reporter assay, cells were transiently co-transfected with miRNA (miR-451 mimics or scrambled-miR-451 negative control) and reporter vectors (wild-type reporter vectors or mutant-type reporter vectors), using Lipofectamine™2000. Luciferase activities were measured using a Dual-Luciferase assay kit (Promega) according to the manufacturer's instructions at 48 h post-transfection. All experiments were performed in triplicate.

Statistical analysis

Statistical testing was conducted with the assistance of SPSS 17.0 software. All data are expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to analyze data. Results were considered significant when P values were < 0.05.

RESULTS

CDKN2D and MAP3K1 are direct targets of miR-451

We based on the following criteria to search for the direct target of miR-451: the target should have oncogenic property and regulate the cell migration and invasion. Among these targets of miR-451 predicted by the bioinformatics algorithms (TargetScan and miRBase), we selected CDKN2D and MAP3K1. The 3′-UTR of CDKN2D contains the seed regions for miR-451 at the position of base 240 nt - 246 nt (Figure 1A). Similarly, the 3′-UTR of MAP3K1 contains the seed regions for miR-451 at the position of base 6270 nt - 6278 nt (Figure 1B).

Subsequent Western blot analysis indeed showed that CDKN2D and MAP3K1 expression was down-regulated in EC9706 and KYSE150 cells following transfection with the miR-451 mimics (Figure 1C and D). In order to test the specific regulation through the seed region, we constructed a reporter vector which consists of the luciferase coding sequence followed by the 3′-UTR of CDKN2D and MAP3K1. Wild type (pmirGLO-CDKN2D-3′-UTR, pmirGLO-MAP3K1-3′-UTR) or mutated sequences (pmirGLO-CDKN2D-mut 3′-UTR, pmirGLO-MAP3K1-mut 3′-UTR) within the seed region sites were cloned into the pmirGLO reporter vector. We used a Dual-Luciferase reporter system containing either wild-type or mutant 3′-UTRs of CDKN2D and MAP3K1, respectively. Co-transfection experiments showed that miR-451 significantly decreased the luciferase activity of wild type in EC9706 and KYSE150 cells ($P < 0.05$; Figure 1E and F), but this was not observed in mutant type ($P > 0.05$; Figure 1E and F). These data indicate that miR-451 negatively regulates CDKN2D and MAP3K1 expression by directly binding to putative binding sites in the 3′-UTR. Our results thus demonstrated that CDKN2D and MAP3K1 are direct targets of miR-451.

CDKN2D and MAP3K1 overexpression reverses the effect of miR-451

To explore the function of CDKN2D and MAP3K1 in EC9706 cells, we constructed pcDNA3.1-CDKN2D and pcDNA3.1-MAP3K1 lacking the 3′-UTR, and then they were transfected into EC9706 cells. Western blot assay showed that transfection of miR-451 mimics inhibited

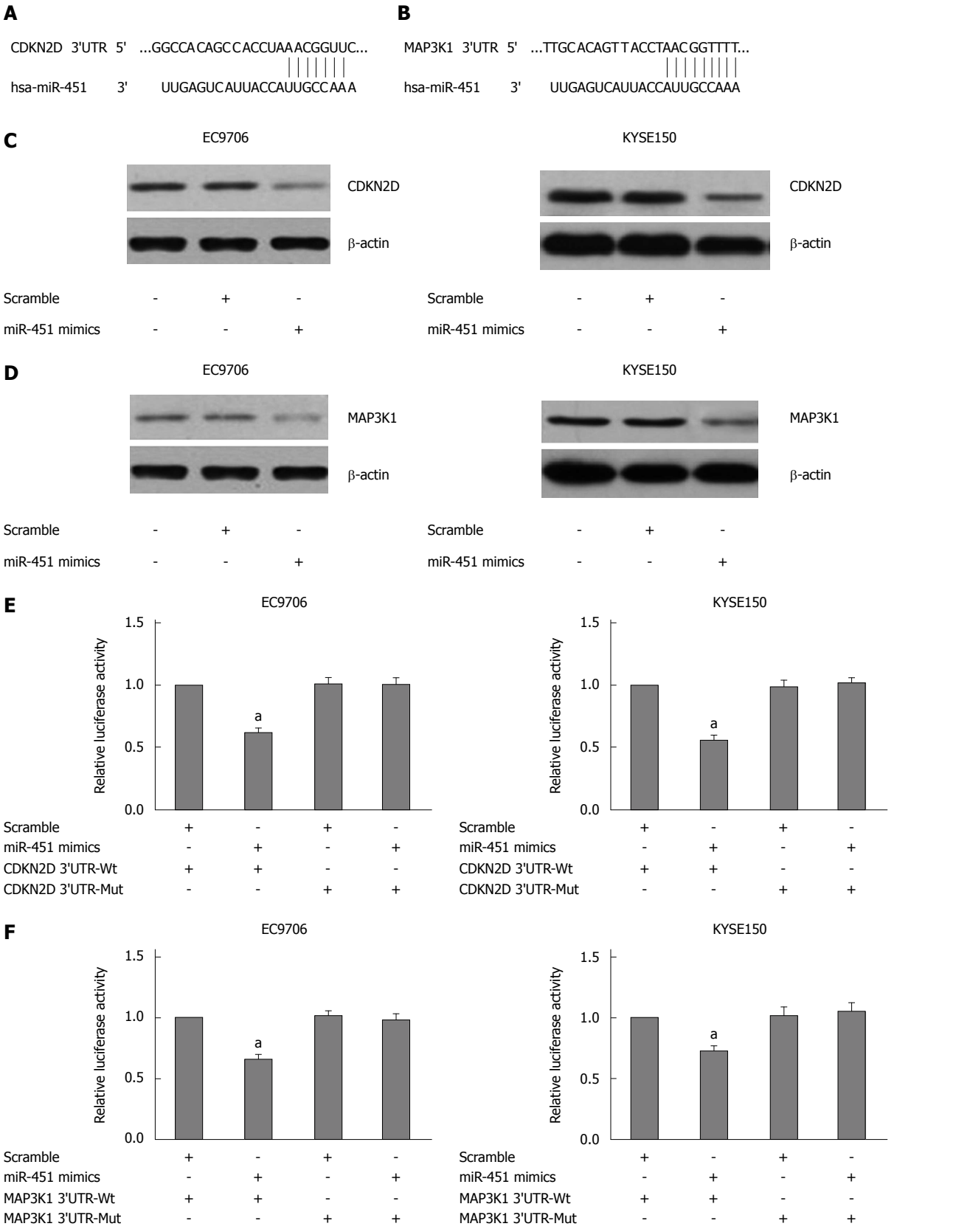


Figure 1 CDKN2D and MAP3K1 are direct targets of miR-451 in EC9706 and KYSE150 cells. A: The putative miR-451 binding sequence for the CDKN2D 3'-untranslated region (3'-UTR). The 3'-UTR of CDKN2D contains a seed region for miR-451; B: The putative miR-451 binding sequence for the MAP3K1 3'-UTR. The 3'-UTR of MAP3K1 contains a seed region for miR-451; C: Western blot analysis of CDKN2D expression in transfected cells. Transfection of miR-451 mimics resulted in a significant reduction of CDKN2D protein expression in EC9706 and KYSE150 cells. β-actin was used as a reference; D: Western blot analysis of MAP3K1 expression in transfected cells. Transfection of miR-451 mimics resulted in a significant reduction of MAP3K1 protein expression in EC9706 and KYSE150 cells. β-actin was used as a reference; E: MiR-451 significantly decreased the luciferase activity of CDKN2D 3'-UTR-Wt in EC9706 and KYSE150 cells; F: MiR-451 significantly decreased the luciferase activity of MAP3K1 3'-UTR-Wt in EC9706 and KYSE150 cells ($^aP < 0.05$ *vs* control group).

the expression of CDKN2D and MAP3K1, respectively (Figure 2A). Co-transfection of pcDNA3.1-CDKN2D and miR-451 abrogated the effects of miR-451 on CDKN2D expression (Figure 2A). Similarly, co-transfection of pcDNA3.1-MAP3K1 and miR-451 abrogated the effects of miR-451 on MAP3K1 expression (Figure 2A).

In the colony formation assays we found that exogenous expression of miR-451 decreased cell colony formation numbers (Figure 2B and C). Subsequently, we exogenously expressed recombinant CDKN2D lacking the 3'-UTR sequence (pcDNA3.1-CDKN2D) or MAP3K1 lacking the 3'-UTR sequence (pcDNA3.1-MAP3K1) in EC9706 cells. Cells transfected with pcDNA3.1-CDKN2D or pcDNA3.1-MAP3K1 alone showed significantly increased cell colony formation numbers (Figure 2B and C). When we, however, co-transfected cells with pcDNA3.1-CDKN2D or pcDNA3.1-MAP3K1 and miR-451, the expression of CDKN2D and pcDNA3.1-MAP3K1 lacking the 3'-UTR sequence were found to reverse the anti-proliferation of miR-451 (Figure 2B and C). From these results we conclude that expression of CDKN2D and MAP3K1 could partially reverse the anti-proliferation function of miR-451.

Our apoptosis assay indicated that exogenous expression of miR-451 increased cell apoptosis induced by serum starvation (Figure 2D and E). Subsequently, we exogenously expressed recombinant CDKN2D lacking the 3'-UTR sequence (pcDNA3.1-CDKN2D) or MAP3K1 lacking the 3'-UTR sequence (pcDNA3.1-MAP3K1) in EC9706 cells. Cells transfected with pcDNA3.1-CDKN2D alone did not show significantly decreased levels of apoptosis (Figure 2D). However, cells transfected with pcDNA3.1-MAP3K1 alone showed significantly decreased levels of apoptosis compared to the blank control (Figure 2E), and that when we co-transfected cells with pcDNA3.1-MAP3K1 and miR-451, the expression of MAP3K1 lacking the 3'-UTR sequence was found to reverse the pro-apoptotic functions of miR-451 (Figure 2E).

In the transwell assays we found that exogenous expression of miR-451 decreased cell invasiveness (Figure 2F and G). Subsequently, we exogenously expressed recombinant CDKN2D lacking the 3'-UTR sequence (pcDNA3.1-CDKN2D) or MAP3K1 lacking the 3'-UTR sequence (pcDNA3.1-MAP3K1) in EC9706 cells. Cells transfected with pcDNA3.1-MAP3K1 alone showed significantly increased cell invasiveness (Figure 2G). When we, however, co-transfected cells with pcDNA3.1-CDKN2D and miR-451, the expression of CDKN2D lacking the 3'-UTR sequence was found to reverse the anti-migration functions of miR-451 (Figure 2F). Similarly, when we co-transfected cells with pcDNA3.1-MAP3K1 and miR-451, the expression of MAP3K1 lacking the 3'-UTR sequence was found to reverse the anti- migration functions of miR-451 (Figure 2G).

Cell cycle analysis showed that administration of miR-451 mimic oligonucleotides significantly increased the percentage of cells in the G1 phase and decreased the percentage of cells in the S phase (Figure 2H and I). When we co-transfected cells with pcDNA3.1-CDKN2D and miR-451, the expression of CDKN2D lacking the 3'-UTR sequence was found to reverse G1 arrest of miR-451 (Figure 2H). When we co-transfected cells with pcDNA3.1-MAP3K1 and miR-451, the expression of MAP3K1 lacking the 3'-UTR sequence was not found to reverse G1 arrest of miR-451 (Figure 2I).

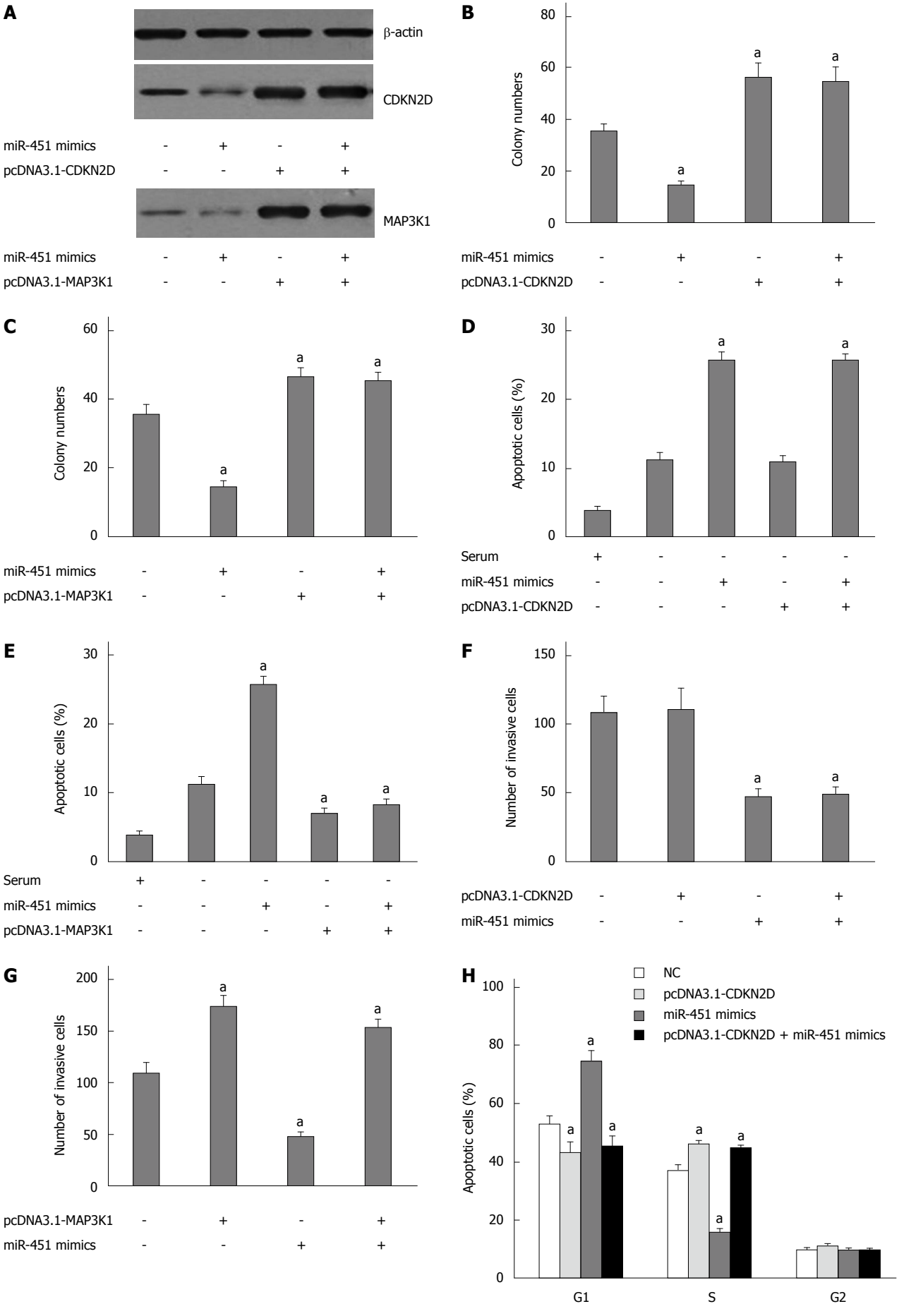
MiR-451 inhibits the proliferation of EC9706 cells by targeting CDKN2D and MAP3K1

To further explore the biological significance of CDKN2D**,** MAP3K1 and miR-451 in EC9706 cells, CDKN2D-siRNAs, MAP3K1-siRNAs and miR-451 mimics were transfected into EC9706 cells. Western blot assay showed that transfection of CDKN2D-siRNAs, MAP3K1-siRNAs and miR-451 mimics inhibited the expression of CDKN2D and MAP3K1, respectively (Figure 3A).

CCK8 assay showed that CDKN2D, MAP3K1 silencing and miR-451 overexpression inhibited the cell proliferation (Figure 3B and C). For EC9706 cells transfected with si-CDKN2D, the inhibition was more obvious than cells transfected with si-MAP3K1 (Figure 3B and C). Compared to the NC group, co-transfection of si-CDKN2D and si-MAP3K1 also significantly inhibited the proliferation of EC9706 cells (Figure 3D). In addition, for co-transfected cells with si-CDKN2D and si-MAP3K1, the inhibitory effects were similar to those for cells overexpressing miR-451 (Figure 3D). Furthermore, colony formation assay obtained the similar results that CDKN2D, MAP3K1 silencing and miR-451 overexpression reduced EC9706 cell colony numbers (Figure 3E).

Invasion assay showed that knockdown of MAP3K1 and the overexpression of miR-451 repressed the invasion capacities of EC9706 cells (Figure 3G). For cells transfected with si-MAP3K1, the numbers of invasive cells were less than cells transfected with si-CDKN2D (Figure 3G). To investigate the effect of si-CDKN2D, si-MAP3K1 and miR-451 on apoptosis, we performed apoptosis assay. As showed in Figure 3F, CDKN2D, MAP3K1 silencing and the overexpression of miR-451 induced EC9706 cell apoptosis significantly compared to the blank control. For EC9706 cells transfected with si-MAP3K1, the apoptotic cells were more than those for cells transfected with si-CDKN2D. For co-transfected cells with si-CDKN2D and si-MAP3K1, the apoptosis effects were similar to those for cells overexpressing miR-451.

Cell cycle analysis showed that knockdown of CDKN2D and the overexpression of miR-451 significantly increased the percentage of cells in the G1 phase and decreased the percentage of cells in the S phase (Figure 3H). Altogether, these results confirm that miR-451 inhibits the proliferation of EC9706 by targeting CDKN2D and MAP3K1.


A
β-actin
CDKN2D
miR-451 mimics - + - +
pcDNA3.1-CDKN2D - - + +
MAP3K1
miR-451 mimics - + - +
pcDNA3.1-MAP3K1 - - + +
B
Colony numbers
80
60
40
20
0
a
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pcDNA3.1-CDKN2D - - + +
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Apoptotic cells (%)
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a
Serum + - - - -
miR-451 mimics - - + - +
pcDNA3.1-CDKN2D - - - + +
E
Apoptotic cells (%)
30
20
10
0
a
a
a
a
Serum + - - - -
miR-451 mimics - - + - +
pcDNA3.1-MAP3K1 - - - + +
F
Number of invasive cells
150
100
50
0
a
a
a
a
pcDNA3.1-CDKN2D - + - +
miR-451 mimics - - + +
G
Number of invasive cells
200
150
100
50
0
a
a
a
a
pcDNA3.1-MAP3K1 - + - +
miR-451 mimics - - + +
H
NC
pcDNA3.1-CDKN2D
miR-451 mimics
pcDNA3.1-CDKN2D + miR-451 mimics
Apoptotic cells (%)
100
80
60
40
20
0
a
a
a
a
a
a
G1
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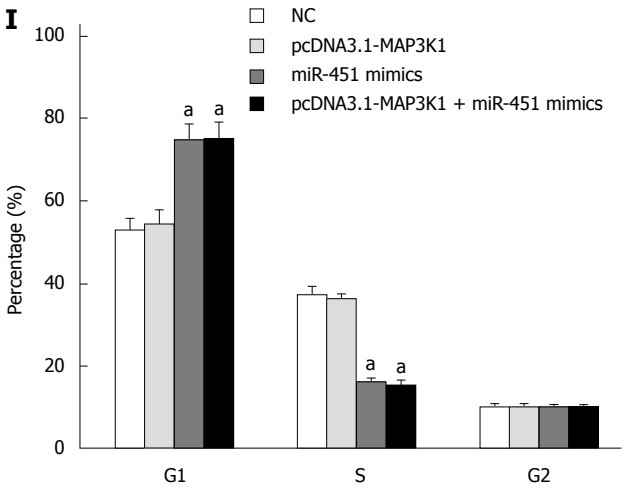

Figure 2 CDKN2D and MAP3K1 overexpression reverses the effect of miR-451. A: CDKN2D and MAP3K1 protein levels were detected by Western blot assay. Western blot assay showed that transfection of miR-451 mimics inhibited the expression of CDKN2D or MAP3K1. Co-transfection of pcDNA3.1-CDKN2D or pcDNA3.1-MAP3K1and miR-451 abrogated the effects of miR-451 on CDKN2D or MAP3K1 expression. β-actin was used as a reference; B: The expression of CDKN2D could partially reverse the anti-proliferation function of miR-451. Colony formation assays were performed. $^{a}P < 0.05$ *vs* control group; C: The expression of MAP3K1 could partially reverse the anti-proliferation function of miR-451. Colony formation assays were performed. $^{a}P < 0.05$ *vs* control group; D: The expression of CDKN2D did not reverse the pro-apoptotic function of miR-451. Cells were transfected with pcDNA3.1-CDKN2D (not including 3'-UTR) or (and) miR-451. The cell apoptosis was assessed using flow cytometry assay; E: The expression of MAP3K1 reversed the pro-apoptotic function of miR-451. Cells were transfected with pcDNA3.1-MAP3K1 (not including 3'-UTR) or (and) miR-451. The cell apoptosis was assessed using flow cytometry assay; F: The expression of CDKN2D reversed the anti-migration function of miR-451. Cells were transfected with pcDNA3.1-CDKN2D (not including 3'-UTR) or (and) miR-451. The cell invasion was assessed using transwell assay; G: The expression of MAP3K1 reversed the anti-migration function of miR-451. Cells were transfected with pcDNA3.1-MAP3K1 (not including 3'-UTR) or (and) miR-451. The cell invasion was assessed using transwell assay; H: The expression of CDKN2D reversed G1 arrest of miR-451. Cells were transfected with pcDNA3.1-CDKN2D (not including 3'-UTR) or (and) miR-451. The cell cycle was assessed using flow cytometry assay; I: The expression of MAP3K1 did not reverse G1 arrest of miR-451. Cells were transfected with pcDNA3.1-MAP3K1 (not including 3'-UTR) or (and) miR-451. The cell cycle was assessed using flow cytometry assay ($^{a}P < 0.05$ *vs* control group).

DISCUSSION

CDKN2D (p19^{INK4d}), a negative regulator of the cell cycle, is located on chromosome 19p13. The protein encoded by this gene is a member of the INK4 family of cyclin-dependent kinase inhibitors. This protein has been shown to form a stable complex with CDK4 or CDK6, and prevent the activation of the CDK kinases, thus functioning as a cell growth regulator that controls cell cycle G1 progression. The abundance of the transcript of this gene was found to oscillate in a cell-cycle dependent manner with the lowest expression at mid G1 and a maximal expression during S phase. The negative regulation of the cell cycle involving this protein was shown to participate in repressing neuronal proliferation, as well as spermatogenesis[15-20]. Little is known of its role in cancer development and prognosis. CDKN2D expression in cancers has been examined in only a few studies and, to date, it has not been linked to cancer development.

Mitogen-activated protein kinases (MAPKs) are key mediators of evolutionarily conserved signaling networks that play an essential role in multiple aspects of cell physiology[21,22]. MAP3K1 or MEKK1 (MEK kinase 1) is a 196-kDa serine-threonine kinase that belongs to the MAP3K family and the STE superfamily[22,23]. MAP3K1 was originally identified as the mammalian homolog of the yeast MAP3Ks Ste11 and Byr2 that function in pheromone responsive signaling. Studies have demonstrated that MAP3K1 functions in cell survival, apoptosis, and cell motility/migration in multiple normal and tumor cell types[24,25].

In previous studies we have reported that miR-451 expression in ESCC tissues were significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in esophageal carcinoma[13,14]. In this study, we identified CDKN2D and MAP3K1 as the direct and functional targets of miR-451, which facilitated our understanding of the mechanisms underlying ESCC progression. Additionally, a further study indicated that CDKN2D and MAP3K1 overexpression reversed the effect of miR-451, and that miR-451 inhibited the proliferation of EC9706 by targeting CDKN2D and MAP3K1. The study demonstrates that miR-451 prefers to act as a potential target for the treatment of ESCC in the future.

MiRNAs have been shown to be important in the development and maintenance of normal cellular function, and an alteration in expression of miRNAs can result in human cancer initiation and tumor progression. MiRNAs can regulate target genes by increasing mRNA decay or by repressing translation. Each miRNA has the potential to target hundreds of genes that harbor in their 3'-UTR sequences complementary to the seed region of the miRNA[26-29]. In the study, for co-transfected cells with si-CDKN2D and si-MAP3K1, the inhibitory effects are similar to

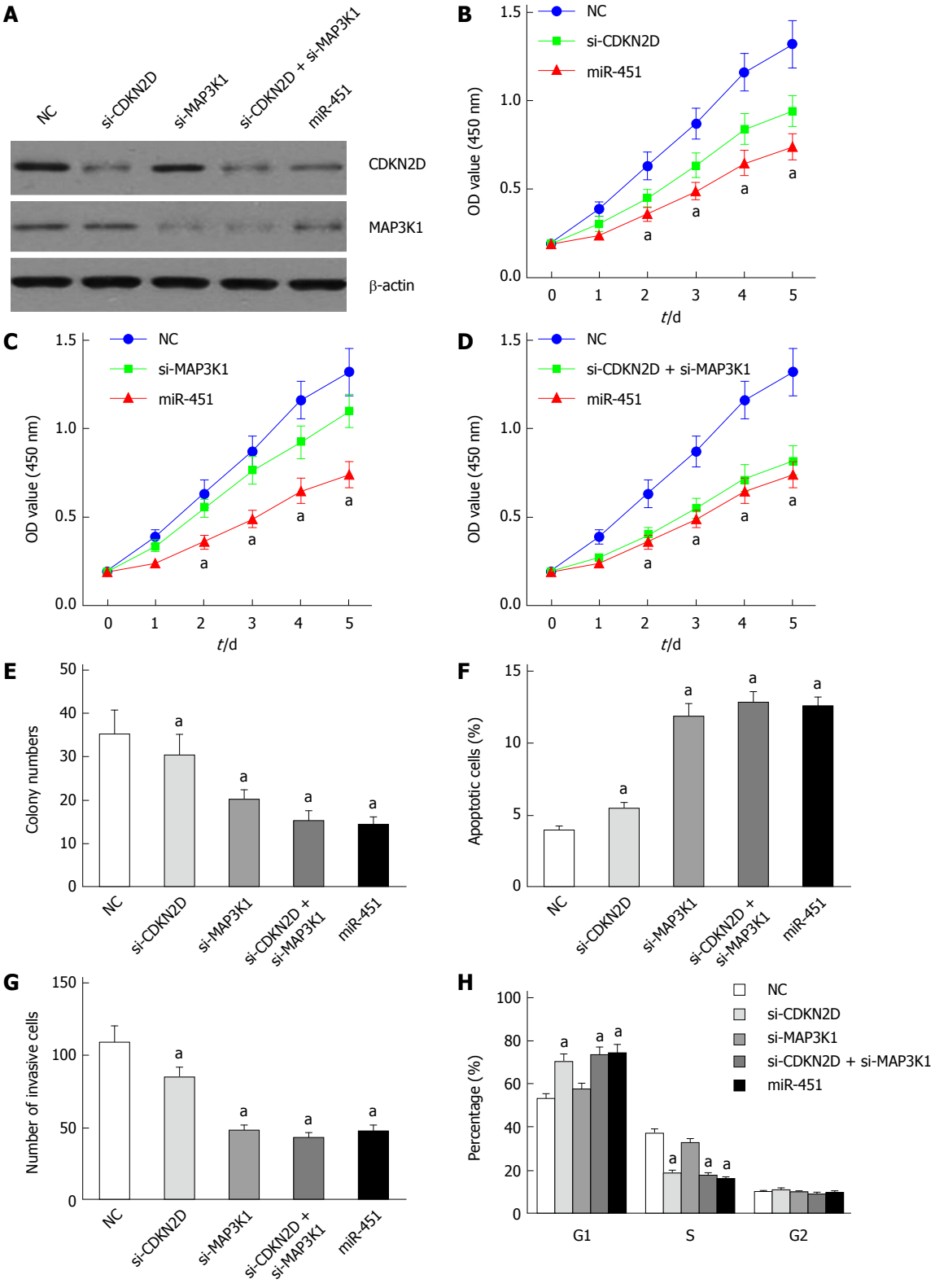

Figure 3 MiR-451 inhibits the proliferation of EC9706 cells by targeting CDKN2D and MAP3K1. A: CDKN2D and MAP3K1 protein levels were detected by Western blot assay. Western blot assay showed that transfection of CDKN2D-siRNAs, MAP3K1-siRNAs and miR-451 mimics inhibited the expression of CDKN2D and MAP3K1, respectively; B: CDKN2D silencing and miR-451 overexpression inhibited the cell proliferation. CCK8 array was used to assess EC9706 proliferation; C: MAP3K1 silencing and miR-451 overexpression inhibited the cell proliferation. CCK8 array was used to assess EC9706 proliferation; D: Co-transfection of si-CDKN2D and si-MAP3K1 inhibited the proliferation of EC9706 cells. CCK8 array was used to assess EC9706 proliferation; E: CDKN2D, MAP3K1 silencing and miR-451 overexpression reduced the growth of colonies of EC9706 cells. Colony formation assay was used; F: CDKN2D, MAP3K1 silencing and miR-451 overexpression induced EC9706 cell apoptosis. The cell apoptosis was assessed using flow cytometry assay; G: CDKN2D, MAP3K1 silencing and miR-451 overexpression suppressed EC9706 cell invasiveness. The cell invasion was assessed using transwell assay; H: CDKN2D silencing and the overexpression of miR-451 significantly increased the percentage of cells in the G1 phase and decreased the percentage of cells in the S phase ($^aP < 0.05$ *vs* control group). The cell cycle was assessed using flow cytometry assay.

those for cells overexpressing miR-451.

However, miRNAs may function according to a combinatorial circuits model, in which a single miRNA may target multiple mRNAs, and several coexpressed miRNAs may target a single mRNA. Recent studies have suggested that the biological concept of "one hit-multiple targets" could be used in clinical therapeutics[30]. If the primary molecular defect of a disease is in the expression of a miRNA, the expression of several critical protein targets could be deregulated. In that case, one might recover the normal phenotype of the cells by normalizing the miRNA expression. Although individual targets responsible for observed phenotypes have been proposed for many miRNAs, it is likely that a specific miRNA may function through cooperative down-regulation of multiple targets. Thus, other target genes of miR-451 may also contribute to tumorigenesis.

In conclusion, we have identified that miR-451 inhibited the proliferation, invasion and induced the apoptosis of ESCC cells *in vitro* and *in vivo* by directly targeting CDKN2D and MAP3K1. MiR-451 might be a novel prognostic biomarker and a potential target for the treatment of ESCC in the future.

COMMENTS

Background

Esophageal squamous cell carcinoma (ESCC) is one of the most lethal malignancies worldwide. ESCC is the 8th most common cancer and the 6th leading cause of cancer-related death. The traditional treatments for ESCC include chemotherapy and radiation therapy. However, many patients who are treated with such traditional therapy still experience disease progression, which suggests that ESCC is resistant to traditional therapy. In human cancer, microRNAs (miRNAs) can act as oncogenes or tumour suppressor genes during tumourigenesis.

Research frontiers

Recently miR-451 has been reported to be induced during zebrafish, mouse, and human erythroid maturation as a key factor involved in regulating erythrocyte differentiation. It was also reported that miR-451 might function as a tumor suppressor and modulate MDR1/P-glycoprotein expression in human cancer cells.

Innovations and breakthroughs

In previous studies the authors have reported that miR-451 expression in ESCC tissues were significantly reduced, and that upregulated expression of miR-451 induced apoptosis and suppressed cell proliferation, invasion and metastasis in esophageal carcinoma. However, the underlying molecular mechanisms remain unclear. In this study, the authors supposed and showed that CDKN2D and MAP3K1 are the targets of miR-451 by the bioinformatics algorithms (TargetScan and miRBase). Moreover, they found that CDKN2D and MAP3K1 contributed to ESCC malignancy.

Applications

The data suggest that miR-451 has potential values as a prognostic marker and a therapeutic target for ESCC.

Terminology

MiRNAs are small, endogenous noncoding RNAs that have been identified as post-transcriptional regulators of gene expression. MiRNAs exert their functions through imperfect base-pairing with the 3'-untranslated region of target mRNAs.

Peer-review

The manuscript is basically good. This is an *in vitro* study that addressed the mechanism of tumor suppressive functions of a miRNA, miR-451. The authors authentically conducted the required experiments using esophageal cancer cells and revealed that miR-451 targeted CDKN2D and MAP3K1 and worked tumor-suppressively through the inhibition of the two kinases. The findings are expected to contribute to the development of molecularly targeted therapy for esophageal cancer.

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ORIGINAL ARTICLE

Basic Study

Effect of nuclear factor-κB and angiotensin Ⅱ receptor type 1 on the pathogenesis of rat non-alcoholic fatty liver disease

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Author contributions: Tan DY and Li CP designed the research; Shi HY performed the research; Zhong XL analyzed the data; all authors contributed to the writing and revising of the manuscript; Tan DY and Li CP contributed equally to the paper.
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Ethics approval: The study was reviewed and approved by the Affiliated Hospital of Luzhou Medical College Institutional Review Board.
Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Affiliated Hospital of Luzhou Medical College (IACUC protocol number: [2012009]).
Conflict-of-interest: We declare that there is no conflict of interest to disclose.
Data sharing: No additional data are available.

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Abstract

AIM: To investigate the roles of nuclear factor (NF)-κB and angiotensin Ⅱ receptor type 1 (AT1R) in the pathogenesis of non-alcoholic fatty liver disease (NAFLD).

METHODS: Forty-two healthy adult male Sprague-Dawley rats were randomly divided into three groups: the control group (normal diet), the model group, and the intervention group (10 wk of a high-fat diet feeding, followed by an intraperitoneal injection of PDTC); 6 rats in each group were sacrificed at 6, 10, and 14 wk. After sacrifice, liver tissue was taken, paraffin sections of liver tissue specimens were prepared, hematoxylin and eosin (HE) staining was performed, and pathological changes in liver tissue (*i.e.*, liver fibrosis) were observed by light microscopy. NF-κB expression in liver tissue was detected by immunohistochemistry, and the expression of AT1R in the liver tissue was detected by reverse transcription-polymerase chain reaction (RT-PCR). The data are expressed as mean ± SD. A two-sample *t* test was used to compare the control group and the model group at different time points, paired *t* tests were used to compare the differences between the intervention group and the model group, and analysis of variance was used to compare the model group with the control group. Homogeneity of variance was analyzed with single factor analysis of variance. H variance analysis was used to compare the variance. $P < 0.05$ was

considered statistically significant.

RESULTS: The NAFLD model was successful after 6 wk and 10 wk. Liver fibrosis was found in four rats in the model group, but in only one rat in the intervention group at 14 wk. Liver steatosis, inflammation, and fibrosis were gradually increased throughout the model. In the intervention group, the body mass, rat liver index, serum lipid, and transaminase levels were not increased compared to the model group. In the model group, the degree of liver steatosis was increased at 6, 10, and 14 wk, and was significantly higher than in the control group ($P < 0.01$). In the model group, different degrees of liver cell necrosis were visible and small leaves, punctated inflammation, focal necrosis, and obvious ballooning degeneration were observed. Partial necrosis and confluent necrosis were observed. In the model group, liver inflammatory activity scores at 6, 10, and 14 wk were higher than in the control group ($P < 0.01$). Active inflammation in liver tissue in the intervention group was lower than in the model group ($P < 0.05$). HE staining showed liver fibrosis only at 14 wk in 4/6 rats in the model group and in 1/6 rats in the intervention group. NF-κB positive cells were stained yellow or ensemble yellow, and NF-κB was localized in the cytoplasm and/or nucleus. The model group showed NF-κB activation at 6, 10, and 14 wk in liver cells; at the same time points, there were statistically significant differences in the control group ($P < 0.01$). Over time, NF-κB expression increased; this was statistically lower ($P < 0.05$) at 14 weeks in the intervention group compared to the model group, but significantly increased ($P < 0.05$) compared with the control group; RT-PCR showed that AT1R mRNA expression increased gradually in the model group; at 14 wk, the expression was significantly different compared with expression at 10 weeks as well as at 6 weeks ($P < 0.05$). In the model group, *AT1R* mRNA expression was significantly higher than at the same time point in the control group ($P < 0.01$).

CONCLUSION: With increasing severity of NAFLD, NF-κB activity is enhanced, and the inhibition of NF-κB activity may reduce *AT1R* mRNA expression in NAFLD.

Key words: Non-alcoholic fatty liver disease; Nuclear factor-κB; Angiotensin Ⅱ receptor type 1; Rats, Liver fibrosis

Core tip: Angiotensin Ⅱ receptor type 1 (AT1R) is closely associated with the process of non-alcoholic fatty liver disease (NAFLD) fibrosis. As the nuclear transcription factor which is closely related to the tissue inflammation and fibrosis, when the activity of nuclear factor (NF)-κB was inhibited, *AT1R* mRNA expression was reduced, and the degrees of inflammation and fibrosis gradually reduced, indicating that NF-κB might play a key role throughout the course of NAFLD and that the NF-κB inhibitor might be effective in the treatment of the disease, while the exact mechanism still requires further study.

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INTRODUCTION

With social and economic development, the incidence of non-alcoholic fatty liver disease (NAFLD) has increased over the years, to the point that it has become a common chronic liver disease that endangers human health. Worldwide epidemiological surveys have shown that the incidence rate of adult NAFLD is 20%-33%; this rate could reach up to 75% in obese individuals or those with type 2 diabetes. A recent study found that the disease also exhibits a trend of developing in younger people; therefore, NAFLD has gained more attention in Western countries and other regions of the world[1-3]. Pathological changes are mainly due to inflammation, necrosis, or apoptosis in liver cells, leading to steatohepatitis, liver fibrosis, and cirrhosis.

Nuclear factor-κB (NF-κB) is a nuclear protein factor that participates in the regulation of a variety of protein genes and causes disease through the induction of cytokines, which are related to immunity, inflammation, and fibrosis; NF-κB thus plays an important role in inflammation and the immune response[4-6]. Some studies have shown that the liver renin-angiotensin-aldosterone system (RAAS) is closely related to the development of liver fibrosis[7]. Therefore, an angiotensin Ⅱ receptor type 1 (AT1R) antagonist could inhibit the development of experimental liver fibrosis[8,9]. In this study, pyrrolidine dithioformate (PDTC) was used to inhibit the activity of NF-κB in NAFLD rats, with the aim to explore the changes in angiotensin (Ang) Ⅱ and *AT1R* mRNA expression, and to investigate the regulation of NF-κB towards Ang Ⅱ *AT1R* mRNA expression in NAFLD rats.

MATERIALS AND METHODS

Grouping and modeling

Thirty-six healthy adult SPF-level male SD rats, weighing 150 g ± 30 g, were randomly divided into the control group ($n = 18$, fed a normal diet) and the model group ($n = 18$, fed a high-fat diet: 2% cholesterol, 0.5% sodium cholate, 0.2% propylthiouracil, 5% sucrose, 10% lard, and 82.3% basal diet, freshly prepared by the experimental Animal Center at Luzhou Medical

College). The rats had free access to water. Six rats of each group were killed after 6, 10, and 14 wk. Serum and liver tissues were isolated and stored at -80 ℃. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Luzhou Medical College.

Light microscopy

The liver tissues were fixed with 10% paraformaldehyde for 1 d, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). Liver histopathological changes were observed by light microscopy.

Radioimmunoassay

The radioimmunoassay kit and radioimmunoassay counter were used to perform the detection. NF-κB protein expression detection was performed by immunohistochemistry according to the instructions of the SP immunohistochemical staining kit and DAB staining kit (Shengzhen Chemical Co. Ltd., Shengzhen, China). NF-κB mainly appeared as brown staining in the cell membrane or cytoplasm, and the nuclear membrane was occasionally stained. Five high-power fields were imaged from each section (with > 500 cells in each field) to calculate the positive rate (*i.e.*, number of positive cells/total cells × 100%).

Reverse transcription-polymerase chain reaction

Total RNA was extracted according to the instructions of the RNA extraction kit. The integrity of the RNA was analyzed, the concentration and purity were measured, and then the extracted RNA was diluted to the same concentration in accordance with the measured concentration; 0.5 μg was prepared for reverse transcription (RT)-polymerase chain reaction amplification according to the instructions in the First Strand cDNA Synthesis Kit (Beijing Biomed Co. Ltd., Beijing, China). The upstream primer sequence of the *AT1R* gene was: 5′-ACG TGT CTC AGC ATC GAC CGC TAC C-3′ and the downstream primer sequence was: 5′-AGA ATG ATA AGG AAA GGG AAC AAG AA-3′. The upstream primer of the internal reference β-actin gene was: 5′-GAG GGA AAT CGT GCG TGA C-3′ and the downstream primer was: 5′-CTG GAA GGT GGA CAG TGA G-3′. The length of the targeted *AT1R* mRNA amplification fragment was about 278 bp, and the RT reaction parameters were: 30 ℃ for 10 min, 42 ℃ for 20 min, 99 ℃ for 5 min, and 4 ℃ for 5 min. Then, 1 μL of the cDNA product, 1 μL of the upstream and downstream primers (10 μM), 12.5 μL of 2 × MasterMix, and 9.5 μL of ddH_2O were mixed together to a total volume of 25 μL for the PCR. The reaction parameters were: AT1R: pre-denaturation at 94 ℃ for 5 min, denaturation at 94 ℃ for 35 s, annealing at 53 ℃ for 30 s, and extension at 72 ℃ for 1 min; after 30 cycles, extension was performed at 72 ℃ for 5 min.

Statistical analysis

SPSS17.0 statistical software was used, with $P < 0.05$ considered to indicate statistical significance. The data are expressed as the mean ± SD, and comparisons among different time points for the control and model groups were made using analysis of variance. Homogeneity of variance test was also performed, and data sets with homogeneity of variance were then submitted to single-factor variance analysis, while those without homogeneity of variance were submitted to non-parametric tests.

RESULTS

Light microscopy observation

The morphology of the liver tissue in the control group after 6, 10, and 14 wk was normal based on HE staining. In the model group, after 6 wk, liver cells within the hepatic lobules exhibited more than 33% fatty degeneration, with no significant inflammatory cell infiltration in the portal area and acinus. There was no significant formation of collagen fibers and fibrosis, indicating the stage of liver cell steatosis. After 10 wk, the steatotic liver cells in the model group accounted for more than 50% of the total cells, and in some rats the ratio reached more than 66%, with significant ballooning degeneration in the acinar 3 band. Spotty hepatocellular necrosis could be seen in the acinus, and inflammatory cells appeared in the portal area, indicating non-alcoholic hepatitis. After 14 wk, various degrees of lobular and portal inflammation could be seen in the model group, and extensive ballooning degeneration could be seen in the acinar 3 band. At the same time, periportal degeneration, spotty necrosis, and focal necrosis were also found, with partial necrosis being integrated into a larger area. Inflammation was apparent in and around the portal area. Four rats exhibited hepatocellular fibrosis. In the intervention group, lobular inflammation could be seen after 14 wk and spotty necrosis appeared in the acinus, with some inflammation in the periportal area. One rat exhibited hepatocellular fibrosis (Figure 1).

Radioimmunoassay results

Throughout the model, the serum Ang Ⅱ concentration gradually increased, and was higher than the concentration in the control group at the same time; the differences were statistically significant ($P < 0.05$). The Ang Ⅱ concentration in the intervention group was lower than in the model group after 14 wk, but higher than the level in the control group ($P < 0.05$) (Table 1).

NF-κB protein expressions

Yellow or buffy yellow-stained NF-κB, located in the cytoplasm and/or the nucleus, identified NF-κB

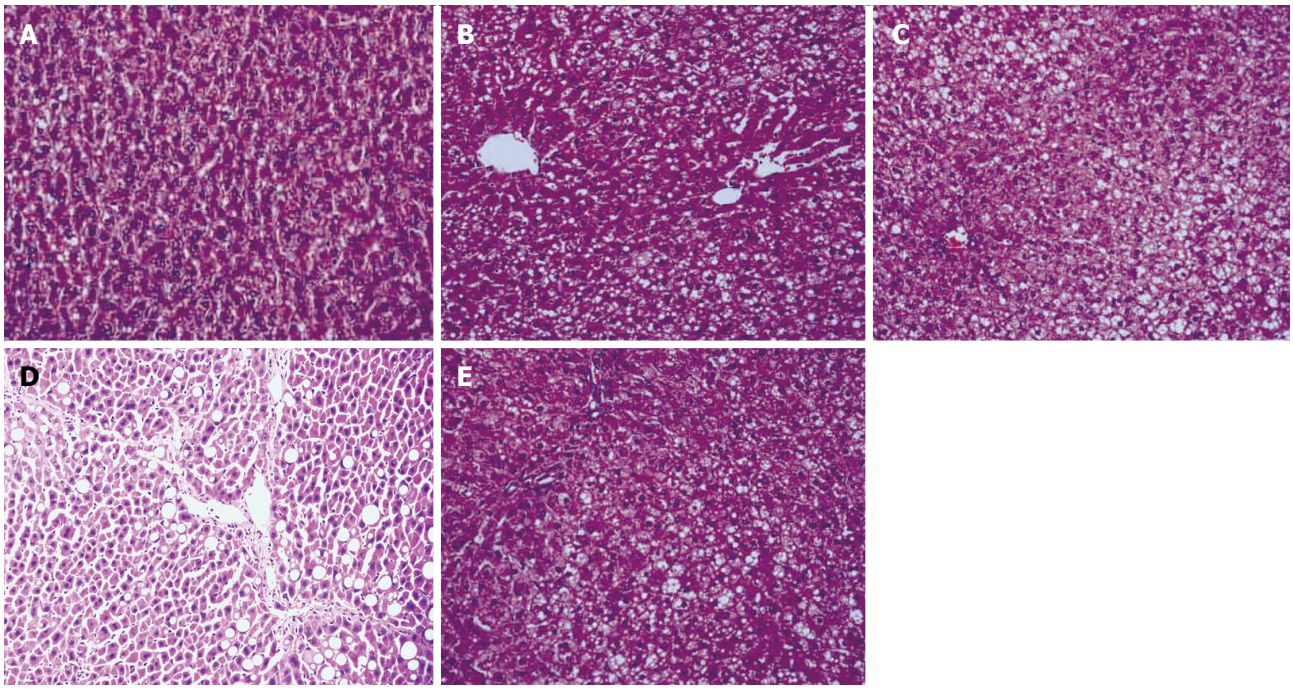

Figure 1 Histopathological results in the liver (hematoxylin and eosin staining, magnification × 100). A: The control group; B: The model group at the end of the 6th week; C: The model group at the end of the 10th week; D: The model group at the end of the 14th week; E: The intervention group at the end of the 14th week.

Table 1 Serum Ang Ⅱ expression in each group (mean ± SD)

Groups	6th week	10th week	14th week
Normal	390.68 ± 15.34	424.54 ± 7.66	475.78 ± 10.51
Model	929.61 ± 463.21[a,b,c]	1244.61 ± 232.81[a,c]	1567.04 ± 273.22[a,d]
Intervention			1349.04 ± 433.22[a]

[a]$P < 0.01$ *vs* the control group; [b]$P < 0.05$ *vs* the model group in the 10th week; [c]$P < 0.05$ *vs* the model group in the 14th week; [d]$P < 0.05$ *vs* the intervention group in the 14th week.

Table 2 Nuclear factor-κB protein expression in rat hepatocytes from each group (mean ± SD, *n* = 6)

Groups	6th week	10th week	14th week
Normal	4.48 ± 0.34	4.26 ± 0.66	4.78 ± 0.74
Model	29.61 ± 3.21[a,b,c]	44.61 ± 2.81[a,c]	67.04 ± 3.22[a,d]
Intervention			49.04 ± 3.22[a]

[a]$P < 0.01$ *vs* the control group; [b]$P < 0.05$ *vs* the model group in the 10th week; [c]$P < 0.05$ *vs* the model group in the 14th week; [d]$P < 0.05$ *vs* the intervention group in the 14th week.

positive cells. In the model group, apparent NF-κB activation could be seen after 6, 10, and 14 wk in the hepatocytes, and expression increased throughout the model. In the intervention group, NF-κB expression after 14 wk was significantly decreased when compared with that in the model group, although still higher when compared with the control group (Figure 2; Table 2).

Expression of AR1T mRNA

The relative expression level of *AR1T* mRNA in the model group at each time point was higher than in the control group, and the relative expression level gradually increased throughout the model. The relative expression in the intervention group after 14 wk was higher than that in the control group at the same time point, but lower than that in the model group at the same time point.

DISCUSSION

With improvements in living standards and social change, the incidence of NAFLD has risen. The pathogenesis of NAFLD is driven by oxidative stress and lipid peroxidation damage, leading to the dysfunction of liver cells and mitochondrial activity, the activation and proliferation of hepatic stellate cells (HSC), and increased release of inflammatory cytokines, followed by inflammation, necrosis, and apoptosis, which then develops into steatohepatitis, fibrosis, and cirrhosis[10-12].

The RAAS is an important neuroendocrine system, as it maintains the balance of blood pressure, water, and electrolytes; Ang Ⅱ is the most important bioactive substance in the RAAS. Some studies have shown that there was also RAAS in the partial liver[13]. Recent studies have also found that liver fibrosis is connected with RAAS activation[14]. The biological effects of Ang Ⅱ are exerted by acting on specific receptors. The Ang Ⅱ receptors include four G protein-coupled receptor subtypes, namely AT1R, AT2R, AT3R, and AT4R; AT1R and AT2R are the best understood. Yang *et al*[15] found that the TGF-β content in wild-type mouse liver tissue is higher than in AT1Ra-deficient

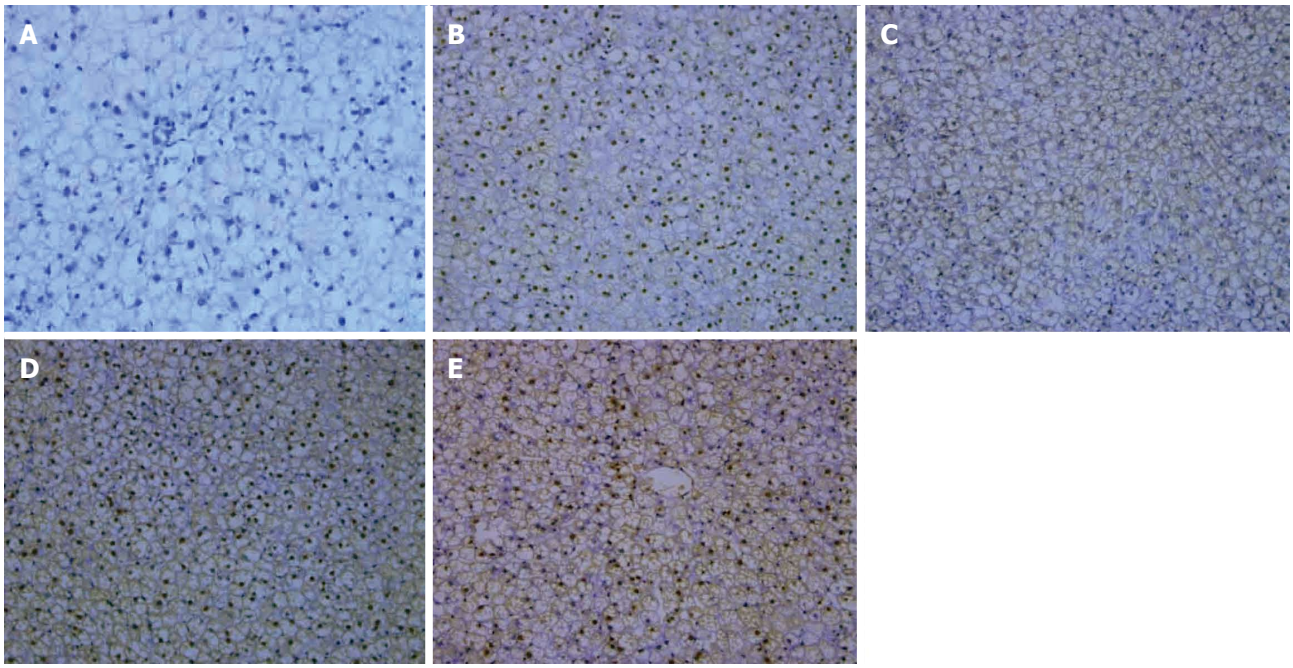

Figure 2 Immunohistochemical staining for nuclear factor-κB protein expression (SP, magnification × 200). A: The control group; B: The model group at the end of the 6th week; C: The model group at the end of the 10th week; D: The model group at the end of the 14th week; E: The intervention group at the end of the 14th week.

Table 3 Angiotensin Ⅱ receptor type 1 mRNA expression in rat hepatocytes from each group (mean ± SD, *n* = 6)

Group	6th week	10th week	14th week
Normal	0.33 ± 0.11	0.41 ± 0.08	0.53 ± 0.15
Model	0.54 ± 0.15[a,b,c]	0.76 ± 0.20[a,c]	1.02 ± 0.27[a,d]
Intervention			0.80 ± 0.20[a]

[a]$P < 0.01$ *vs* the control group; [b]$P < 0.05$ *vs* the model group in the 10th week; [c]$P < 0.05$ *vs* the model group in the 14th week; [d]$P < 0.05$ *vs* the intervention group in the 14th week.

mice, and the degree of inflammation and fibrosis was much greater in wild-type than in AT1Ra-deficient mice, suggesting that AT1R plays an important role in the process of liver fibrosis. In the current study, it was shown that *AT1R* mRNA expression increased in liver tissue during the process of liver steatosis, and the processes of inflammation and fibrosis aggravated this. Most biological effects of Ang Ⅱ are mediated by AT1R through a series of signal transduction pathways. At the same time, it can also act as a pro-inflammatory cytokine in various inflammatory processes. The results shown here indicate that AT1R is involved in the development of NAFLD from steatosis to hepatic fibrosis. When damaging factors affect the liver, this stimulates *AT1R* mRNA transcription, and when Ang Ⅱ binds to AT1R, it conducts a signal via the G protein-coupled pathway and the mitogen-activated protein kinase pathway, thereby activating downstream factors and promoting inflammation. During the stages of steatosis and hepatitis, AT1R accelerates the synthesis of inositol phosphate, which causes the sarcoplasmic reticulum to release calcium into the cytoplasm. This has an important role in the inflammatory response and oxidative stress, resulting in liver cell degeneration and necrosis through multiple ways, and thus leading to hepatic steatosis and liver cell inflammation. In the stage of liver fibrosis, AT1R promotes the transcription of proto-oncogenes, inducing mesenchymal cells and fibroblasts to proliferate abnormally, leading to increased collagen synthesis and fibrosis[16]. Meanwhile, AT1R activation also induces hepatic stellate cell proliferation and upregulates TGF-β1 levels[17], thus playing an important role in liver fibrosis.

Recent studies have found that the activation of NF-κB is closely related to the process of liver inflammation and fibrosis. The key to NF-κB activation is IκB degradation. During the second stage of NAFLD, the products of reactive oxygen species and lipid peroxidation significantly increase, which activates the NF-κB complex; first, through the mitogen-activated protein kinase (MAP kinase, MAPK) family, serine kinase (IKK) catalyzes the phosphorylation[18-22]. When the IKK complex combines with membrane coupled receptors, two types of autophosphorylation occur to activate the serine kinase[23-26]. Subsequently, under the effect of IKK, IκB is phosphorylated and, via pantothenic acid, the proteasome, and other mediators, the 3D structure of activated IκB is destroyed, exposing the amino acid sequence which is then recognized by the pantothenic acid ligase. Pantothenic acidification then occurs, and NF-κB is thus hydrolyzed and activated. Increased activity of NF-κB promotes the expression of COX-2, IL-6, and IL-8 by HSC, which increase liver inflammation, and further activates NF-κB through inflammatory mediators, thereby maintaining HSC activation and

eventually leading to the occurrence of liver fibrosis. Ang Ⅱ induces NF-κB, resulting in the activation of a large number of target genes, such as cytokines, chemokines, adhesion molecules, and TNF-α. Ji *et al*[27-29] found that the intrahepatic RAAS system can mediate hepatic fibrosis by activating the NF-κB and AP-1 pathways, and AECI and ARB may play an anti-liver fibrosis role by inhibiting NF-κB activity. Activated NF-κB can also act on angiotensinogen, further affecting the local hepatic RAAS system and leading to liver fibrosis. In the intervention group in this experiment, it was found that by inhibiting NF-κB activity, the pathological changes were better than in the model group, and AT1R mRNA expression was significantly lower than in the model group, which further indicates the presence of this kind of relationship.

AT1R is closely associated with the process of NAFLD fibrosis[30,31]. Since this nuclear transcription factor is closely related to tissue inflammation and fibrosis, when the activity of NF-κB was inhibited, *AT1R* mRNA expression was reduced, and the degree of inflammation and fibrosis gradually reduced as well, indicating that NF-κB might play a key role throughout the course of NAFLD and an NF-κB inhibitor might be effective in the treatment of this disease. However, the exact mechanism still needs further study.

COMMENTS

Background

The pathogenesis of non-alcoholic fatty liver diseases (NAFLD) is not clear. Many studies have shown that nuclear factor (NF)-κB and the angiotensin Ⅱ receptor type 1 (AT1R) may participate in its pathogenesis.

Research frontiers

NF-κB participates in the regulation of a variety of protein genes, and thus plays an important role in inflammation and the immune response. An AT1R antagonist could inhibit the development of experimental liver fibrosis, but the role of this pathway in NAFLD is not clear.

Innovations and breakthroughs

Pyrrolidine dithioformate (PDTC) was used to inhibit the activity of NF-κB in NAFLD rats with the aim to explore the changes in angiotensin Ⅱ (Ang Ⅱ) and *AT1R* mRNA expression, and to investigate the regulation of NF-κB regarding Ang Ⅱ and *AT1R* mRNA expression in NAFLD rats.

Applications

NF-κB might play a key role throughout the course of NAFLD, and an NF-κB inhibitor might be effective in the treatment of this disease. It would be helpful to develop a new drug for the treatment of NAFLD.

Terminology

NF-κB is a nuclear protein factor that can cause disease by inducing cytokines which are related to immunity, inflammation, and fibrosis, thus playing an important role in inflammation and the immune response.

Peer-review

AT1R is closely associated with the process of NAFLD fibrosis. NF-κB might play a key role throughout the course of NAFLD, and an NF-κB inhibitor might be effective in the treatment of this disease.

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ORIGINAL ARTICLE

Basic Study

Effect of microRNA-1 on hepatocellular carcinoma tumor endothelial cells

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Author contributions: Hu C performed the majority of experiments, analyzed the data and wrote the paper; Shen SQ critically revised the manuscript, and approved the final version of the manuscript submitted for publication; Chen ZB designed the study; Cui ZH performed the minority of experiments; and Li W contributed analytic tools.

Ethics approval: All experiments involving animals and human subjects were designed and performed in compliance with the relevant laws regarding the humane care and use of subjects.

Institutional animal care and use committee: All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Renmin Hospital of Wuhan University (IACUC protocol number: SYXK 2014-0013).

Conflict-of-interest: We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Data sharing: No additional data are available.

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Abstract

AIM: To investigate the effect of microRNA-1 (miR-1) on tumor endothelial cells (TECs) of human hepatocellular carcinoma (HCC).

METHODS: MiR-1 specific short hairpin RNA (shRNA) was synthesized and cloned into a recombinant lentiviral vector. TECs were then infected by the miRNA-1-shRNA recombinant lentivirus. TECs were divided into three groups: a control (CON) group consisting of normal TECs without lentiviral infection, a negative control (NC) group consisting of normal TECs infected with a negative control virus, and a micro-down (MD) group consisting of normal TECs infected with the miR-1-inhibition virus containing the target gene. Silencing of miR-1 expression was quantified *via* quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The proliferation of TECs was detected using MTT (Thiazolyl Blue Tetrazolium Bromide) assay; the observations were continued for 5 d, and the optical density value at 490 nm was detected every day. Apoptosis was detected *via* flow cytometry using Annexin V-APC single staining. The migration and invasion of TECs were detected using transwell assays.

RESULTS: Lentiviral miR-1 shRNA was successfully transduced into TECs, and specifically silenced the expression of miR-1. The results of qRT-PCR showed that the expression of miR-1 was significantly decreased in the MD group ($2^{-\Delta\Delta Ct}$ = 0.57 ± 0.14) compared with the CON group ($2^{-\Delta\Delta Ct}$ = 1) and the NC group ($2^{-\Delta\Delta Ct}$ = 1.05 ± 0.13) (P < 0.01). The results of MTT assay showed that the cell proliferation was all significantly inhibited in the MD group in the 5 days compared with the CON and NC groups (P < 0.01). The results of flow cytometry showed that the apoptosis was significantly increased in the MD group (6.32% ± 0.33%) compared with the CON

group (2.03% ± 0.30%) and the NC group (2.18% ± 0.15%) (P < 0.01). The ability of cell migration was significantly inhibited in the MD group (62.0 ± 5.48) compared with the CON group (99.8 ± 3.11) and the NC group (97.2 ± 3.70) (P < 0.01). The ability of invasion of TECs was also significantly inhibited in the MD group (29.8 ± 2.39) compared with the CON group (44.6 ± 3.36) and the NC group (44.4 ± 5.17) (P < 0.01).

CONCLUSION: MiR-1 might be a potential tumor activator. Inhibiting its expression could decrease proliferation, induce apoptosis, and inhibit the migration and invasion of TECs of human HCC.

Key words: Tumor endothelial cells; Hepatocellular carcinoma; Short hairpin RNA; MicroRNA-1

Core tip: Our study demonstrated that microRNA-1 (miR-1) might be a potential tumor activator. Inhibition of the expression of miR-1 could decrease the proliferation, induce the apoptosis, and inhibit the migration and invasion of tumor endothelial cells of human hepatocellular carcinoma.

Hu C, Shen SQ, Cui ZH, Chen ZB, Li W. Effect of microRNA-1 on hepatocellular carcinoma tumor endothelial cells. *World J Gastroenterol* 2015; 21(19): 5884-5892 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5884.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5884

INTRODUCTION

Currently, primary liver cancer, which consists predominantly of hepatocellular carcinoma (HCC), is the fifth most prevalent cancer worldwide, the second most frequent cause of cancer death in men, the seventh most commonly diagnosed cancer and the sixth leading cause of cancer death in women[1]. The only treatment that offers a potential for curing patients with HCC is surgical resection or liver transplantation. However, only a small number of patients can receive surgical treatment due to the limitations such as the stage of the carcinoma, the number and the size of the nodules, and the liver function. Furthermore, no effective treatment is presently established once surgical treatment cannot be performed, especially for advanced HCC patients[2]. Therefore, identifying novel therapeutic targets for the treatment of HCC is urgently required.

Recent evidence highlighted that tumor microenvironment plays an important role in the initiation, progression and metastasis of cancer[3]. Cancer is an ensemble production in which tumor cells act as the leading villains and the tumor stroma, blood vessels, infiltrating inflammatory cells and a variety of associated tissue cells in tumor microenvironment play the role of supporting player to aid the malignant progression of cancer[4]. The nontumor cells in the tumor microenvironment can be modified by the cancer cells to produce a variety of growth factors, chemokines, and matrix-degrading enzymes that enhance the proliferation and invasion of the tumor. Recent experiments have suggested various approaches to target different cell types in the tumor microenvironment such as the tumor stroma[5], tumor vasculature[6] and immune cells[7]. Therefore, an increasing number of experts believe that the treatment strategy for cancer targeting to the tumor microenvironment may produce unexpected effects.

HCC is a solid cancer with a rich blood supply. Angiogenesis is crucial in the occurrence, development and prognosis of this cancer. Its growth, invasion and metastasis are all closely associated with angiogenesis. Therefore, regulation of tumor angiogenesis can control tumor growth[8]. During the development of the tumor from the avascular stage to the vascular stage, angiogenesis is regulated by angiogenic growth factors and their inhibitors, and once this balance is disturbed, angiogenesis can be accelerated[9]. Therefore, identifying the specific molecular markers of tumor vascular endothelial cells can provide a new basis for antiangiogenic therapy targeting the tumor neovasculature in HCC.

MicroRNAs (miRNAs) are a class of non-coding, small RNA molecules consisting of 20-22 nucleotides that regulate gene expression at the post-transcriptional level[10]. They regulate the expression of downstream target genes at the protein level, thus playing important regulatory roles in cellular pathways[11]. Many recent studies have suggested that abnormal expression of miRNA target genes is associated with the initiation and development of various types of cancer[12,13]. MiR-1 has been reported as a down-regulated miRNA in various human malignancies and has a tumor suppressive function[14-17]. However, Liu *et al*[18] found that miR-1 was markedly up-regulated in the serum of gastric cancer patients. Our previous studies also found that miR-1 was up-regulated in tumor endothelial cells (TECs) of human HCC[19]. Therefore, to investigate the effect of miR-1 on TECs of human HCC, we inhibited the expression of miR-1 in TECs using antisense oligonucleotides and observed the effect on biological behaviors including proliferation, apoptosis, migration, and invasion of TECs.

MATERIALS AND METHODS

Cells

TECs of human HCC, human hepatic sinusoidal endothelial cells (HSECs), and 293T cells were purchased from Shanghai Xinran Biotechnology (Shanghai, China) and cultured in DMEM (Dulbecco'

s Modified Eagle's Medium) high-glucose medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin under 5% CO_2 in a humidified incubator at 37 ℃.

Construction of lentiviral vectors

A DNA template and oligonucleotides corresponding to miRNA-1 were targeted. The oligonucleotide sequences were designed and synthesized as follows: miR-1-inhibition-F: 5'- TGGAATGTAAAGAAGTATGTAT- 3', and miR-1-inhibition-R: 5'- ATACATACTTCTTTACATTCCA-3'. The combined sequences of the enhanced green fluorescent protein (EGFP) gene and the miRNA-1-inhibitor were cloned into the Age Ⅰ and EcoR Ⅰ sites of the GV159 vector (Genechem, Biotechnology, China) containing a CMV-driven GFP reporter. All constructed plasmids were confirmed *via* sequence analysis. All plasmids were transfected into 293T cells using a packaging vector mix (Invitrogen). Eight hours after transfection, the culture medium was replaced with complete culture medium. After another 48 h of culture, the cell culture supernatant rich in lentiviral particles was collected and filtered by using a 0.45 μm filter which allows only viruses, and not cells to pass through. Then, the filtrate was centrifuged to obtain a high titer, concentrated lentiviral solution. The virus titer was determined and calibrated in 293T cells.

Infection of TECs by lentiviruses

The experimental cells were divided into 3 groups: a control (CON) group consisting of normal TECs without lentiviral infection, a negative control (NC) group consisting of normal TECs infected with a negative control virus, and a micro-down (MD) group consisting of normal TECs infected with the miR-1-inhibition virus containing the target gene. Cells under good culture conditions from each experimental group were inoculated into 6-well plates one day prior to viral infection. On the day of infection, lentiviruses were added to each group of cells to perform the infection experiments. Three days after infection, the expression of GFP was observed under a fluorescence microscope, and the proportion of cells with positive fluorescence was higher than 90%. When cells became confluent, they were harvested and examined.

Quantitative reverse transcription-polymerase chain reaction for detection of mature miR-1 expression

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to detect the expression of miR-1 in the different groups and the U6 small nuclear RNA was used as the internal standard control. Total RNA was harvested from cells of different groups using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. For reverse transcription, 5 μg of total RNA was converted to cDNA using a TaqMan MicroRNA Reverse Transcription Kit (Fermentas) according to the manufacturer's protocol. The resulting cDNA was diluted 1:10 and used for PCR with 4 μL of miR-1 or U6 TaqMan primers and SYBR Green/Flourescein qPCR Master Mix (Fermentas) in the ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA). The sequences of the primers were as follows: miR-1 forward: 5'-TGCGCTGGAATGTAAAGAAGTA-3'; miR-1 reverse: 5'-CCAGTGCAGGGTCCGAGGTATT-3'; U6 forward: 5'-CTCACTTCGGCAGCACATA-3'; U6 reverse: 5'-AACTCTTCACGATTTTGTCTGTC-3'. PCR amplification from cDNA was performed in a final volume of 25 μL, and the cycling parameters were preheat at 95 ℃ for 10 min, then 40 cycles of 95 ℃ for 30 s and 60 ℃ for 1 min, followed by a melting curve analysis. The specificity of the PCR amplification was confirmed *via* a dissociation curve analysis. All reactions were performed in triplicate. The ΔCt data were collected automatically and -ΔΔCt was calculated using the following formula:-ΔΔCt = ΔCt of CON group-ΔCt of the NC or MD group. The relative expression of the target gene was calculated using $2^{-\Delta\Delta Ct}$[20].

Detection of TEC proliferation using MTT assay

Cells from each group that were in the logarithmic phase were digested with trypsin and resuspended in complete culture medium. The cells were counted using a hemocytometer, and the cell density used for inoculation was determined according to the growth rate (usually 2000 cells/well). Each group had 3-5 duplicate wells with 100 μL of cell suspension in each well; a total of five 96-well plates were used, and the observations were continued for 5 d. When adding cells to the tissue culture plates, the cell number in each well was consistent. After the cells were plated, they were cultured at 37 ℃ in a 5% CO_2 incubator. During the period starting from the second day of plating to 4 h before the termination of culture, 10 μL of 5 mg/mL MTT (Thiazolyl Blue Tetrazolium Bromide) was added to each well without changing the medium. After 4 h, the culture medium was discarded, and 100 μL DMSO was added to each well to stop the reaction. After vortexing for 5-10 min, the optical density (OD) value was detected at 490 nm using a microplate reader, and the results were statistically analyzed.

Flow cytometry detection of TEC apoptosis by Annexin V-APC single staining

Apoptosis was determined using an Annexin-V-APC apoptosis detection kit (eBioscience, San Diego, CA, United States). The cell culture supernatant from each group was collected into 5 mL centrifuge tubes after the cells were infected for 5 d. The cells were washed once with D-Hanks and digested with trypsin; the digestion was stopped by the addition of culture supernatant. The cells were harvested and collected into the same 5 mL centrifuge tube, and each group had three duplicate wells. Then, the cells were collected *via* centrifugation at 1500 rpm for 5 min and

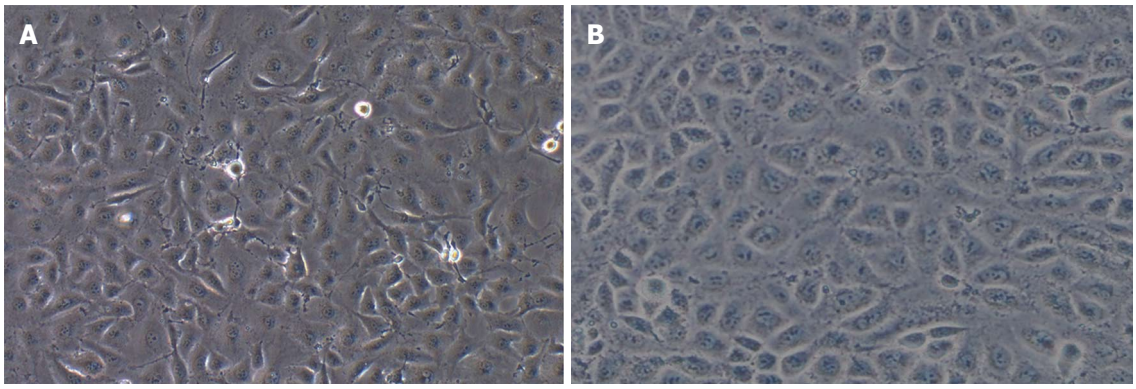

Figure 1 Comparison of the morphology of tumor endothelial cells and hepatic sinusoidal endothelial cells. A: The morphology of tumor endothelial cells (magnification × 100); B: The morphology of hepatic sinusoidal endothelial cells (magnification × 100).

washed twice in PBS. Next, cells were resuspended in binding buffer, and cell suspensions were collected, stained with 5 μL Annexin V-APC at room temperature for 15 min in the dark, and then transferred into flow cytometry tubes for detection using a FACSCalibur (BD Biosciences, San Jose, CA, United States).

The excitation and emission wavelengths of the Annexin V-APC fluorescent signals were 633 nm and 660 nm, respectively. The signals were detected in logarithmic mode at FL4. Annexin V-APC was set as the horizontal axis. Cells staining positive for Annexin V-APC were considered apoptotic (the right upper quadrant and right lower quadrant).

Transwell migration assay

A total of 3 × 10^4 cells were resuspended in serum-free DMEM after transfection and placed in the top portion of a Transwell chamber with 8-μm pores (Millipore). The lower portion of the chamber contained 10% FBS as a chemoattractant. The chambers were incubated at 37 ℃ in 5% CO_2 for 12 h. Non-migrating cells on the top of the membrane were removed with cotton swabs. Cells that migrated to the bottom of the insert were fixed with 95% ethanol, stained with 0.2% crystal violet (Beyotime, China), and counted and photographed under magnification × 100. Five random fields were analyzed in each chamber.

Transwell invasion assay

A total of 3 × 10^4 cells were resuspended in serum-free DMEM after transfection and placed in the top portion of a Transwell chamber with 8-μm pores (Millipore) and coated with 30 mg/cm^2 matrigel extracellular matrix (ECM) gel (Sigma-Aldrich, United States). The lower portion of the chamber contained 10% FBS as a chemoattractant. The chambers were incubated at 37 ℃ in 5% CO_2 for 24 h. Cells on the top of the membrane were removed with cotton swabs. Cells that invaded the bottom of the insert were fixed with 95% ethanol, stained with 0.2% crystal violet (Beyotime, China), and counted and photographed under × 100 magnification. Five random fields were analyzed in each chamber.

Statistical analysis

All data are expressed as the mean ± SD. SPSS 17.0 software was used for statistical analyses. Differences among groups were assessed using an unpaired Student's *t*-test. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Cell morphology

Observations under a microscope revealed that most of the TECs showed a thin and long "filamentous rod-shaped" morphology, and some showed a triangle or a polygon shape with different sizes (Figure 1A), whereas HSECs showed a uniform, typical, "cobblestone-like" morphology (Figure 1B).

Determination of lentivirus titers after infection of TECs

Three days after infection, the expression of GFP was observed under a fluorescence microscope. A1, B1 and C1 in Figure 2 show the morphology of cells in the CON, NC and MD groups, respectively, under the bright field and A2, B2 and C2 show the same fields under the green fluorescence field. The CON group showed no green fluorescence (Figure 2A2); the NC group (Figure 2B2) and the MD group (Figure 2C2) showed obvious green fluorescence. The results indicated that the lentiviral shRNA was successfully constructed and the proportion of cells with positive fluorescence was over 90%.

MiR-1 expression in cells of three groups

As observed in Figure 3, the expression of miR-1 was significantly decreased in the MD group ($2^{-\Delta\Delta Ct}$ = 0.57 ± 0.14) compared with the CON group ($2^{-\Delta\Delta Ct}$ = 1) and the NC group ($2^{-\Delta\Delta Ct}$ = 1.05 ± 0.13) ($P < 0.01$). There was no significant difference between the NC group and the CON group ($P > 0.05$).

Detection of TEC proliferation by MTT assay

As observed in Figure 4, cell proliferation was all significantly inhibited in the MD group for the 5 d (the

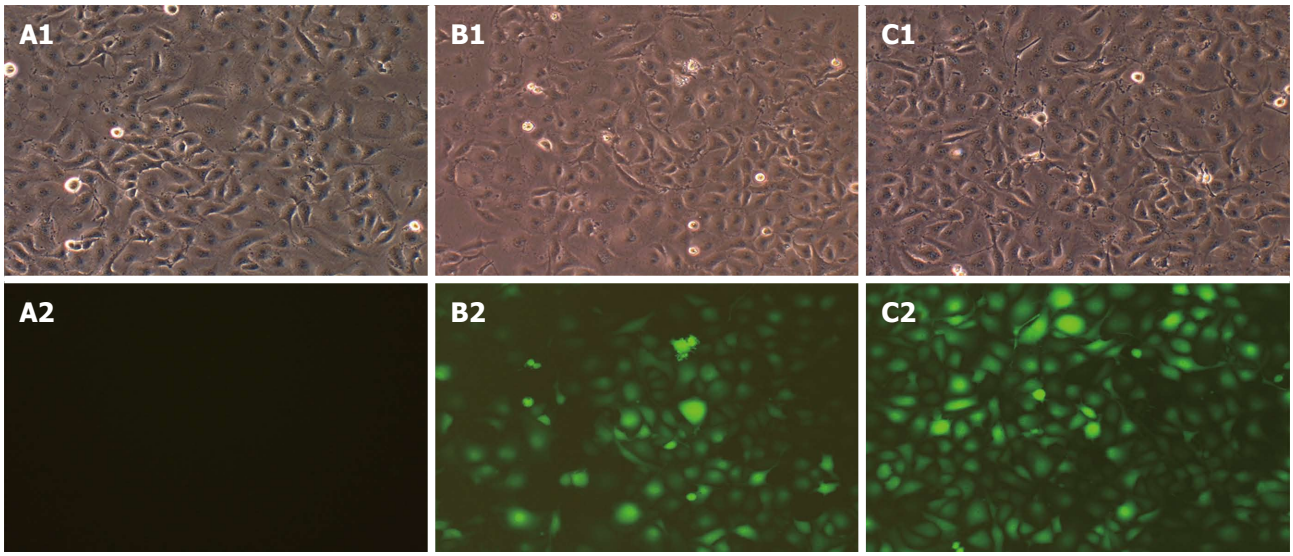

Figure 2 Determination of lentivirus titers after infection of tumor endothelial cells. The morphology of cells in the three groups under bright field and green fluorescence field (A1: CON 100 × B; B1: NC 100 × B; C1: MD 100 × B; A2: CON 100 × G; B2: NC 100 × G; C2: MD 100 × G). B and G indicate the bright field and the green fluorescence field, respectively. MD: Micro-down; NC: Negative control; CON: Control.

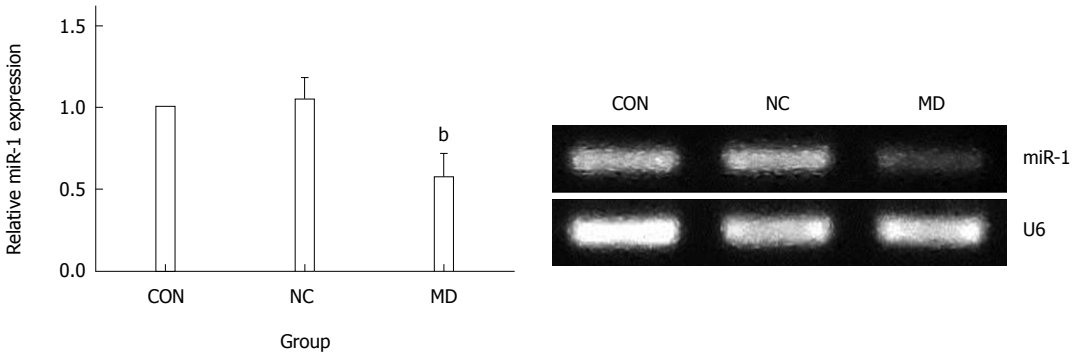

Figure 3 MiR-1 expression in cells of the three groups. The expression of miR-1 was significantly decreased in the MD group compared with the NC and CON groups ($P < 0.01$). There was no significant difference between the NC group and CON group ($P > 0.05$). $^{b}P < 0.01$ *vs* CON and NC group. MD: Micro-down; NC: Negative control; CON: Control.

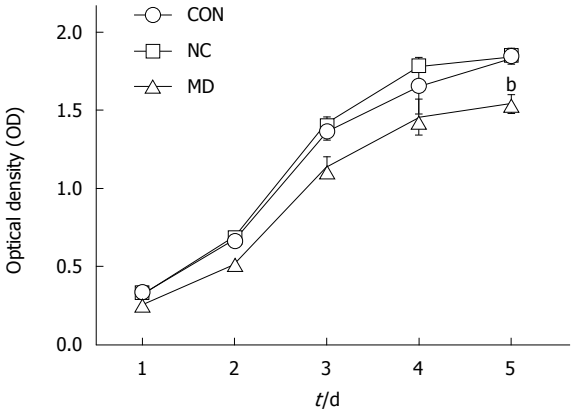

Figure 4 Detection of tumor endothelial cell proliferation by MTT assay. Cell proliferation was significantly inhibited in the MD group compared with the CON and NC groups ($P < 0.01$), while there was no significant difference between the CON and NC groups ($P > 0.05$). $^{b}P < 0.01$ *vs* CON and NC groups.

OD490 of days 1-5 was 0.258 ± 0.005, 0.518 ± 0.029, 1.140 ± 0.054, 1.457 ± 0.106, and 1.543 ± 0.047) compared with the CON group (the OD490 of days 1-5 was 0.328 ± 0.005, 0.672 ± 0.008, 1.362 ± 0.044, 1.653 ± 0.173, and 1.829 ± 0.031) and the NC group (the OD490 of days 1-5 was 0.322 ± 0.011, 0.695 ± 0.007, 1.419 ± 0.031, 1.778 ± 0.037, and 1.843 ± 0.039) ($P < 0.01$), whereas cell proliferation in the CON and NC groups was not affected ($P > 0.05$).

Flow cytometric detection of apoptosis in each group after transduction

Five days after transduction, the confluence of each group of cells was approximately 90%. As observed in Figure 5, there was no significant difference between the CON group (2.03% ± 0.30%) and the NC group (2.18% ± 0.15%) ($P > 0.05$), whereas the MD group

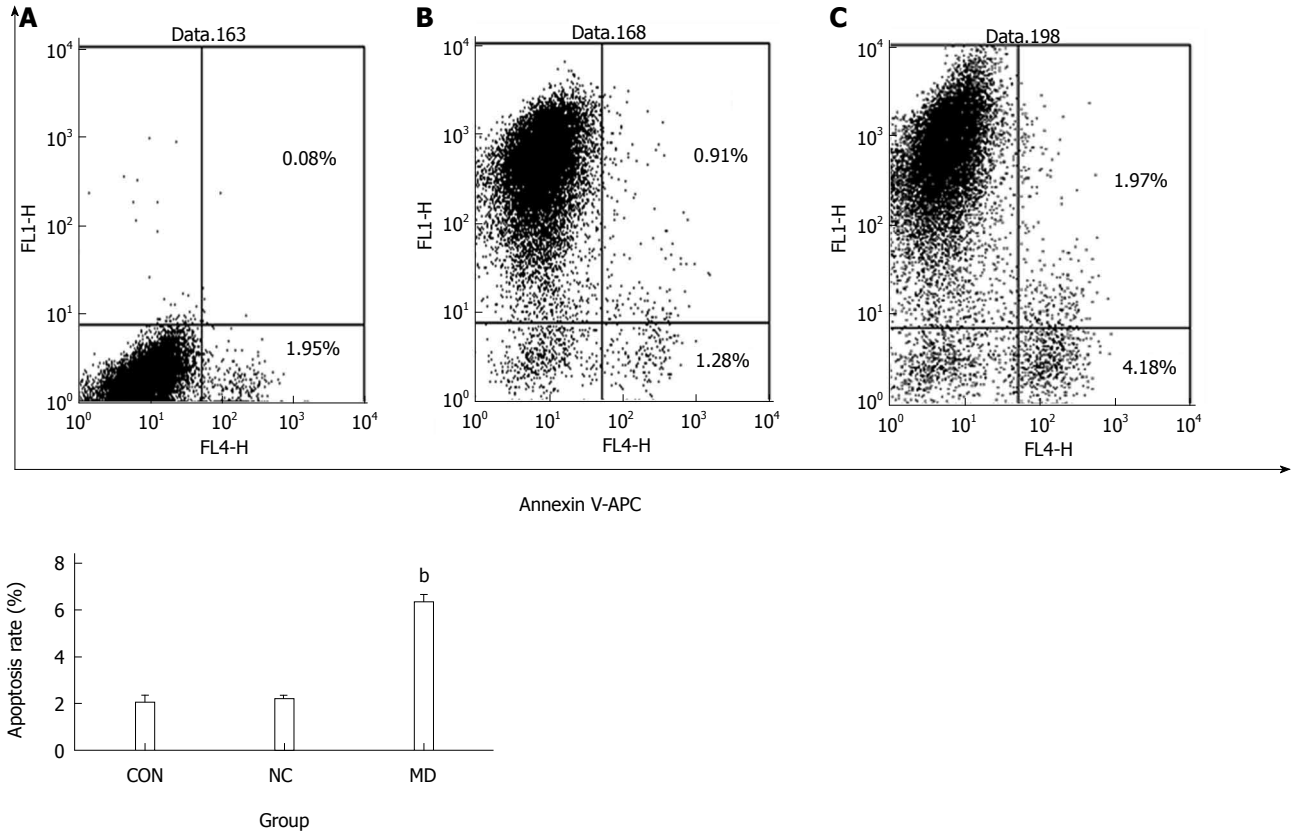

Figure 5 Flow cytometric detection of apoptosis in each group after transduction. A: Flow cytometry results of the CON group; B: Flow cytometry results of the NC group; C: Flow cytometry results of the MD group. Cells staining positive for Annexin V-APC were considered apoptotic (the right upper quadrant and right lower quadrant). $^{b}P < 0.01$ *vs* CON and NC groups. MD: Micro-down; NC: Negative control; CON: Control.

(6.32% ± 0.33%) showed significant apoptosis compared with the other groups ($P < 0.01$).

Transwell migration assay in each group after transduction

As shown in Figure 6, the number of cells that migrated to the lower portion of the chamber in the CON, NC group, and MD groups was 99.8 ± 3.11, 97.2 ± 3.70, and 62.0 ± 5.48, respectively. The results showed that the migration of TECs was significantly inhibited in the MD group compared with the CON and NC groups ($P < 0.01$). There was no significant difference between the CON group and the NC group ($P > 0.05$).

Transwell invasion assay in each group after transduction

As observed in Figure 7, the number of the cells that invaded the lower portion of the chamber in the CON, NC, and MD groups was 44.6 ± 3.36, 44.4 ± 5.17, and 29.8 ± 2.39, respectively. The results showed that the invasion of TECs was significantly inhibited in the MD group compared with the CON and NC group ($P < 0.01$). There was no significant difference between the CON group and the NC group ($P > 0.05$) (Figure 7).

DISCUSSION

HCC is one of the most common malignant tumors of the digestive system. More and more studies demonstrated that angiogenesis can influence tumor genesis and growth[21,22]. Therefore, if we can selectively inhibit or directly kill the TECs of HCC, this may provide a more effective and less toxic therapeutic method for HCC patients. Thus, the identification of specific markers for HCC TECs and the investigation of specific targeted therapies against TECs have become new hot spots in the field of tumor therapy[23]. MiRNAs are involved in the regulation of diverse cellular processes, such as proliferation, differentiation, cellular migration and apoptosis[24-27]. MiR-1 has initially been described as a regulator of myogenesis[28]. Subsequently, it has been found that miR-1 is dysregulated in many diseases especially in tumors. In this study, we explored the effect of suppressing the expression of miRNA-1 on biological behavior of TECs of HCC and the following major findings were obtained from our study: suppressing the expression of miRNA-1 could inhibit proliferation, promote apoptosis and inhibit migration and invasion of TECs of HCC.

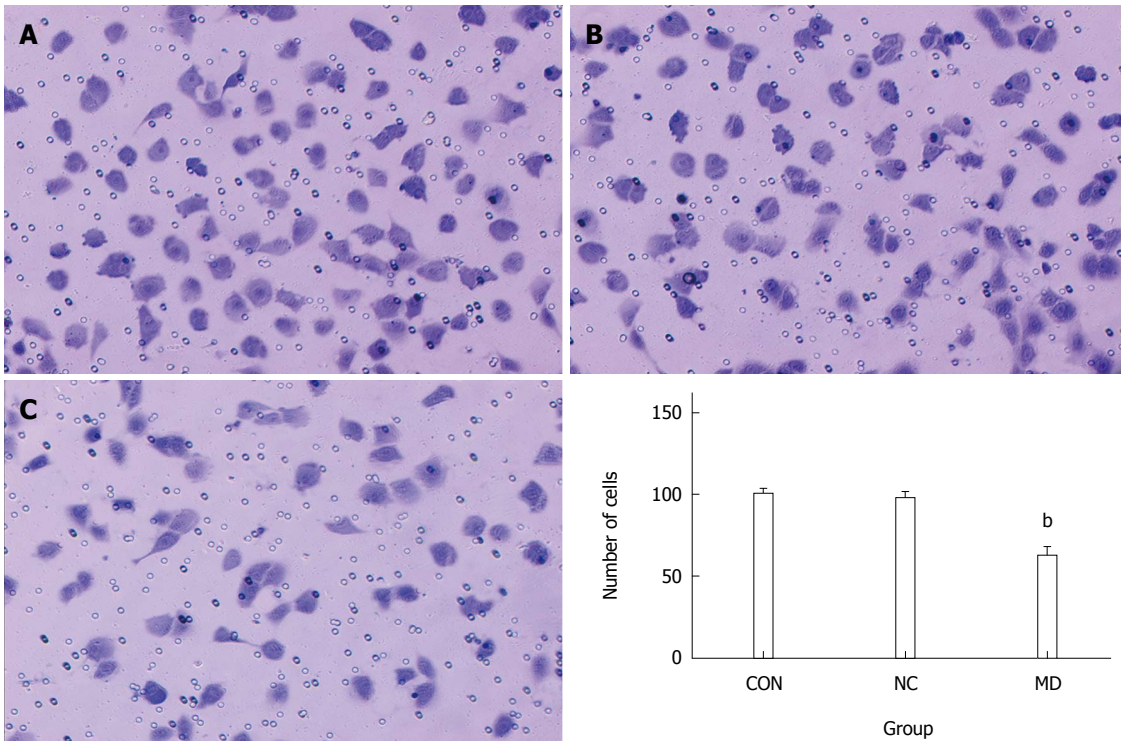

Figure 6 Transwell migration assay. The number of cells migrated to the lower portion of the chamber was significantly decreased in the MD group compared with the CON and NC groups ($P < 0.01$). There was no significant difference between the CON and NC groups ($P > 0.05$). $^{b}P < 0.01$ *vs* CON and NC groups. A: CON group; B: NC group; C: MD group. MD: Micro-down; NC: Negative control; CON: Control.

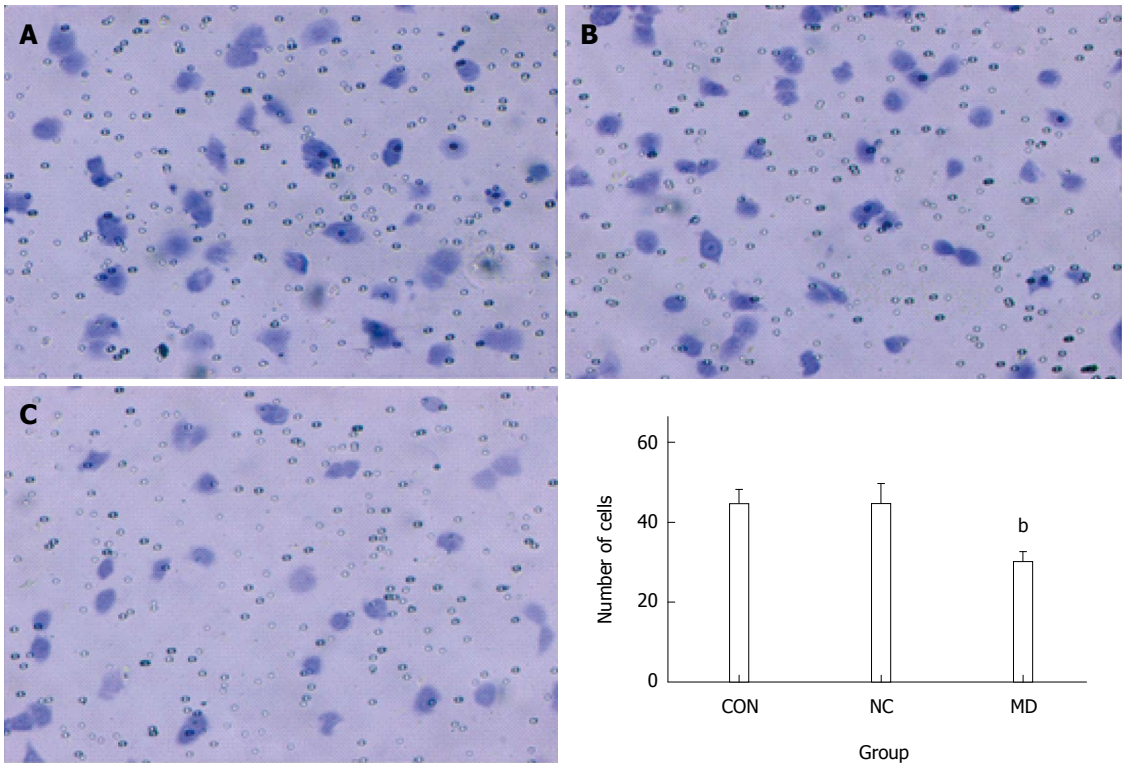

Figure 7 Transwell invasion assay. The number of cells invading to the lower portion of the chamber was significantly decreased in the MD group compared with the CON and NC groups ($P < 0.01$). There was no significant difference between the CON and NC groups ($P > 0.05$), $^{b}P < 0.01$ *vs* CON and NC groups. A: CON group; B: NC group; C: MD group. MD: Micro-down; NC: Negative control; CON: Control.

RNA interference (RNAi) is a posttranscriptional gene silencing mechanism that has emerged as a powerful method for silencing gene expression[29]. However, its two greatest disadvantages, inefficient delivery and transient effects, prohibit its application in chronic degenerative diseases. Lentivirus can easily integrate into the host genome and stably encode shRNA to overcome these drawbacks[30]. As a result, miR-1 shRNA was explored as a means to silence endogenous miR-1 expression in the present study. The transduction rate was almost 90%.

It is well known that tumorigenesis is due to an imbalance between cell proliferation and apoptosis[31]. Therefore, in the present study, we observed the effect of miR-1 on the proliferation and apoptosis of TECs. The results of MTT assay indicated that inhibition of the expression of miR-1 could significantly decrease TEC proliferation, and the difference was the most significant on day 5. Therefore, based on the initial MTT assay results, day 5 presented the most significant proliferation inhibition and was used as the time point for apoptosis detection. The results of apoptosis detection also showed that inhibition of the expression of miR-1 could significantly induce apoptosis in TECs. The death-inducing signaling complex pathway and the mitochondrial pathway are the two major pathways of cellular apoptosis that have been identified[32]. The mechanism of regulation of miR-1 for apoptosis in TECs was not identified in this study and we will conduct further research to reveal the potential mechanism.

Tumor invasion and metastasis are important causes of morbidity and death for liver cancer patients. Blood vessels play an important role in the invasion and metastasis of HCC. Our experiments concerning *in vitro* migration and invasion showed that inhibition of the expression of miR-1 could significantly inhibit the migration and invasion of TECs of human HCC. However, Weiwei *et al*[33] found that overexpression of miR-1 could inhibit the invasion and migration of HepG2 cells. Therefore, to investigate the effect of miR-1 on the invasion and metastasis of HCC, *in vivo* experiments will need to be performed in our further research.

In summary, our study demonstrated that miR-1 might be a potential tumor activator. Inhibition of the expression of miR-1 could decrease proliferation, induce apoptosis, and inhibit migration and invasion of TECs of human HCC. The mechanism of the effect described above still requires further research. We have found some potential target genes of miR-1 such as gap junction protein (*GJA1*), tankyrase (*TNKS2*), monocyte to macrophage differentiation-associated 2 (*MMD2*) using the software TargetScan and we will use methods such as luciferase activity assay and Northern blot to confirm these genes. In addition, we will also perform *in vivo* experiments to observe the effect of miR-1 on the growth, invasion and metastasis of HCC.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is a solid cancer with a rich blood supply. Angiogenesis is crucial in the occurrence, development and prognosis of this cancer. Therefore, if we can selectively inhibit or directly kill the tumor endothelial cells (TECs) of HCC, it may provide a more effective and less toxic therapeutic method for HCC patients. MiR-1 is dysregulated in many diseases especially in tumor. Therefore, it is meaningful to research the effect of miR-1 to TECs of HCC.

Research frontiers

Current studies on microRNAs (miRNAs) are mostly focused on tumor cells. Thus far, few studies have described the miRNAs in TECs of human HCC. The underlying mechanism was also investigated to provide new targets and a theoretical basis for the anti-angiogenic gene therapy of HCC.

Innovations and breakthroughs

Current studies on miRNAs are mostly focused on tumor cells. This study for the first time focused on the role of TECs in HCC and explored the effect of miR-1on biological behavior of TECs of HCC.

Applications

MiR-1 can be used as a new target for gene therapy and can provide effective and specific reference indicators for HCC anti-angiogenic therapy and prognosis evaluation.

Terminology

MiRNAs are non-coding, small RNAs that regulate gene expression at the posttranscriptional level. RNA interference is a posttranscriptional gene silencing mechanism that has emerged as a powerful method for silencing gene expression.

Peer-review

This study described the effect of suppression of miRNA-1 on several biological functions, aiming its future use as a potential tumor suppressor. The results of this study are very interesting and are promising.

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ORIGINAL ARTICLE

Retrospective Cohort Study

Validation of aspartate aminotransferase to platelet ratio for diagnosis of liver fibrosis and prediction of postoperative prognosis in infants with biliary atresia

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Data sharing: Technical appendix, statistical code, and dataset are available from the corresponding author at xiangliuhaiying@aliyun.com. Participants gave informed consent for data sharing.

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Abstract

AIM: To validate the value of aspartate aminotransferase to platelet ratio index (APRI) in assessment of liver fibrosis and prediction of postoperative prognosis of biliary atresia (BA) infants from Mainland China.

METHODS: Medical records of 153 BA infants who were hospitalized from January 2010 to June 2013 were reviewed. The efficacy of APRI for diagnosis of liver fibrosis was assessed using the receiver operator characteristic (ROC) curve compared to the

pathological Metavir fibrosis score of the liver wedge specimens of 91 BA infants. The prognostic value of preoperative APRI for jaundice persistence, liver injury, and occurrence of cholangitis within 6 mo after KP was studied based on the follow-up data of 48 BA infants.

RESULTS: APRI was significantly correlated with Metavir scores (r_s = 0.433; P < 0.05). The mean APRI value was 0.76 in no/mild fibrosis group (Metavir score F0-F1), 1.29 in significant fibrosis group (F2-F3), and 2.51 in cirrhosis group (F4) (P < 0.001). The area under the ROC curve (AUC) of APRI for diagnosing significant fibrosis and cirrhosis was 0.75 (P < 0.001) and 0.81 (P = 0.001), respectively. The APRI cut-off of 0.95 was 60.6% sensitive and 76.0% specific for significant fibrosis diagnosis, and a threshold of 1.66 was 70.6% sensitive and 82.7% specific for cirrhosis. The preoperative APRI in infants who maintained jaundice around 6 mo after KP was higher than that in those who did not (1.86 ± 2.13 *vs* 0.87 ± 0.48, P < 0.05). The AUC of APRI for prediction of postoperative jaundice occurrence was 0.67. A cut-off value of 0.60 showed a sensitivity of 66.7% and a specificity of 83.3% for the prediction of jaundice persistence. Preoperative APRI had no significant association with later liver injury or occurrence of cholangitis.

CONCLUSION: Our study demonstrated that APRI could diagnose significant liver fibrosis, especially cirrhosis in BA infants, and the elevated preoperative APRI predicts jaundice persistence after KP.

Key words: Aspartate aminotransferase to platelet ratio index; Biliary atresia; Cirrhosis; Liver fibrosis; Prognosis

Core tip: There is still controversy over the role of aspartate aminotransferase to platelet ratio index (APRI) in the diagnosis of liver fibrosis and prognosis in biliary atresia. In this paper, we confirmed that APRI could help diagnose significant liver fibrosis, especially cirrhosis in a cohort of infants with biliary atresia from Mainland China, and preoperative APRI also could predict jaundice persistence after Kasai portoenterostomy. These results may have implications in the management of this disease.

Yang LY, Fu J, Peng XF, Pang SY, Gao KK, Chen ZR, He LJ, Wen Z, Wang H, Li L, Wang FH, Yu JK, Xu Y, Gong ST, Xia HM, Liu HY. Validation of aspartate aminotransferase to platelet ratio for diagnosis of liver fibrosis and prediction of postoperative prognosis in infants with biliary atresia. *World J Gastroenterol* 2015; 21(19): 5893-5900 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5893.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5893

INTRODUCTION

Biliary atresia (BA) is a severe, cholestatic disease of infancy of unknown cause characterized by fibrous obliteration of inter- and extra-hepatic biliary tree. In untreated cases, it would cause death by 2 years of age. It occurred with an incidence of 1 in 5000 to 1 in 19000 live births, and was more common in Asian countries[1-3]. Etiology and molecular networks underpinning such an expeditious fibrogenic process have not been well established. Immune and nonimmune factors were implicated in the pathogenesis of BA, and the resultant cholestasis and oxidative stress appeared to be the main triggers of hepatic fibrosis in this disease[4,5]. Early Kasai portoenterostomy (KP) is the only and palliative therapy with the hope of restoring bile flow to the gastrointestinal tract to alleviate injury of the liver caused by cholestasis and to prolong the survival with the native liver, but it cannot stop the progress of inflammation around bile ducts and hepatic fibrosis. Nearly one third of cases may develop persistent jaundice, recurrent cholangitis and other symptoms a short term post-operation and had worse outcome. Variable liver fibrosis, cirrhosis, and liver failure may still happen[6-8]. When KP had failed and severe complications occurred, liver transplantation is the only effective treatment. Hepatic fibrosis is not only a universal and prominent feature of BA, but also the most important predictor of outcome post KP. Therefore, assessment of hepatic fibrosis is critical for the management after KP[9-12].

In BA, assessment of liver fibrosis had traditionally been accomplished by subjective visual analysis of trichrome staining of liver tissue obtained at the time of KP or liver transplantation[13,14]. It cannot meet the needs of early and continual liver status assessment in BA patients, though pathological diagnosis is believed as the gold standard for determining the degree of liver fibrosis[15]. Less invasive techniques developed in adults had been applied in pediatric patients with chronic liver diseases[13,16,17]. Among the serological indicators, aspartate aminotransferase to platelet ratio index (APRI) is the simplest model based on aspartate transaminase (AST) and platelet count from routine hematological and biochemical tests and had obtained attention in BA patients during the last several years[18-21]. However, controversial results have been released when assessing the value of APRI in liver fibrosis diagnosis in BA children and post KP outcome prediction. Regarding that the biased results might be caused by small sample size in previous studies, we validated the value of APRI in assessment of liver

fibrosis and prediction of postoperative prognosis of BA infants from Mainland China.

MATERIALS AND METHODS

Case enrollment

The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of Guangzhou Women and Children's Medical Center in Guangzhou, China. Enrollment was proposed to all consecutive infants with diagnosed BA confirmed by cholangiogram at abdomen surgical or laparoscopic biliary duct exploration, who admitted to our center between January 2010 and June 2013. Basic information including sex, age at operation, and routine biochemical and hematological parameters were collected. Follow-up data which could evaluate persistence of jaundice (total bilirubin > 34 μmol/L), acute liver injury (ALT > 35 U/L), and occurrence of cholangitis within 6 mo after KP were collected for analysis of short-term outcomes.

Patients with incomplete data, or younger than 15 days, or diagnosed with co-occurring heart disease were excluded, because their platelet counts and AST levels might be influenced.

Calculation of APRI

APRI was calculated using the formula of Wai *et al*[18]: [AST/upper limit of normal (ULN)]/platelet count (expressed as platelets × 10^9/L) × 100. AST and platelets tested within 1 wk before the operation were used in the analysis. Results closest to the date of operation were chosen if more than one set of laboratory data was available.

The ULN of AST was verified before calculation. AST levels of 150 age- and sex-matched healthy infants were tested according to document C28-A3 of the Clinical and Laboratory Standards Institute (CLSI)[22]. Their AST values showed normal distribution with 95% confidence interval (CI) of 28-60 U/L. Therefore, 60 U/L was used as the ULN of AST for APRI calculation in this study.

Liver histology and quantification of liver fibrosis

Liver sections of the enrolled patients were retrieved from sample bank in department of pathology and reviewed blindly by two experienced pathologists. Liver wedge tissue samples were obtained during surgical exploration or KP. Biopsy specimens were fixed in 10% neutral formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE), and Masson's trichrome. The Metavir scoring system was applied to quantify liver fibrosis based on the architectural features of portal fibrosis, of which a 0-4 scale was staged as follows: F0 = no fibrosis, F1 = portal fibrosis without septa, F2 = portal fibrosis with rare septa, F3 = numerous septa and bridging fibrosis without cirrhosis, and F4 = cirrhosis[23]. Scoring F0-F1 was considered as no/mild fibrosis, F2-F3 as significant fibrosis, and F4 as cirrhosis.

Statistical analysis

Analyses were performed using IBM SSPS 19.0. Quantitative data are shown as mean ± SD unless otherwise stated. Continuous variables were compared using the Student's *t*-test or ANOVA analysis. Correlation was evaluated by the Spearman correlation coefficient. The diagnostic efficacy of APRI was assessed using the receiver operator characteristic (ROC) curve. All of the possible cut-off values were associated with the probability of a true positive (sensitivity) and a true negative (specificity). The best cut-off point was defined as the highest value of sensitivity plus specificity. Values of the area under the ROC curves (AUC) near 1.0 indicated high diagnostic accuracy. *P* values less than 0.05 were regarded as statistically significant. The statistical methods of this study were reviewed by Jing Gu from the Department of Medical Statistics and Epidemiology, School of Public Health, Sun Yat-sen University.

RESULTS

One hundred and fifty-three BA infants (92 boys and 61 girls) were diagnosed at an average age of 85 ± 38 d (range: 1-241 d). Among them, 45.8% (70/153) underwent KP with a mean age of 73 ± 23 d at operation. After the exclusion criteria were considered, 91 and 48 BA infants who fulfilled the requirements of the clinical and laboratory data were entered into the study of APRI in liver fibrosis and postoperative prognosis, respectively.

Correlation between APRI and severity of fibrosis

Ninety-one infants, 55 boys and 36 girls, with a median age at surgery of 83 ± 36 d (range: 19-224 d), were enrolled for analysis of correlation between APRI and liver fibrosis severity. Metavir scores were F0 in 2, F1 in 23, F2 in 35, F3 in 21, and F4 in 10 patients. Clinical characteristics of the patients are summarized in Table 1. APRI in BA patients correlated with Metavir scores (r_s = 0.433; $P < 0.05$), as well as original AST (r_s = 0.266; $P < 0.05$) and PLT (-0.346; $P < 0.05$). Additionally, the age at the time of surgery and total bile acids (the cholestatic parameter) showed a significant positive correlation with the degree of fibrosis (r_s = 0.289, 0.332, respectively; $P < 0.01$ for each). Comparison of clinical characteristics between these individuals showed a statistical significance for APRI by Metavir stage ($P < 0.001$); the median APRI in F4 was significantly different from any stage of F0-F3 with followed the least significant difference post-hoc test (P = 0.000-0.007) but no statistical difference in the stages of F0 to F3 (P = 0.050-0.703). When categorizing F0-F4 into groups of no/mild fibrosis (F0-F1), significant fibrosis (F2-F3) and cirrhosis

Table 1 General characteristic of infants with biliary atresia by Metavir stage *n* (%)

Parameter, mean ± SD	All	Metavir stage					
	(*n* = 91)	F0 (*n* = 2)	F1 (*n* = 23)	F2 (*n* = 35)	F3 (*n* = 21)	F4 (*n* = 10)	*P* value
Age (d)	83 (36)	45 (37)	70 (37)	84 (22)	85 (34)	110 (62)	0.027
AST (U/L)	285 (182)	233 (259)	221 (114)	310 (230)	307 (169)	306 (125)	NS
ALT (U/L)	179 (105)	127 (152)	175 (128)	197 (104)	152 (78)	198 (94)	NS
ALP (U/L)	541 (221)	366 (235)	511 (207)	569 (220)	518 (242)	597 (225)	NS
γ-GT (U/L)	895 (685)	892 (841)	751 (632)	1009 (810)	972 (610)	662 (404)	NS
TP (g/L)	58.3 (4.8)	57.6 (13.9)	57.9 (5.7)	58.8 (4.2)	58.7 (4.0)	56.5 (4.5)	NS
ALB (g/L)	40.0 (4.7)	41.4 (3.9)	40.0 (3.8)	40.6 (5.4)	40.0 (4.2)	38.2 (5.3)	NS
TBIL (μmol/L)	202.8 (75.8)	313.5 (44.6)	192.5 (71.9)	193.5 (60)	222.5 (100.6)	195.4 (65.6)	NS
DBIL (μmol/L)	147.6 (55.2)	133.6 (92.6)	138.9 (52.9)	144.2 (41.3)	170.3 (75.6)	134.9 (43.1)	NS
TBA (μmol/L)	166.7 (68.5)	71.9 (36.9)	133.0 (53.8)	175.2 (65.6)	194.9 (64.0)	177.2 (84.5)	0.007
PLT (× 10^9/L)	447 (162)	713 (127)	498 (126)	454 (151)	435 (155)	279 (165)	0.000
INR	1.03 (0.26)	1.69 (1.06)	1.01 (0.13)	1.00 (0.11)	1.00 (0.12)	1.18 (0.59)	0.001
AST/ALT	1.84 (1.13)	2.14 (0.52)	1.84 (1.80)	1.64 (0.65)	2.21 (1.06)	1.66 (0.52)	NS
APRI	1.28 (1.05)	0.50 (0.52)	0.79 (0.45)	1.26 (0.93)	1.35 (0.93)	2.51 (1.68)	0.000

SD: Standard deviation; NS: Not significant; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; γ-GT: Gamma-glutamyl transpeptidase; TP: Total protein; ALB: Albumin; TBIL: Total bilirubin; DBIL: Direct bilirubin; TBA: Total bile acids; INR: International normalized ratio; APRI: Aspartate aminotransferase to platelet ratio index. Reference intervals: AST (5-60 U/L), ALT (3-35 U/L), ALP (118-390 U/L), γ-GT (13-57 U/L), TP (60-80 g/L), ALB (35-50 g/L), TBIL (2-17 μmol/L), DBIL (0-7 μmol/L), TBA (0-15 μmol/L), PLT (140-440 × 10^9/L), INR (0.8-1.5).

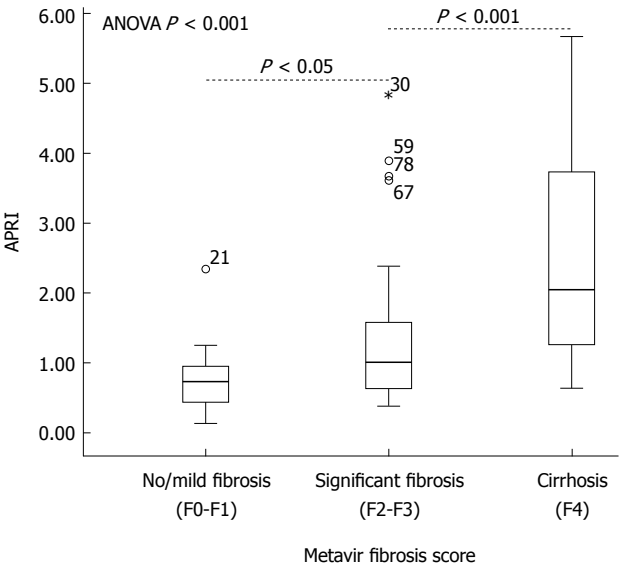

Figure 1 Aminotransferase to platelet ratio index by Metavir score of liver fibrosis in infants with biliary atresia. Round dots represent values larger than the upper quartile plus 1.5 times the interquartile range. Asterisks represent values larger than the upper quartile plus 3.0 times the interquartile range. Metavir fibrosis scores of F0-F4 were categorized into groups of no/mild fibrosis (F0-F1, *n* = 25), significant fibrosis (F2-F3, *n* = 56) and cirrhosis (F4, *n* = 10). The APRI in the three groups significantly differed from each other by ANOVA analysis (*P* < 0.001).

(F4) by the degree of fibrosis, no/mild fibrosis was present in 27.5% of the biopsies, significant fibrosis in 61.5% and cirrhosis in 11.0%. The mean APRI values in groups of no/mild fibrosis, significant fibrosis, and cirrhosis were 0.76, 1.29, and 2.51, respectively (ANOVA *P* < 0.001; Figure 1). The APRI in the three groups significantly differed from each other and increased with the progression of liver fibrosis.

Based on two different thresholds of significant fibrosis (F2-F3) and cirrhosis (F4), ROC curves of APRI for the 91 subjects were constructed as F0-F1 *vs* F2-F4, and F0-F3 *vs* F4 (Figure 2). The area under the ROC curve (AUC) of APRI for diagnosing significant fibrosis (F2-F3) and cirrhosis (F4) were 0.75 (*P* < 0.001) and 0.81 (*P* = 0.001), respectively. For significant fibrosis, an APRI cut-off of 0.95 was 60.6% sensitive and 76.0% specific. A threshold of 1.66 was 70.6% sensitive and 82.7% specific for cirrhosis. The accuracies of APRI for diagnosing significant fibrosis and cirrhosis in the BA infants were 64.8% and 82.4%, respectively.

Prediction of postoperative outcomes

Forty-eight BA infants who had followed-up record were included in the prediction of postoperative outcomes. Twenty-four of them (50%) showed persistent jaundice 6 mo after KP and had higher preoperative APRI than the jaundice-free infants (1.86 ± 2.13 *vs* 0.87 ± 0.48, *P* < 0.05). The marginal cut-off value for preoperative APRI was 0.60 (AUC = 0.67; *P* < 0.05) in predicting persistence of jaundice, with a sensitivity of 66.7% and a specificity of 83.3%. The APRI value significantly predicted the persistence of jaundice after operation with an odds ratio of 7.0 (95%CI: 1.6-29.9) and accuracy of 68.9%. Clearance of jaundice was not achieved in any of the children with APRI > 2.0. 18/48 (37.5%) who developed cholangitis within 6 mo after KP, showed no difference in preoperative APRI from those did not (1.33 ± 1.20 *vs* 1.39 ± 1.84, *P* > 0.05). This suggested that there was no association between preoperative APRI and the development of postoperative cholangitis.

With a cut off value of APRI = 0.95, 24 (50%) patients were in the group of significant fibrosis and the others in non-significant fibrosis group. The significant fibrosis group had higher AST and ALT levels

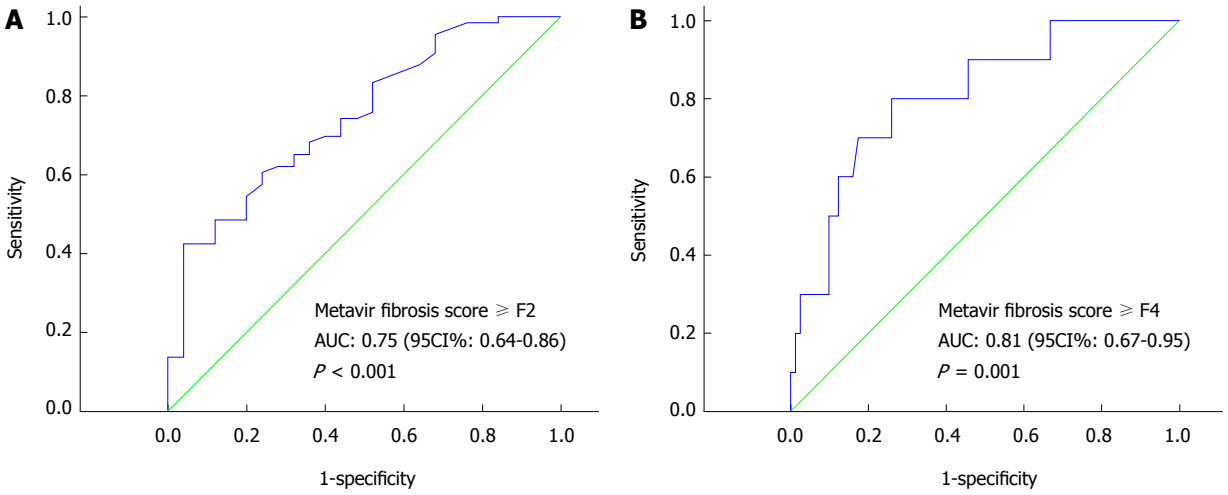

Figure 2 Receiver-operating characteristics curve for aminotransferase to platelet ratio index in the diagnosis of significant liver fibrosis or cirrhosis in biliary atresia. A: Receiver-operating characteristics (ROC) curve of F0-F1 *vs* F2-F4. The area under the ROC curve (AUC) of APRI for diagnosing significant fibrosis (⩾ F2) was 0.75 (95%CI: 0.64-0.86, $P < 0.001$); B: ROC curve of F0-F3 *vs* F4. The area under the ROC curve (AUC) of APRI for diagnosing liver cirrhosis (= F4) was 0.81 (95%CI: 0.67-0.96, $P = 0.001$).

Table 2 Comparison of liver injury parameters in biliary atresia patients according to aminotransferase to platelet ratio index cut-off value *n* (%)

Parameter	APRI for significant fibrosis (cut-off = 0.95)				APRI for cirrhosis (cut-off = 1.66)			
	At KP		6 mo after KP		At KP		6 mo after KP	
	⩽ 0.95 (*n* = 24)	> 0.95 (*n* = 24)	⩽ 0.95 (*n* = 24)	> 0.95 (*n* = 24)	⩽ 1.66 (*n* = 38)	> 1.66 (*n* = 10)	⩽ 1.66 (*n* = 38)	> 1.66 (*n* = 10)
AST (U/L)	194 (83)	496 (721)[1]	210 (188)	149 (93)	234 (92)	785 (1077)[2]	183 (159)	165 (102)
ALT (U/L)	136 (73)	293 (327)[1]	163 (128)	126 (96)	164 (100)	407 (474)[2]	139 (112)	168 (121)

All data are shown as mean (SD). [1]Compared with non-significant fibrosis group, $P < 0.05$; but it decreased to the same levels as non-significant fibrosis group. There were no obvious changes of AST and ALT in non-significant fibrosis group before and after KP. [2]Compared with non-cirrhosis group, $P < 0.01$; but it decreased to the same levels as non-cirrhosis group. There also were no obvious changes of AST and ALT in non-cirrhosis group before and after KP. APRI: Aminotransferase to platelet ratio index.

(parameters of liver injury) at the time of KP than the non-significant fibrosis group ($P < 0.05$ for each, Table 2). Six months after KP, these parameters decreased to the same levels as the non-significant fibrosis group. There was no obvious change of AST and ALT in non-significant fibrosis group before and after KP. With a cut off value of APRI = 1.66, 10 (26.3%) patients were in the cirrhosis group. At the time of KP, infants in the cirrhosis group also had much higher AST and ALT than those without ($P < 0.01$ for each). But six months after KP, these two parameters of those infants decreased to the same as in the non-cirrhosis group, and that before surgery. The change of AST and ALT before and after KP for all patients had no significant correlation with APRI ($P > 0.05$ for each).

DISCUSSION

APRI is an indirect biochemical marker of hepatic fibrosis, based on limited expense and widespread available laboratory parameters, reflecting alterations in hepatic function. It was developed by Wai *et al*[18] in 2003 when the correlation of serum AST and platelet counts with fibrosis was found in adult patients with chronic hepatitis C. It has been introduced to detect fibrosis and cirrhosis in adult patients with other liver diseases with promising results[24,25]. There are little data available on the use of APRI in pediatric patients mainly with chronic hepatitis (B or C), NAFLD, and intestinal failure[22,26,27]. Until 2007, association of APRI with BA was first described, and it was first reported to significantly correlate with fibrosis stages and have a significant AUC for the diagnosis of cirrhosis of 0.73 on biopsies from 33 children with a group of chronic liver diseases including nine presenting with BA[20]. Then, in a series of 35 BA children, Kim *et al*[19] observed a significant correlation between APRI and the degree of hepatic fibrosis ($r = 0.77$), and AUC of 0.92 and 0.91 for the determination of Metavir ⩾ F3 and F4, respectively. However, it could not be proved when Lind *et al*[21] studied 31 BA patients. Nonetheless, the latest study showed that APRI at a cut-off of 1.22 (AUC = 0.83) could predict macroscopic cirrhosis with a sensitivity of 75% and a specificity of 84%[28], when macroscopic assessment of fibrosis were done in a larger population involving 260 BA infants.

Our study is the first to validate the performance of APRI in assessing liver fibrosis with a cohort of BA infants from Mainland China, a popular area with high morbidity for this disease[5,12]. Our findings validated that APRI could diagnose significant fibrosis and cirrhosis but with less accuracy than initially described for BA patients. In our study, APRI significantly correlated with Metavir stages in BA infants and increased with the progression of fibrosis, similar to that previously reported in pediatric liver diseases including BA[20]. We also observed that APRI was not sensitive enough to differentiate Metavir stages except cirrhosis and indicated the presence of mild fibrosis as Lind *et al*[21] reported. This was possibly related to the basis of APRI on the routine laboratory parameters which did not directly reflect fibrogenesis but rather the current state of hepatic damage due to fibrosis or cirrhosis. In spite of this, APRI could identify significant fibrosis and cirrhosis at AUC of 0.75 and 0.81, respectively, in the present study, which showed similar performance for staging hepatitis C-related fibrosis in a recent meta-analysis by Lin *et al*[29]. The cut-offs and diagnostic efficacy including sensitivity, specificity and accuracy were different from other studies about BA patients, which could be explained due to the difference in the number of patients enrolled and AST ULN used for calculation of APRI. Thereby, our study showed that APRI could be used as a non-invasive alternative index for assessing the severity of liver fibrosis in BA, especially for diagnosing significant fibrosis and cirrhosis.

BA is a progressive disease consistent with the effects of on-going cholangitis, cholestasis and fibrosis. APRI has provided a 5-year prognostic value in patients with chronic hepatitis in a prospective study[30]. The good correlation of APRI with the presence of varices in elder children post KP had been found by several studies[19,31,32]. And this correlation with poorer outcomes after surgery had been brought forward to preoperative APRI but no agreed conclusions have been drawn[19,21]. In the latest study, the infants that achieved clearance of jaundice had significantly lower median APRI at the time of KP, and jaundice persisted in any child with an initial APRI of > 3.0 and required a liver transplant to survive[28]. The survival rate of the native liver in BA infants with low APRI was significantly improved compared to those with a high APRI. With a similar rate of jaundice clearance to the above-mentioned study, our study showed that preoperative APRI (> 0.60) could predict jaundice persistence with an odds ratio of 7.0, but not worsened liver injury or occurrence of cholangitis after KP. Jaundice clearance was not achieved in any child with an APRI > 2.0. Therefore, determination of APRI before KP may help clinicians by providing beneficial information in decision of therapy and management of BA after surgery.

The present study has the following advantages compared to previous studies. First, the association of APRI originated parameters AST and PLT with Metavir fibrosis scores was verified, which strongly provided important preconditions for feasibility of the index in assessing liver fibrosis in BA. The results dispelled the suspicions whether APRI could apply in such fibrosis that dramatically progresses to cirrhosis within a few weeks after birth and may represent another type of fibrosis distinguishing what is involved in other chronic liver diseases. Second, a verified and age-matched ULN of AST was used in the calculation of APRI. The aminotransferase ULN varying with age and methods is thought to be a major disadvantage of APRI[33]. Different ULN used in the subjects with same age were noticed in previous studies, which may be partly responsible for the difference in results. It is worth noting that using a uniform ULN may benefit the comparability between different studies and clinical application of APRI. One limitation of this study is that this is a retrospective study including patients from a single center. The efficacy of cut-off values remains to be further validated in clinical prospective and multicenter studies. Moreover, other important postoperative outcomes resulting from liver fibrosis, including EV/GV and portal hypertension, were not focused in a short-term follow-up in the present study. This may limit the clarification of APRI for postoperative prognosis, since initial APRI had little predictive value for future variceal formation reported recently and several studies described that it could predict EV/GV in elder BA children because of short follow-up time[29]. This could be considered in a prospective study.

In conclusion, APRI is effective in diagnosing significant liver fibrosis, especially cirrhosis, in BA infants. The elevated preoperative APRI also could predict jaundice persistence after KP. Since the method is convenient, inexpensive, and non-invasive, it will benefit the management of BA infants particularly in regions with limited healthcare resources.

COMMENTS

Background

Biliary atresia (BA) is the most common cholestatic liver disease which results in progressive sclerosing fibrous obliteration of the extra-hepatic bile ducts in early infancy. Kasai portoenterostomy (KP) is the standard surgical management for BA patients. Full evaluation of liver fibrosis and determination of the prognosis after KP may be beneficial to clinical practice. There still lacks an appropriate non-invasive marker.

Research frontiers

Aspartate aminotransferase to platelet ratio index (APRI) is the simplest serological model for evaluating liver fibrosis. It has been used to assess liver fibrosis and cirrhosis in adult patients and even children with liver-related diseases with promising results. It also has been found to be a predictor of poorer outcomes post KP. However, it is controversial that APRI could be valuable in diagnosing liver fibrosis and predicting postoperative prognosis after surgery for BA children.

Innovations and breakthroughs

This is the first study to investigate the diagnostic performance of APRI in assessing liver fibrosis and predicting postoperative prognosis in a larger cohort of infants with BA in Mainland China. The authors validated the association of

APRI-originated parameters (AST and platelet count) with the degree of liver fibrosis in BA, which provided important preconditions for the use of APRI in this disease. Moreover, a verified and age-matched upper limit of normal of AST was used for calculation of APRI. This study suggests that APRI is effective in diagnosing significant liver fibrosis, especially cirrhosis, in infants with BA and also has predictive value in determining persistence of jaundice after KP.

Applications

APRI may decrease the need for staging liver biopsy specimens in highly suspected BA infants or in monitoring of fibrosis. It can help clinicians to select proper therapeutic strategy in the management of BA infants.

Terminology

APRI is an indirect invasive parameter for assessing liver fibrosis. It does not reflect fibrogenesis directly but rather the current state of hepatic damage due to fibrosis or cirrhosis. It is calculated using the formula: (AST/upper limit of normal)/platelet count (expressed as platelets × 10^9/L) × 100.

Peer-review

The authors investigated the diagnostic performance of APRI in assessing liver fibrosis and predicting postoperative prognosis in a larger cohort of infants with BA in Mainland China. They found that it could identify significant fibrosis and cirrhosis in BA infants at the area under the receiver operator characteristic curve of 0.75 and 0.81, respectively, and preoperative APRI (> 0.60) could predict jaundice persistence but not worsened liver injury or occurrence of cholangitis after KP. The results are interesting and may help to make decisions in selection of therapeutic strategy and management of BA infants after KP.

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ORIGINAL ARTICLE

Retrospective Study

Prognostic value and clinical correlations of 18-fluorodeoxyglucose metabolism quantifiers in gastric cancer

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Informed consent: All study participants, or their legal guardian, provided informed written consent prior to study enrollment.
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Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at grabinska.kinga@gmail.com.

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Abstract

AIM: To investigate the correlations of pre-treatment positron emission tomography-computer tomography (PET-CT) metabolic quantifiers with clinical data of unstratified gastric cancer (GC) patients.

METHODS: Forty PET-CT scans utilising 18-fluorodeoxyglucose in patients who received no prior treatment were analysed. Analysis involved measurements of maximum and mean standardised uptake volumes (SUV), coefficient of variation (COV), metabolic tumour volumes and total lesion glycolysis of different thresholds above which the tumor volumes were identified. The threshold values were: SUV absolute value of 2.5, 30% of SUVmax, 40% of SUVmax, and liver uptake-based (marked 2.5, 30, 40 and liv, respectively). Clinical variables such as age, sex, clinical stage, performance index, weight loss, tumor histological type and grade, and CEA and CA19.9 levels were included in survival analysis. Patients received various treatment modalities appropriate to their disease stage and the outcome was defined by time to metastasis (TTM) and overall survival (OS). Clinical and metabolic parameters were evaluated by analysis of

variance, receiver operating characteristics, univariate Kaplan-Meier, and multivariate Cox models. $P < 0.05$ was considered statistically significant.

RESULTS: Significant differences were observed between initially disseminated and non-disseminated patients in mean SUV (6.05 *vs* 4.13, $P = 0.008$), $TLG_{2.5}$ (802 cm^3 *vs* 226 cm^3; $P = 0.031$), and TLG_{30} (436 cm^3 *vs* 247 cm^3, $P = 0.018$). Higher COV was associated with poor tumour differentiation (0.47 for G3 *vs* 0.28 for G1 and G2; $P = 0.03$). $MTV_{2.5}$ was positively correlated to patient weight loss (< 5%, 5%-10% and > 10%: 40.4 cm^3 *vs* 123.6 cm^3 *vs* 181.8 cm^3, respectively, $P = 0.003$). In multivariate Cox analysis, TLG_{30} was prognostic for OS (HR = 1.001, 95%CI: 1.0009-1.0017; $P = 0.047$) for the whole group of patients. In the same model yet only including patients without initial disease dissemination TLG_{30} (HR = 1.009, 95%CI: 1.003-1.014; $P = 0.004$) and $MTV_{2.5}$ (HR = 1.02, 95%CI: 1.002-1.036; $P = 0.025$) were prognostic for OS; for TTM TLG_{30} was the only significant prognostic variable (HR = 1.006, 95%CI: 1.001-1.012; $P = 0.02$).

CONCLUSION: PET-CT in GC may represent a valuable diagnostic and prognostic tool that requires further evaluation in highly standardised environments such as randomised clinical trials.

Key words: Stomach neoplasmas; Positron-emission tomography; ^{18}Fluorodeoxyglucose; Neoplasm staging; Distant metastasis

Core tip: This study is one of the first to investigate the potential use of such a variety of radiotracer quantifiers, demonstrating their ability to differentiate locally advanced and disseminated tumours. This broad analysis can be utilized in clinical use to identify groups of patients with an unfavourable tumour prognosis who could possibly benefit from more aggressive treatment. Our database is being continuously updated and we plan to validate our findings in a larger and more homogeneous cohort.

Grabinska K, Pelak M, Wydmanski J, Tukiendorf A, d'Amico A. Prognostic value and clinical correlations of 18-fluorodeoxyglucose metabolism quantifiers in gastric cancer. *World J Gastroenterol* 2015; 21(19): 5901-5909 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5901.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5901

INTRODUCTION

Gastric adenocarcinoma is the third leading cause of cancer death in both sexes worldwide (723000 deaths, 8.8% of the total). The highest estimated mortality rates are in Eastern Asia (24 per 100000 in men, 9.8 per 100000 in women), and the lowest in Northern America (2.8 and 1.5, respectively). High mortality rates are also present in both sexes in Central and Eastern Europe and in Central and South America. Gastric cancer exhibits a high mortality to incidence ratio, which indirectly demonstrates the low curability of gastric cancer, suggesting that there is still significant room for improvement in its diagnosis and therapy[1].

Unspecific symptoms construed as indigestion or peptic ulcer disease are often ignored and contribute to late detection. The diagnosis of gastric cancer largely relies on endoscopy, which has been proven effective both medically and epidemiologically. However, due to changing approach to treatment of gastric cancer, which varies between local, locoregional, and metastatic disease, endoscopy should be accompanied by imaging modalities that help fully identify disease stage. This additional imaging has traditionally been achieved through contrast-enhanced computer tomography of the abdomen[2,3]. Recent studies have demonstrated high specificity and sensitivity of fusion positron emission tomography with computed tomography and its superiority over former methods in gastric cancer in detection of distant metastatic sites. However, reports demonstrate both advantages and limitations[4-7].

Currently, there are a number of reports indicating high usability of positron emission tomography-computer tomography (PET-CT) in determining disease stage[8-11], identification of recurrence following surgery[12], response to chemotherapy[8,9], prognostic value of ^{18}Fluorodeoxyglucose (^{18}FDG) metabolism quantifiers, risk of nodal and distant spread of tumour[11] and correlation between ^{18}FDG uptake and tumour histopathology or grade[6].

Some of the limitations of PET-CT in gastric cancer as described by various studies include insufficient uptake in signet-ring histological subtype as well as in nodal and peritoneal metastasis. Insufficient uptake is also a problem for tumours less than 30 mm in diameter or those limited submucosa. The dependence of sensitivity on tumour infiltration level within the stomach wall has been reported for PET-CT. Reports have shown sensitivity of 44% in T1 and 92% in T2-T4 tumours.

However, false positive results with high tracer uptake are often observed in chronic mucosal inflammation[5]. Optimal study pre-conditioning (adequate filling of the gastric and dilating its wall) can increase imaging sensitivity[13,14].

Our study analysed the pattern of commonly measured and potentially clinically significant ^{18}FDG metabolism quantifiers in PET-CT fusion studies in a cohort of previously untreated patients with gastric cancer and its correlations to clinical data. We also investigated the quality of the ^{18}FDG variables in an attempt to select the variables most suitable for measurement in stomach cancer. The present study is

one of a few studies that have evaluated such a large number of PET/CT parameters in gastric cancer. Earlier studies concentrated only on the SUV max value. Our analysis focused in particular on the differences between patients with local and metastatic disease and the possible impact of disease on their overall survival and time-to-metastasis.

MATERIALS AND METHODS

Patients

All patients received no treatment prior to the examination and had undergone abdominal computed tomography with contrast-enhancement before PET/CT. The intention of the examination was to initially assess stage of disease for optimal selection of different treatment modalities. This study was approved by the local ethics committee (committee number-KB/493-59/09) in accordance with the Helsinki Declaration of 1975, as revised in 2000. The study protocol (established upon our institution experience) was uniform for all patients as follows: after a 6-h fast, patients were intravenously administered 7-15 mCi activity of ^{18}FDG (0.1 mCi per kg body weight). CT data were acquired on exhaust breath phase not exceeding 9.6 s; PET acquisition times ranged from 17 to 20 min. All metabolic quantifiers were analysed on Siemens® Syngo.via™ PET-CT-dedicated workstations. Glucose metabolism-related factors that could possibly affect interpretation of results (blood glucose level at time of study and incidence of diabetes mellitus) were uniformly distributed in all groups. Clinical characteristics of the patients are summarised in Table 1.

^{18}FDG metabolism quantification

We retrospectively analyzed a set of 40 ^{18}FDG PET-CT scans performed in Maria Skłodowska-Curie Memorial Institute of Oncology between 2008 and 2014 by one of two hybrid PET-CT scanners (Philips® Gemini XL and Siemens® Biograph™ mCT) in patients who had histologically confirmed gastric cancer.

Both scanners were periodically calibrated against the same electronic phantom probe that guarantees identical baseline SUV readouts of the reference radiotracer activity. PET-CT studies were performed randomly 1 h after administration of ^{18}FDG. Acquired DICOM images were analysed on Siemens Syngo.via PET-CT workstations. The following parameters were assessed for each primary gastric tumour: maximum standard uptake volume (SUV_{max}), mean SUV (SUV_{mean}) and metabolic tumour volume (MTV). The latter was measured with four different thresholds, varying by the SUV above which voxels inside the three-dimensional region of interest (ROI) covering the visible tumour were considered the metabolic volume. The following metabolic volumes were listed: $MTV_{2.5}$ (threshold: SUV = 2.5), MTV_{30} ($\geqslant$ 30% of SUV_{max}), MTV_{40} ($\geqslant$ 40% of SUV_{max}), MTV_{liv} [$\geqslant$ mean SUV of the patient's liver + 2 standard deviations (SD)]. These values were measured directly on PET-CT workstations. Also analysed were the following composite parameters: Total lesion glycolysis (TLG = SUV_{mean}* MTV) and coefficient of variation (COV = SD/SUV_{mean}).

Statistical analysis

Statistical calculations were performed using Statistica 10 Software (StatSoft, Inc.). The group was compared with the independent sample *t*-test, analysis of variance or the Mann-Whitney *U*-test. Univariate ROC curve model[15] was used for assessment of the confidence of ^{18}FDG metabolism quantifiers to identify metastatic and local disease. The impact of ^{18}FDG metabolism quantifiers on overall survival (OS) and time-to-metastasis (TTM) was analysed by Cox and Kaplan-Meier models. Overall survival was defined as time from date of PET-CT study to patient death and (TTM) was defined as time from PET-CT study to first follow-up visit at which tumour dissemination was confirmed (by pathologic examination of specimen, most commonly probatory peritoneal biopsies or by clinically evident lesions in imaging studies); TTM was only assessed for patients who were not initially disseminated. Results within 95%CI ($P < 0.05$) were considered statistically significant.

RESULTS

^{18}FDG metabolism quantifiers and clinical variables

Analysis of ^{18}FDG metabolism quantifiers revealed significant differences between three particular clinical groups of patients with stomach cancer. $MTV_{2.5}$ was related to level of weight loss relative to starting weight: the average volume varied significantly among groups with: (1) less than 5% weight loss; and (2) 5% to 10% weight loss; (3) more than 10% weight loss: 40.4 cm^3 *vs* 123.6 cm^3 *vs* 181.8 cm^3, respectively (P = 0.003, Figure 1).

Other significant metabolic variables included COV (0.21 *vs* 0.44 *vs* 0.44; P = 0.03) and TLG_{liv} (4.63 cm^3 *vs* 17.45 cm^3 *vs* 19.52 cm^3, P = 0.03). Another finding was the difference among different tumour grades with respect to COV, with higher COV observed in poorly differentiated G3 tumours than in better differentiated G1 and G2 tumours (0.46 *vs* 0.28, P = 0.03). Finally, almost all metabolic quantifiers differed between T1-T3 and T4 clinical stages. Tumour metabolic volumes ($MTV_{2.5}$: 102.4 cm^3 *vs* 217.3 cm^3, P = 0.009; MTV_{40}: 46.7 cm^3 *vs* 107.5 cm^3, P = 0.0007; MTV_{liv} 57.8 cm^3 *vs* 166.3 cm^3, P = 0.002) and the total lesion glycolysis volumes ($TLG_{2.5}$ 543.6 cm^3 *vs* 1827.1 cm^3, P = 0.004; TLG_{30}: 394.3 cm^3 *vs* 1086.1 cm^3, P = 0.005; TLG_{liv}: 12 cm^3 *vs* 26.1 cm^3, P = 0.01) increased with clinical stage. A summary of this analysis is presented in Table 2.

^{18}FDG metabolism quantifiers in local vs metastatic disease

A comparative analysis of the primary tumour metabolism

Table 1 Clinical patient characteristics *n* (%)

Variable	Overall (*n* = 40) number	Locally advanced (*n* = 23) number	Disseminated (*n* = 17) number	*P* value
Age: median/range (yr)	63/37-79	69/37-79	62/42-79	0.38
Sex				0.66
Male	31 (77)	17 (74)	14 (82)	
Female	9 (23)	6 (26)	3 (18)	
Performance status				0.73
0	18 (45)	11 (48)	7 (41)	
1-2	22 (55)	12 (52)	10 (59)	
Weight loss				0.61
< 5%	11 (28)	7 (30)	4 (24)	
5%-10%	12 (30)	7 (30)	5 (29)	
> 10%	17 (42)	9 (40)	8 (47)	
Tumour location				0.85
Upper third	20 (50)	12 (52)	8 (47)	
Middle third	15 (37.5)	8 (35)	7 (41)	
Lower third	5 (12.5)	3 (13)	2 (12)	
Tumour clinical stage (AJCC 2010)				0.39
cT1-T3	32 (80)	20 (87)	12 (71)	
cT4	8 (20)	3 (13)	5 (29)	
Nodal involvement (AJCC 2010)				0.014
cN0	20 (50)	16 (70)	4 (23)	
cN1-N3	20 (50)	7 (30)	13 (77)	
Histology				0.45
Intestinal type	32 (80)	17 (74)	15 (88)	
Diffuse type	8 (20)	6 (26)	2 (12)	
Histological grade				0.71
G1-G2	12 (30)	8 (35)	4 (24)	
G3	18 (45)	9 (39)	9 (52)	
Not specified	10 (25)	6 (26)	4 (24)	
CA19.9 median (range), IU/mL	10.65 (2-159674)	9.43 (2.0-12571)	11.07 (2.0-159674)	0.10
CEA median (range), IU/mL	2.86 (0.5-514)	2.09 (0.5-117)	9.04 (0.7-514)	0.17

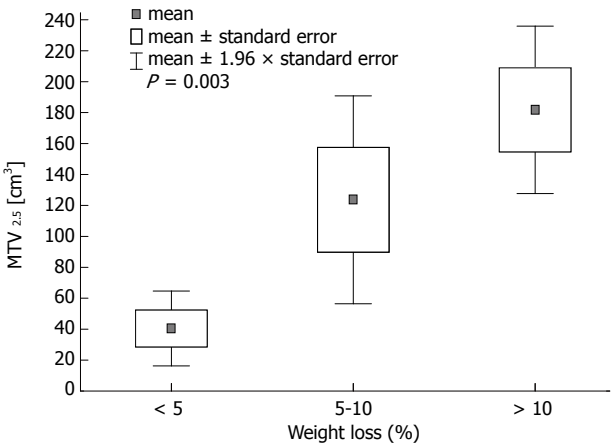

Figure 1 Box plot of $MTV_{2.5}$ values of patients grouped by extent of weight loss.

of patients with local and disseminated disease revealed a statistically significant difference in SUV_{mean} between the two groups: 4.13 *vs* 6.05, respectively, (*P* = 0.008). $TLG_{2.5}$ and TLG_{30} also varied between local and disseminated disease: 225.87 cm^3 *vs* 802.17 cm^3 (*P* = 0.03) for $TLG_{2.5}$ and 247.33 cm^3 *vs* 435.61 cm^3 (*P* = 0.01) for TLG_{30}. Comparisons for all parameters are presented in Table 3.

Analysis of all metabolic quantifiers using ROC curve model named TLG_{30}, $TLG_{2.5}$, MTV_{40} and SUV_{mean} as fairly reliable quantifiers to identify tumour dissemination. Results, including identification of optimal cut-off values (best weighed between specifity and sensitivity) for each parameter are displayed in Table 4. Parameters are displayed in order according to overall grade, which is based on AUC (area under curve[15])-see table descriptions.

^{18}FDG metabolism quantifiers as survival predictors

Clinical and metabolic variables were analysed using Cox multivariate models. Factors that significantly affected OS were as follows: male sex (HR = 0.13, 95%CI: 0.007-0.41; *P* = 0.005), initial tumour site in antrum (HR = 0.08, 95%CI: 0.007-0.39; *P* = 0.008), and TLG_{30} (HR = 1.001, 95%CI: 1.0009-1.0017; *P* = 0.047). The Kaplan-Meier univariate model comparing two groups of patients stratified by the ROC-calculated threshold for TLG_{30} confirmed TLG_{30} parameter to be a significant prognostic factor for OS (Figure 2). Predictably, there were more patients with disseminated disease who had TLG_{30} volumes above the threshold than non-disseminated ones (15/17 *vs* 9/23, *P* = 0.008). Notably though, the initial disease dissemination itself was not a significant survival predictor in univariate Kaplan-Meier model.

A separate analysis of OS and TTM was performed for patients in whom the PET-CT study identified local disease (*n* = 23). For OS, TLG_{30} was as well an independent prognostic factor (HR = 1.009, 95%CI: 1.003-1.014; *P* = 0.004), along with $MTV_{2.5}$ (HR =

Table 2 Distribution of ^{18}Fluorodeoxyglucose metabolism quantifiers in various clinical patient subgroups

	SUV_{max}	SUV_{mean}	$MTV_{2.5}(cm^3)$	$MTV_{30}(cm^3)$	$MTV_{40}(cm^3)$	$MTV_{liv}(cm^3)$	COV	$TLG_{2.5}(cm^3)$	$TLG_{30}(cm^3)$	$TLG_{40}(cm^3)$	$TLG_{liv}(cm^3)$
Sex											
Male	12.97	5.00	133.19	105.19	60.81	86.26	0.41	866.70	551.5	16.14	13.99
Female	10.88	4.76	98.91	85.45	52.06	56.03	0.28	571.58	384.9	10.21	9.22
P value	0.51	0.79	0.43	0.65	0.63	0.39	0.18	0.52	0.48	0.29	0.30
Performance status											
0	11.16	4.39	109.79	124.00	68.8	65.79	0.37	582.67	570.26	15.14	13.35
1-2	13.60	5.41	138.31	81.73	50.69	90.63	0.39	978.35	478.41	11.09	15.99
P value	0.36	0.17	0.44	0.24	0.24	0.40	0.87	0.3	0.65	0.29	0.59
Weight loss											
< 5%	7.71	4.31	40.4[1]	61.38	38.98	23.98	0.21[1]	209.67	243.88	7.52	4.63[1]
5%-10%	12.68	4.56	123.6[1]	90.26	57.73	88.61	0.44[1]	678.12	431.41	14.17	17.45[1]
> 10%	15.47	5.63	181.8[1]	133.62	72.48	108.90	0.44[1]	1268.7	747.12	15.52	19.52[1]
P value	0.051	0.28	0.003[1]	0.24	0.19	0.053	0.03[1]	0.059	0.096	0.20	0.027[1]
Tumour location											
Upper third	13.93	5.15	129.16	79.95	52.18	79.18	0.43	850.20	418.48	12.98	15.41
Middle third	11.11	4.83	142.17	74.72	54.09	88.87	0.29	878.94	727.09	13.95	15.01
Lower third	10.96	4.46	82.93	59.68	37.85	52.31	0.41	364.75	256.94	9.51	11.77
P value	0.56	0.82	0.67	0.19	0.22	0.75	0.24	0.68	0.2	0.77	0.89
AJCC tumour clinical stage											
cT1-T3	11.67	4.61	102.4[1]	87.24	46.7[1]	57.8[1]	0.39	543.6[1]	394.3[1]	11.61	12[1]
cT4	15.82	6.28	217.3[1]	154.79	107.5[1]	166.3[1]	0.34	1827.1[1]	1086.1[1]	18.24	26.10[1]
P value	0.21	0.07	0.009[1]	0.13	0.0007[1]	0.002[1]	0.63	0.004[1]	0.005[1]	0.16	0.01[1]
AJCC nodal involvement											
cN0	11.32	4.49	97.33	70.88	46.16	66.48	0.40	600.96	376.09	11.11	12.04
cN1-N3	13.68	5.41	153.62	130.61	71.52	91.43	0.36	999.63	668.3	14.73	17.58
P value	0.37	0.22	0.12	0.1	0.10	0.38	0.58	0.29	0.14	0.34	0.26
Histology											
Intestinal type	13.34	5.17	138.79	106.51	60.93	91.90	0.40	902.61	560.06	13.85	17.01
Diffuse type	9.13	4.06	72.19	77.68	50.47	29.67	0.29	391.05	328.19	9.16	5.98
P value	0.21	0.24	0.14	0.53	0.59	0.09	0.31	0.28	0.38	0.32	0.07
Histological grade											
G1-G2	13.16	4.83	118.67	64.91	39.76	73.64	0.28[1]	632.14	309.77	10.07	17.06
G3	11.99	5.20	122.11	133.85	71.31	70.96	0.47[1]	950.73	702.29	14.20	10.14
P value	0.61	0.73	0.91	0.14	0.36	0.89	0.03[1]	0.59	0.11	0.42	0.16

Average metabolic parameter values are presented. [1]Metabolic quantifiers exhibited statistically significant differences among clinical subgroups (described earlier in the text). SUV: Standard uptake volume; MTV: Metabolic tumour volume; COV: Coefficient of variation; TLG: Total lesion glycolysis.

Table 3 Comparison of average ^{18}Fluorodeoxyglucose metabolism quantifiers between metastatic and local tumours

Metabolic quantifier	Overall (n = 40)	Locally advanced (n = 23)	Disseminated (n = 17)	*P* value
SUV_{max}	10.95	10.36	12.78	0.130
	4.14-47.74	4.14-22.66	2.73-47.74	
SUV_{mean}[1]	4.95[1]	4.13[1]	6.05[1]	0.008[1]
	1.94-13.83[1]	1.94-6.38[1]	2.78-13.83[1]	
COV	0.34	0.38	0.35	0.360
	0-1.12	0.03-1.12	0-0.80	
$MTV_{2.5}(cm^3)$	91.36	77.16	135.55	0.058
	0.96-688.63	6.91-379.2	0.96-688.63	
$MTV_{30}(cm^3)$	69.16	58.36	91.97	0.130
	12.79-668.60	12.79-275.3	21.82-668.60	
$MTV_{40}(cm^3)$	46.05	31.85	56.64	0.110
	8.13-208.36	8.13-208.22	14.34-208.36	
$MTV_{liv}(cm^3)$	47.31	31.56	87.12	0.140
	0-352.25	1.18-337.38	0-352.25	
$TLG_{2.5}(cm^3)$[1]	445.07[1]	225.87[1]	802.17[1]	0.031[1]
	2.62-5984[1]	15.13-2419.9[1]	2.62-5984[1]	
$TLG_{30}(cm^3)$[1]	352.65[1]	247.33[1]	435.61[1]	0.018[1]
	46.7-2913[1]	46.7-1756.4[1]	113.9-2913[1]	
$TLG_{40}(cm^3)$	8.35	7.61	8.82	0.760
	0.56-49.26	1.41-45.77	0.56-49.26	
$TLG_{liv}(cm^3)$	10.5	6.6	13.81	0.950
	0-70.42	0.4-70.42	0-34.15	

[1]Parameters exhibiting a statistically significant difference (as described earlier in text). SUV: Standard uptake volume; MTV: Metabolic tumour volume; COV: Coefficient of variation; TLG: Total lesion glycolysis.

Table 4 Analysis of accuracy of metabolic quantifiers to differentiate between local and disseminated tumours by ROC model

Metabolic quantifier	AUC	SE	95%CI	Cutoff value	Sensitivity of cutoff value	Specifity of cutoff value	Overall mark	*P* value
$TLG_{30}(cm^3)$[1]	0.78[1]	0.07[1]	0.63-0.92[1]	390.53[1]	65%[1]	78%[1]	Fair[1]	0.0006[1]
SUV_{mean}[1]	0.73[1]	0.08[1]	0.58-0.89[1]	6.87[1]	35%[1]	100%[1]	Fair[1]	0.0900[1]
$TLG_{2.5}(cm^3)$[1]	0.72[1]	0.08[1]	0.55-0.88[1]	802.17[1]	53%[1]	83%[1]	Fair[1]	0.0340[1]
$MTV_{40}(cm^3)$[1]	0.71[1]	0.08[1]	0.55-0.87[1]	37.25[1]	88%[1]	61%[1]	Fair[1]	0.0010[1]
$MTV_{30}(cm^3)$	0.69	0.08	0.53-0.86	83.54	65%	74%	Poor	0.0020
$MTV_{2.5}(cm^3)$	0.68	0.09	0.51-0.84	132.55	59%	74%	Poor	0.0600
$MTV_{liv}(cm^3)$	0.67	0.09	0.50-0.84	87.12	53%	74%	Poor	0.1000
SUV_{max}	0.61	0.09	0.43-079	14.44	47%	78%	Poor	0.4000
$TLG_{liv}(cm^3)$	0.61	0.09	0.43-0.79	7.42	71%	56%	Poor	0.3000
$TLG_{40}(cm^3)$	0.50	0.09	0.31-0.68	49.26	6%	100%	Useless	-
COV	0.50	0.09	0.27-0.63	1.13	0%	96%	Useless	-

[1]Parameters rated as "fair" or better. Overall mark by AUC value: 1.0 ideal, 0.99-0.9 excellent, 0.89-0.8 good, 0.79-0.7 fair, 0.69-0.51 poor, 0.5 useless (ideally random). SUV: Standard uptake volume; MTV: Metabolic tumour volume; COV: Coefficient of variation; TLG: Total lesion glycolysis; AUC: Area under curve.

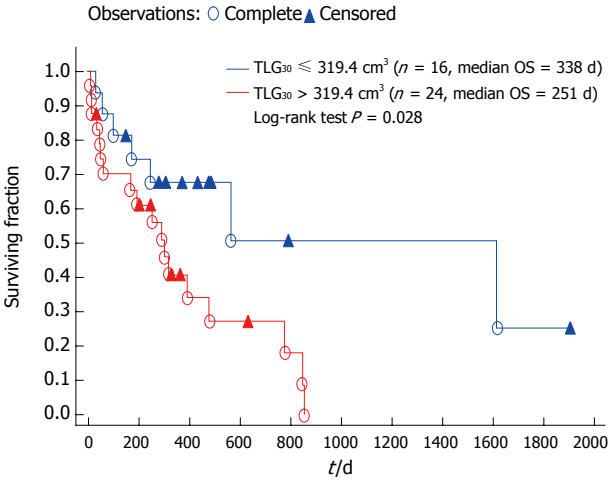

Figure 2 Kaplan-Meier curves for overall survival of patient groups stratified by ROC-calculated threshold for TLG_{30}.

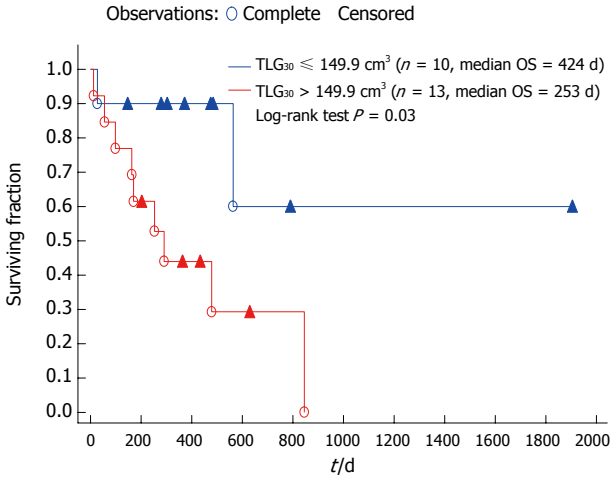

Figure 3 Kaplan-Meier curves for overall survival of patient groups stratified by ROC-calculated threshold for TLG_{30} (including only patients who were not disseminated initially).

1.02, 95%CI: 1.002-1.036; *P* = 0.025). OS in the Kaplan-Meier model varied significantly for TLG_{30}-stratified groups (Figure 3; note the different ROC threshold value resulting from lower TLG_{30} values in non-disseminated patients' group). For TTM, the Cox proportional hazard model identified TLG_{30} as the only significant prognostic factor (HR = 1.006, 95%CI: 1.001-1.012; *P* = 0.02). This result was not significant when analysed in univariate Kaplan-Meier model. However, of all of parameters analysed in this model, TLG_{30} threshold was closest to reaching statistical significance (*P* = 0.06).

Other metabolic parameters were not observed to vary significantly across patient subgroups with respect clinical variables listed in Table 1, particularly between metastatic and non-metastatic patients. A summary of the average metabolic quantifier values for these variables is presented in Tables 2 and 3.

DISCUSSION

General prognostic value of PET-CT in gastric cancer

Two reports on usefulness of PET-CT imaging in gastric cancer (GC) have emerged recently, especially regarding its utilisation in disease staging[7,16]. These suggest that, despite its particular limitations in assessment of small, early tumours[17], PET-CT can reliably visualise advanced primary tumours and can detect tumour dissemination to (regional) lymph nodes and distant organs. The latter are often silent on contrast-enhanced CT[4,5,7]. One group reported 94% sensitivity in PET-CT diagnosis in advanced GC (stages Ⅲ and Ⅳ)[18].

In addition to diagnostic value, many researchers have attempted to identify possible prognostic and predictive information derived from radiotracer metabolism quantification. In GC, the majority of available studies focused on SUV_{max} value. One group investigated 271 patients following gastrectomy and identified SUV_{max} > 8.2 as a negative prognostic factor favouring disease recurrence[19]. Another analysed 62 patients with disseminated GC. Their study described primary tumour SUV_{max} < 6.0 as a positive prognostic factor for OS and PFS. This value was calculated using an ROC model, and its significance out-powered

median SUV_{max} value of the whole group (7.2)[8]. In a third study comprising 75 tumours limited to the stomach and 22 disseminated tumours, 25% of all tumours did not exhibit pathologic ^{18}FDG uptake. The authors indirectly explained this by pointing at a significant share of tumours of low clinical stage and poor cellular differentiation (with both showing tendency for low ^{18}FDG-uptake) in their group. In our study, no false negative results occurred. Tumours that did demonstrate pathologic ^{18}FDG uptake, as well as T3/T4 tumours and those above 60 mm in diameter, exhibited a statistically worse OS, whereas a threshold of median SUV_{max} (6.7) was not prognostic for OS. This study is notable for demonstrating the superiority of PET-CT over contrast-enhanced CT in detection of metastatic lymph nodes; PET-CT-positive lymph nodes were a significant prognostic factor, whereas lymph nodes positive on CT were not prognostic[4]. Finally, it was reported that SUV_{max} > 8 was prognostic for worse OS in a group of 35 disseminated GC patients who underwent palliative chemotherapy[9].

In our study, despite a notable inequality in median SUV_{max} between limited and disseminated GC (10.36 *vs* 12.78), no significant difference in SUV_{max} was observed. The ROC model also failed to identify an SUV_{max} value that was significantly prognostic for tumour dissemination. However, we did identify other ^{18}FDG metabolic quantifiers (TLG, MTV, SUVmean; these quantifiers are presented in Table 4) that significantly correlated with clinical variables, some of which have not been described previously in GC.

Clinical stage and grade

This study observed that MTV and TLG of almost all aforementioned thresholds were significantly elevated in T4 tumours. Other studies investigating the correlation between ^{18}FDG metabolism and tumour stage have also demonstrated increased radiotracer uptake. One study reported that increased uptake was associated with worse OS but[4] another study did not[20]. Caution should be taken in the interpretation of our observations due to the significant differences in the sizes of the pT1-3 and pT4 groups (32 *vs* 8) and due to the lack of contrast enhancement of the low-dose CT layer in the PET-CT study. There have been numerous reports on high levels of ^{18}FDG uptake by chronic gastric inflammation, which can significantly increase the number of false positive studies, as well as reports of predictions of inaccurately large tumour volumes in the context of chronic inflammation co-existing (common because *gastritis chronica* is an identified precancerous state)[21]. Dilation of the stomach with neutral fluid prior to the examination has been proposed by some who claim that it helps better visualise tumour borders and distinguishes physiological ^{18}FDG uptake from tumor uptake and involved regional lymph nodes[13,22].

In our study, we observed that COV was significantly higher in poorly differentiated G3 tumours. This parameter has not been reported in GC, yet studies covering different tumour types describe elevated COV as an indicator of tumour heterogeneity and a predictive factor of a worse treatment outcome[23,24]. In our study, due to the variety of treatment modalities, we only investigated the correlation between COV and clinical and pathological variables. Our observation may indicate that tumours with higher COV exhibit a worse response to chemotherapy, consistent with the aforementioned studies. This hypothesis requires further study.

Clinical course, prediction of metastasis and survival

$MTV_{2.5}$, $TLG_{liv,}$ and COV were significantly associated with the degree of weight loss, regardless of clinical stage. This trend may be explained by increased glucose metabolism in large and advanced tumours, leading to cachexia through deteriorating gastric function (through limiting its capacity, absorption and muscular activity) and secretion of pro-cachectic cytokines. No other study has investigated such correlations. One group included BMI in their analysis, but there was no correlation between BMI and tumours with high and low ^{18}FDG uptake or according to lymph node involvement status[11].

Our study analysed ^{18}FDG metabolism of primary tumours in an attempt to identify differences in metabolic quantifiers that differ significantly between metastatic and non-metastatic tumours. These metabolic quantifiers could help to identify patients in whom tumour dissemination is believed to have occurred. These quantifiers would be of particular interest in signet-ring carcinoma, which is characterised by exceptionally low ^{18}FDG uptake (reportedly due to lower cellular density and lower GLUT-1 expression) and peritoneal tumour spread with tumour foci below the limit of PET-CT resolution[25,26]. We have identified six metabolic quantifiers as potentially able to differentiate disseminated and limited disease. The investigated parameters were pre-selected to be suitable for tumours with relatively low radiotracer uptake (*i.e.*, 50% SUV_{max} threshold was omitted from our analysis), and results confirmed the overall superiority of MTV and TLG with centre-weighed 30% SUV_{max} thresholds (78% AUC). Their potential to identify tumours likely to disseminate has not been well established for GC, but other studies in other cancers have described TLG as a useful prognostic biomarker[27-30].

Multi- and univariate analysis of OS and TTM in our study identified TLG_{30} as the most valuable survival predictor of all ^{18}FDG metabolism quantifiers. The results of studies of other tumour types[27] *n* = 81 NSCLC patients[29] *n* = 86 oesophageal carcinoma patients; in both $TLG_{2.5}$ prognostic for OS and recurrence-free survival[30]; *n* = 41 various solid tumours (*n* = 6 GC), TLG predictive for chemotherapy response are consistent with our observations regarding prognosis as well[27,29,30].

Study limitations

Our study was designed as a hypothesis generator to investigate general trends and correlations of ^{18}FDG metabolism quantifiers with the clinical course of GC and should only be regarded as such. Patients were not standardised or stratified according to clinical stage, and they received a variety of treatment modalities (some received none except best supportive care). No minimal follow-up period was introduced (range: 6 d to 5.2 years, median-9.5 mo). Therefore, all results related to the survival analysis should be interpreted with caution. Lack of contrast enhancement in the CT layer in our PET/CT study protocol could also disturb interpretation of local lymph node involvement status (in PET image, lymph nodes often merge into a single high-radiotracer uptake region with the primary tumour) and, consecutively clinical stage.

In conclusion, PET-CT is a useful tool in the diagnosis of gastric cancer. When appropriate study protocol enhancements are applied, PET-CT can be used to more reliably stage patients and, as our study has demonstrated, identify patients with potentially worse prognoses and those at greater risk of peritoneal tumour spread. Radiotracer quantifiers with low ^{18}FDG uptake are preferred for PET-CT; the results of our and other studies indicate that total lesion glycolysis with low radiotracer uptake with 2.5 absolute thresholds and 30% relative thresholds should always be included in the analysis. Initial and, optionally, intra-treatment PET-CT can be a valuable addition to randomised clinical trials to help properly stage patients and to assess radiotracer metabolism changes as a response to therapy. In a clinical trial with a standardised group of patients, the diagnostic, prognostic and possibly predictive values of PET-CT, as well as its limitations, can be more unambiguously confirmed, eventually permitting the widespread use of PET-CT in the therapeutic decision-making in GC patients.

COMMENTS

Background

Gastric cancer (GC) features a high mortality to incidence ratio, which indirectly demonstrates its low curability. Thus, there is still significant room for improvement in its diagnosis and therapy. Fusion 18-fluorodeoxyglucose positron emission tomography with computed tomography [^{18}Fluorodeoxyglucose (^{18}FDG)-positron emission tomography-computer tomography (PET-CT)] is not a routinely used diagnostic method in gastric cancer. However, many recent studies confirm its diagnostic potential, especially in tumours of higher clinical stage quantification.

Research frontiers

In addition to visualising the primary tumour, locoregional and distant metastatic sites, studies also note its prognostic and predictive value.

Innovations and breakthroughs

In context s of predictive and prognostic value, most publications highlight the maximum standardized uptake value (SUV) value of the primary tumour. In our study, in addition to SUV_{max}, the authors analysed numerous radiotracer quantifiers, which have only been mentioned in few or none studies in gastric cancer. Despite a notable inequality in median SUV_{max} between limited and disseminated GC, no significant difference in SUV_{max} was observed. However, we did identify other ^{18}FDG metabolic quantifiers measured in primary tumor (total lesion glycolysis (TLG), metabolic tumor volume (MTV), SUVmean) which allowed to differentiate between patients with local and metastatic disease and had a prognostic significance. The results confirmed the overall superiority of radiotracer uptake quantifiers that are not affected by relatively low radiotracer uptake of the tumour (which is generally the case for GC compared to other tumours), namely MTV and TLG with centre-weighed 30% SUV_{max} thresholds (78% AUC). Their potential to identify tumours likely to disseminate has not been well established for GC, but a number of studies in other cancers have described TLG as a useful prognostic biomarker.

Applications

This broad analysis can be clinically utilized to identify groups of patients with worse tumour prognosis and who could possibly benefit from more aggressive treatment. The study results suggest that PET-CT studies may have a firm place in the therapeutic decision-making in gastric cancer patients.

Terminology

PET/CT is a tool of nuclear medicine. It is a combination of PET, a functional imaging technique that produces a three-dimensional image of metabolic processes in the body and CT, a 3-dimensional X-ray scan performed on the patient during the same session, in the same machine. ^{18}FDG is an analogue of glucose labeled with a short-life radioactive isotope of fluorine, which is injected into a patient before PET scanning. The concentrations of radiotracer visualized indicate tissue metabolic activity by virtue of the regional glucose uptake. Radiotracer uptake above a certain level is considered as suspected for presence of a primary malignant tumor or metastasis, even despite its normal structural image in CT. The SUV is often used in PET imaging for a simple semi-quantitative analysis. The SUV represents the ratio of the radioactivity measured in a spatially defined part of the body at a certain time point to a hypothetically even distribution of the injected radioactivity across the whole body. MTV represents a measurable volume of a given cubic unit whose radioactivity exceeds a threshold assumed to differentiate between a normal tissue and a malignant tumor. TLG is defined as SUVmean x MTV; it represents a product of intensity and volume quantifiers describing the same spatially localized radioactivity.

Peer-review

Prognostic value and clinical correlations of 18-fluorodeoxyglucose metabolism quantifiers in gastric cancer is an excellent paper.

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ORIGINAL ARTICLE

Retrospective Study

Risk factors causing structural sequelae after anastomotic leakage in mid to low rectal cancer

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Author contributions: Ji WB performed the research, analyzed the data, and wrote the paper; Kwak JM designed the research and analyzed the data; Kim J, Um JW, and Kim SH contributed analytical tools and reviewed this study.

Ethics approval: The study was reviewed and approved by the Korea University Anam Hospital Institutional Review Board.

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Data sharing: No additional data are available.

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Abstract

AIM: To investigate the risk factors causing structural sequelae after anastomotic leakage in patients with mid to low rectal cancer.

METHODS: Prospectively collected data of consecutive subjects who had anastomotic leakage after surgical resection for rectal cancer from March 2006 to May 2013 at Korea University Anam Hospital were retrospectively analyzed. Two subgroup analyses were performed. The patients were initially divided into the sequelae (stricture, fistula, or sinus) and no sequelae groups and then divided into the permanent stoma (PS) and no PS groups. Univariate and multivariate analyses were performed to identify the risk factors of structural sequelae after anastomotic leakage.

RESULTS: Structural sequelae after anastomotic leakage were identified in 29 patients (39.7%). Multivariate analysis revealed that diversion ileostomy at the first operation increases the risk of structural sequelae [odds ratio (OR) = 6.741; P = 0.017]. Fourteen patients (17.7%) had permanent stoma during the follow-up period (median, 37 mo). Multivariate analysis showed that the tumor level from the dentate line was associated with the risk of permanent stoma (OR = 0.751; P = 0.045).

CONCLUSION: Diversion ileostomy at the first operation increased the risk of structural sequelae of the anastomosis, while lower tumor location was associated with the risk of permanent stoma in the management of anastomotic leakage.

Key words: Anastomotic leakage; Permanent stoma; Leakage sequelae; rectal cancer; Anastomotic leakage fate

Core tip: This study aimed to find the risk factors causing structural sequelae of anastomotic site after leakage in rectal cancer patients. Anastomotic leakage is one of the most challenging complications. Even after

patients recover from the acute complication phase, they can suffer from its structural sequelae including stricture, fistula, sinus, or permanent stoma. No studies have evaluated the risk factors causing structural sequelae of anastomosis after leakage. Here we report our data about the fate of anastomotic leakage and the risk factors that should be considered after anastomotic leakage in patients with rectal cancer.

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INTRODUCTION

Despite technological advancements in surgical devices, methods, and perioperative management, anastomotic leakage (AL) after colorectal surgery remains a significant problem for patients and surgeons. AL can result in poor surgical, oncological, and functional outcomes. It increases postoperative morbidity and mortality[1,2] as well as local and systemic tumor recurrence or progression[3] and decreases quality of life[4-6]. Even with the proper management of AL, structural sequelae can develop such as prolonged fistula, sinus formation, or stricture. Those complications may cause various symptoms, complicate postoperative management, delay or prevent stomal repair, and postpone adjuvant treatment.

There have been many studies on AL after colorectal surgery as well as its risk factors[7-11]. However, none have examined the prognosis of leakage itself. Here we evaluated the clinical consequences of the anastomosis site after leakage and identified factors influencing poor anastomotic healing after leakage.

MATERIALS AND METHODS

Study cohort and data collection

We performed a retrospective data analysis with prospectively collected data from a cohort of 107 consecutive patients who experienced AL after elective surgical resection for rectal cancer from March 2006 to May 2013 at Korea University Anam Hospital. A total of 809 patients with rectal cancer underwent surgical resection during this period. We included 79 patients in this study and excluded 28 patients who had upper rectal cancer (n = 16), were lost to follow-up, had postoperative mortalities (n = 9), or had another pelvic organ malignancy (n = 1). Follow-up loss was defined as when the patient did not present at the clinic on any of the designated dates during study period.

Two subgroup analyses were performed. First, all included patients were divided into the sequelae and no sequelae groups according to the existence of structural sequelae of AL (fistula, sinus, or stricture). Second, all patients were divided into the permanent stoma (PS) and no PS groups (Figure 1).

Procedures and follow-up

All surgical procedures were performed by three surgeons in a division that specialized in laparoscopic and robotic colorectal surgery. All surgical resections for rectal cancer were performed using a conventional laparoscopic or robotic method. A normal diet was resumed by clinical decision according to the surgeons' preferences. The pathological examinations were performed by pathologists according to the seventh edition on colon and rectal cancer of the American Joint Committee on Cancer. The approach to managing AL was chosen by the surgeons among conservative management with antibiotic therapy, percutaneous abscess drainage (PAD), surgical procedures such as drainage and irrigation, diversion enterostomy with or without primary repair of the leakage site, re-resection with anastomosis, and Hartmann's procedure.

After surgical resection of the rectal cancer, all patients were routinely followed every 3-6 mo during the first 2 years and every 6 mo thereafter. Routine follow-up tests included computed tomography (CT) of the abdomen and pelvis, chest CT, and carcinoembryonic antigen levels. Total colonofiberoscopy or sigmoidoscopy was performed if needed. Patients with AL underwent additional CT, sigmoidoscopy, or contrast study at the physician's discretion.

Definitions

Anastomotic leakage was defined as in previous studies with any grade including abscess in close proximity and was diagnosed based on radiologic and endoscopic findings together with clinical signs such as a change in drainage color or signs of peritonitis that required surgery[12]. Mid to low rectal cancer was defined as rectal cancer located < 10 cm from the dentate line[13]. Hospital stay was defined as the period of time from admission until discharge. Intensive care unit (ICU) transfer was defined as transfer to the ICU during the course of in-hospital management including routine stays after the surgical procedure. Tumor location was defined as the length in centimeters between the lower tumor margin and the dentate line. Multi-organ failure was defined as functional deterioration of two or more vital organs.

Structural sequelae of AL included prolonged fistula, sinus formation, and anastomotic stricture. A fistula was diagnosed when the contrast study findings showed a fistulous tract connected to the intra-abdominal cavity, abdominal organ, or abscess cavity. Sinus formation was diagnosed when the end of a fistulous tract of any length without connection was identified during the examination. Anastomotic stricture

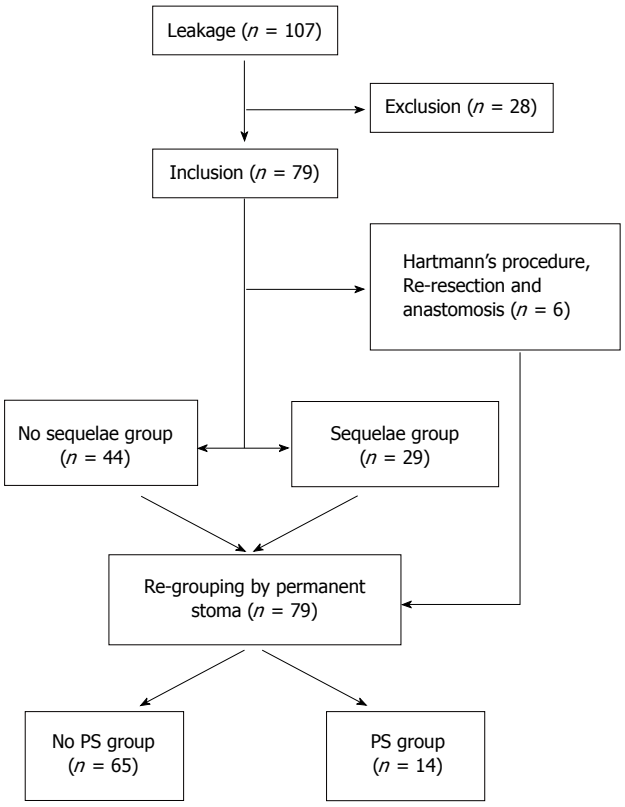

Figure 1 Flow chart of patient selection and subgroups in our study. PS: Permanent stoma.

was defined when the endoscopic findings showed any degree of stenotic lesion at the anastomotic area with or without symptoms. Permanent stoma was any type of enterostomy for the diversion that was not intended to be repaired until the last follow-up visit during the study period.

Statistical analysis

Continuous data were analyzed by Student's *t*-test and categorical data were analyzed by logistic regression test or χ^2 test with Fisher's exact test as needed. Multivariate analysis was performed using a logistic regression test that was set up using univariate associations that were significant at *P* values < 0.05. Survival analysis and the test for cumulative incidence of permanent stoma were performed using Kaplan-Meier survival analysis with a log-rank test. All tests were two-sided and statistical significance was considered at *P* values < 0.05. Statistical analysis was performed using SPSS Statistics for Windows (version 20; IBM, Armonk, NY, United States).

RESULTS

Baseline characteristics

The median follow-up period of the included 79 patients was 37 mo. Disease-related death occurred in five patients, while disease progression or recurrence was observed in 18 patients. The baseline characteristics of the included patients (*n* = 79) are presented in Table 1. There was no statistically significant difference in initial management methods after AL (Tables 2 and 3). A total of 29 patients underwent multiple invasive procedures to manage complications after the initial therapies. Conservative therapy using antibiotics and diet control was initially intended for 32 patients, but 11 of them (34.4%) underwent other management tactics such as PAD or surgical procedures.

Table 1 Baseline characteristics of patients diagnosed with anastomosis leakage after resection of mid to low rectal cancer (*n* = 79)

	n (%)	Surgical method	*n* (%)
Age (yr), median	59		
Gender		Conventional	3 (3.8)
Male	55 (69.6)	Laparoscopic	45 (57.0)
Female	24 (30.4)	Robotic	28 (35.4)
BMI (mean, kg/m^2)	24.18	Conversion	3 (3.8)
ASA score		Anastomosis method	
Ⅰ	34 (43.0)	Hand-sewn	10 (12.8)
Ⅱ	43 (54.4)	Stapling	68 (87.2)
Ⅲ	2 (2.5)	Anastomosis type	
Tumor stage		End-to-end	70 (88.6)
0/Ⅰ	23 (29.1)	End-to-side	5 (6.3)
Ⅱ	24 (30.4)	Colonic J-pouch	4 (5.1)
Ⅲ	24 (30.4)	Neoadjuvant chemoradiotherapy	19 (24.1)
Ⅳ	8 (10.1)	Transfusion	13 (17.3)

Data are expressed as *n* (%), mean or median. BMI: Body mass index; ASA: American Society of Anesthesiologists.

Risk of occurrence of structural sequelae after AL

Among the 79 patients, six were excluded from this comparison because they experienced changes in the anastomosis site by Hartmann's procedure or re-resection and anastomosis at any point during the management period. Structural sequelae after anastomotic leakage, such as prolonged fistula, sinus formation, and stricture, were identified in 29 patients (39.7%). Univariate analysis of the sequelae and no sequelae groups revealed that age, pathological stage (0/Ⅰ/Ⅱ), ileostomy, hospital stay, duration of antibiotic use, and transfusion were significantly different between the two groups (Table 4). Multivariate analysis performed using these variables showed that diversion ileostomy at the first operation increases the risk of complications [odds ratio (OR) = 6.741; 95% confidence interval (CI): 1.404-32.364; *P* = 0.017; Table 4].

Risk analysis of permanent stoma

Among 79 patients, 14 (17.7%) had a permanent enterostomy. None of the patients in the no sequelae group required a permanent stoma, while 15 patients (51.7%) in the sequelae group (*n* = 29) were able to have their stomas repaired after anastomotic complication management.

Sinus formation occurred in eight patients, stricture

Table 2 Univariate analysis of differences between the sequelae and no sequelae groups (n = 73) n (%)

	Sequelae[1]	No sequelae	OR	95%CI		*P* value
				Lower	Upper	
Age, median (yr)	62	55.5				0.037
> 65	11 (37.9)	14 (31.8)	1.459	0.557	3.819	0.442
Gender						
Male	21 (72.4)	30 (68.2)	1.148	0.411	3.212	0.792
BMI, mean	23.99	24.25				0.748
ASA score						
Ⅰ	11	20				
Ⅱ/Ⅲ	18	24	1.259	0.487	3.255	0.635
Operation time, mean (min)	284.49	260.74				0.205
Ileostomy	21 (72.4)	20 (45.5)	3.412	1.254	9.290	0.016
LNM, mean	1.1	1.3				0.698
RLN, mean	22.1	23.6				0.681
Stage						
0/Ⅰ/Ⅱ	22 (75.9)	22 (50.0)				
Ⅲ/Ⅳ	7 (24.1)	22 (50.0)	0.318	0.114	0.890	0.029
Tumor location, median (centimeters from AV)	5	5.5				0.144
Neoadjuvant chemoradiotherapy	9 (31.0)	8 (18.2)	2.137	0.715	6.393	0.174
Surgical method						0.080
Conventional	0	3 (6.9)				
Laparoscopic	14 (48.3)	27 (61.4)				
Robotic	13 (44.8)	14 (31.8)				
Conversion	2 (6.9)	0				
Anastomosis						0.726
Hand-sewn	3 (10.3)	6 (13.6)				
Stapling	26 (89.7)	38 (86.4)				
Anastomosis type						0.766
End-to-end	26 (89.7)	39 (88.6)				
End-to-side	2 (6.9)	2 (4.5)				
Colonic J-pouch	1 (3.4)	3 (6.9)				
Mechanical bowel preparation						
Yes	20 (69.0)	34 (77.3)				
No	9 (31.0)	10 (22.7)	0.784	0.281	2.187	0.642
Initial AL management						0.512
Conservative	12 (41.4)	20 (45.5)				
PAD	7 (24.1)	9 (20.5)				
Diversion only	4 (13.8)	10 (22.7)				
Primary repair + diversion	0	1 (2.3)				
Surgical irrigation + drainage	6 (20.7)	4 (9.1)				
Transfusion	8 (27.6)	4 (9.1)	3.810	1.027	14.137	0.046
Leakage (d)	5.7	4.2				0.054
Hospital stay (d)	48.9	20.9				0.001
Days to start diet (d)	6.3	5.7				0.665
Antibiotics use (d)	25.3	15.0				0.001
ICU transfer	3 (10.3)	3 (6.9)	1.212	0.251	5.852	0.811
Multi-organ failure	3 (10.3)	2 (4.3)	2.538	0.398	16.204	0.325
Postoperative ileus	5 (17.2)	4 (8.7)	2.197	0.536	8.935	0.276

[1]Patients who had structural sequelae after leakage, includes patients with anastomosis fistula, sinus, or stricture. Data are expressed as n (%), mean or median. BMI: Body mass index; ASA: American Society of Anesthesiologists; LNM: Lymph node metastasis; RLN: Retrieved lymph nodes; AV: Anal verge; PAD: Percutaneous abscess drainage; AL: Anastomotic leakage; ICU: Intensive care unit; OR: Odds ratio.

in nine, and fistula in 14. Of these, 87.5% of stomas with sinus formation and 66.7% of those with stricture were closed, but only 35.7% of stomas with prolonged fistula could be closed. A log-rank test revealed a difference in the risk of PS between AL complication types with borderline significance (P = 0.05; Figure 2).

There was a statistically significant difference between the PS and no PS group in terms of tumor location from the anal verge, duration of antibiotic use, ICU transfer, and multi-organ failure (Table 3). Multivariate analysis showed that a tumor location farther from the anal verge was associated with decreased risk of PS (OR = 0.751; 95%CI: 0.567-0.994; P = 0.045) (Table 5).

DISCUSSION

To our knowledge, this is the first study of the structural sequelae of AL to include its prognosis and risk factors. Although several studies have been performed to identify the factors influencing PS[14-16], we identified the risk of structural sequelae that can

Table 3 Univariate analysis of differences between the permanent stoma and no permanent stoma groups (n = 79) n (%)

	PS	No PS	OR	95%CI		*P* value
				Lower	Upper	
Age, median (yr)	58	60				0.772
> 65	4 (28.6)	25 (38.5)	0.250	0.181	2.262	0.488
Gender						
Male	10 (71.4)	45 (69.2)	1.111	0.311	3.971	0.871
BMI, mean	24.29	24.16				0.893
ASA score						
Ⅰ	8 (57.1)	26 (40.0)				
Ⅱ/Ⅲ	6 (42.9)	39 (60.0)	0.500	0.155	1.609	0.245
Surgical time, mean (min)	274.79	269.63				0.827
Ileostomy	11 (78.6)	34 (52.3)	3.343	0.853	13.107	0.083
LNM, mean	2.21	1.06				0.299
RLN, mean	20.57	23.11				0.561
Stage						
0/Ⅰ/Ⅱ	9 (64.3)	23 (58.5)				
Ⅲ/Ⅳ	5 (35.7)	23 (41.5)	0.782	0.236	2.594	0.688
Tumor location, median (centimeters from AV)	3	5				0.029
Neoadjuvant chemoradiotherapy	5 (35.7)	14 (21.5)	2.204	0.584	7.014	0.266
Surgical method						0.438
Conventional	0 (0)	3 (4.6)				
Laparoscopic	10 (71.4)	35 (53.8)				
Robotic	3 (21.4)	25 (38.5)				
Conversion	1 (7.1)	2 (3.1)				
Anastomosis						0.545
Hand-sewn	1 (7.7)	9 (13.8)				
Stapling	12 (92.3)	56 (86.2)				
Anastomosis type						0.129
End-to-end	12 (85.7)	58 (89.2)				
End-to-side	2 (14.3)	3 (4.6)				
Colonic J-pouch	0	4 (6.2)				
Mechanical bowel preparation						
Yes	5 (35.7)	17 (26.2)				
No	9 (64.3)	48 (73.8)	0.638	0.187	2.171	0.471
Initial AL management						0.633
Conservative	4 (28.6)	29 (44.6)				
PAD	4 (28.6)	13 (20.0)				
Diversion only	2 (14.3)	12 (18.5)				
Primary repair and diversion	0	1 (1.5)				
Surgical irrigation and drainage	3 (21.4)	7 (10.8)				
Re-anastomosis and diversion	0	2 (3.1)				
Hartmann's procedure	1 (7.1)	1 (1.5)				
Transfusion	5 (35.7)	10 (15.4)	3.056	0.846	11.036	0.088
Leakage, mean (d)	5.0	4.7				0.768
Hospital stay, mean (d)	45.9	29.1				0.121
Days to start diet, mean (d)	7.5	5.8				0.427
Antibiotics use, mean (d)	26.8	17.5				0.018
ICU transfer	4 (28.6)	5 (7.7)	4.800	1.098	20.990	0.037
Multi-organ failure	3 (21.4)	3 (4.6)	5.636	1.005	31.602	0.049
Postoperative ileus	1 (7.1)	8 (12.3)	0.548	0.063	4.773	0.586

Data are expressed as n (%), mean or median. PS: Permanent stoma; BMI: Body mass index; ASA: American Society of Anesthesiologists; LNM: Lymph node metastasis; RLN: Retrieved lymph nodes; AV: Anal verge; AL: Anastomotic leakage; PAD: Percutaneous abscess drainage; ICU: Intensive care unit; OR: Odds ratio.

occur after leakage as well as the risk of having a PS of patients who underwent surgical resection for mid to low rectal cancer.

Temporary diversion with ileostomy is frequently performed after high-risk anastomosis. Several factors are associated with high-risk anastomosis including preoperative radiotherapy, male gender, low-level anastomosis, co-morbidities, steroid use, and obesity[9-11]. However, the decision to perform diversion ileostomy depends on the surgeon. Although ileostomy after rectal surgery could be considered a subjective variable, diversion turned out to be the single most predictive risk factor of structural sequelae of AL in the current study. Diversion after surgical resection of mid to low rectal cancer can be interpreted to indicate that the anastomosis was unstable for various reasons, including preoperative radiotherapy, difficult procedure due to a deep and narrow pelvis (as is common in male patients), or very a low-level anastomosis.

Diversion for protection against AL or a defunctioning

Table 4 Multivariate analysis of the sequelae and no sequelae groups including variables selected on univariate analysis

	OR	95%CI		*P* value
		Lower	Upper	
Age	1.035	0.979	1.095	0.378
Ileostomy	6.741	1.069	13.372	0.017
Stage (Ⅲ/Ⅳ)	0.292	0.079	1.077	0.064
Antibiotics use (d)	1.016	0.954	1.085	0.613
Hospital stay (d)	1.045	0.987	1.106	0.135
Transfusion	5.760	0.787	42.138	0.085

OR: Odds ratio.

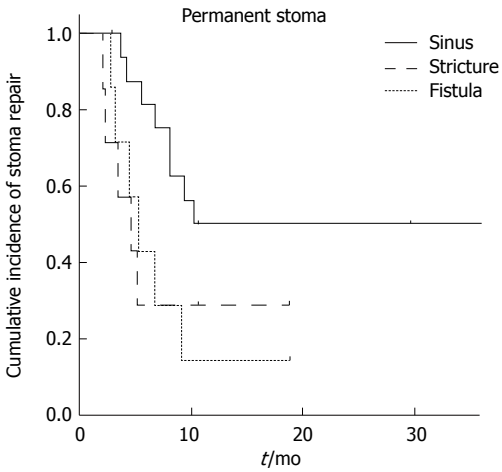

Figure 2 Cumulative incidence of stoma repair according to the anastomotic leakage sequelae type during 3 years following the operation. The log-rank test revealed a difference among the three types of structural sequelae in terms of permanent stoma incidence with borderline significance (P = 0.05).

stoma can reduce the risk of anastomosis failure[17,18]. In a randomized multicenter trial, Matthiessen *et al*[19] reported that a defunctioning stoma could reduce the risk of AL (OR = 3.4; P < 0.001). However, the results of our study showed that if AL had already occurred, diversion was the most predictive factor of structural sequelae of AL. The significant difference in hospital stay and duration of antibiotic use between the sequelae and no sequelae groups on univariate analysis is thought to reflect the association between the development of AL complications and its severity. In addition, transfusion is usually performed when the procedures are difficult for various reasons such as severe adhesion, narrow pelvis, and an advanced cancer lesion.

Dinnewitzer *et al*[16] reported that coloanal anastomosis and anastomotic leakage were risk factors for PS on multivariate analysis. den Dulk *et al*[20] reported that postoperative complications and secondary stoma formation were limiting factors for stoma reversal in patients undergoing total mesorectal excision (TME) for rectal cancer. We found that all protective diversions were repaired in patients who did not have structural sequelae of AL, whereas only 51.7% of patients in the

Table 5 Multivariate analysis of the permanent stoma and no permanent stoma groups with factors selected on univariate analysis

	OR	95%CI		*P* value
		Lower	Upper	
Tumor location	0.751	0.567	0.994	0.045
Antibiotic use	1.036	0.990	1.084	0.125
ICU transfer	4.184	0.277	63.162	0.301
Multi-organ failure	0.685	0.027	17.426	0.528

OR: Odds ratio; ICU: Intensive care unit.

sequelae group had repairable stomas. Our results also showed that a higher cancer lesion location decreased the risk of PS (OR = 0.751; P = 0.045). Univariate analysis showed that ICU transfer and multi-organ failure were associated with PS (Table 3). A patient's postoperative condition might affect the decision to perform stoma repair.

The prognosis of patients who experience AL after colorectal surgery for colorectal cancer is known to be worse than that of those who do not[21-24]. We did not compare the leakage and non-leakage groups, but 5-year progression-free survival was significantly decreased in the patients who could not undergo stoma repair compared to those of patients who could (data now shown). Dekker *et al*[25] showed the importance of the first postoperative year for the prognosis of patients with colorectal cancer. Our finding of a worse 5-year progression-free survival rate of the PS group suggest that post-leakage structural sequelae should be a concern in the consideration of cancer prognosis.

We could not collect data on anorectal function for all of the included patients. Patients who had their stomas repaired might have problems with long-term anorectal function[26,27]. By adding functional data, we would learn more about the prognosis of structural sequelae of AL. Moreover, there were no standard protocols for the choice of management options for the structural sequelae of AL; rather, it depended on each physician's choice[28-30]. Nonetheless, there was no statistically significant difference in management methods between the study groups (Tables 2 and 4).

In conclusion, even with proper management, patients undergoing rectal surgery may experience structural sequelae of anastomotic leakage. Although there are several reasons to perform diversion, our study showed that performing ileostomy significantly increased the risk of structural sequelae of AL and that a lower cancer lesion location was a risk factor for PS.

COMMENTS

Background

Anastomotic leakage is one of the significant complications experienced by patients with rectal cancer. However, even after proper management is provided for anastomotic leakage, patients my still develop structural sequelae

of anastomotic leakage and the symptoms caused by them.

Innovations and breakthroughs

By retrospectively analyzing the data, the authors described the fate of anastomotic leakage and risk factors that cause the structural sequelae of anastomotic leakage.

Peer-review

In this study, the authors reviewed experience with anastomotic leakage in patients with mid to low rectal cancer to identify the risk factors of the structural sequelae of anastomotic leakage and permanent stoma. They concluded that previous diversion ileostomy was a risk factor of the structural sequelae of anastomotic leakage and a low cancer lesion location was a risk factor of permanent stoma.

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ORIGINAL ARTICLE

Retrospective Study

Needle-knife fistulotomy *vs* double-guidewire technique in patients with repetitive unintentional pancreatic cannulations

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Author contributions: Kim SJ collected the clinical information and was the main author of the manuscript; Kang DH designed the study and performed ERCP; Kim HW performed ERCP; Choi CW performed the statistical analysis; Park SB, Song BJ, Hong YM participated in its design and coordination and helped to draft the manuscript; all authors read and approved the final manuscript.

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Ethics approval: The study was reviewed and approved by the Pusan National University Yangsan Hospital Institutional Review Board.

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

Conflict-of-interest: The authors have no conflicts of interest.

Data sharing: Technical appendix, statistical code, and dataset available from the corresponding author at sulsulpul@naver.com. Participants gave informed consent for data sharing.

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Abstract

AIM: To compare the success rates and adverse events of early needle-knife fistulotomy (NKF) and double-guidewire technique (DGT) in patients with repetitive unintentional pancreatic cannulations.

METHODS: From a total of 1650 patients admitted for diagnostic or therapeutic endoscopic retrograde cholangiopancreatography (ERCP) at a single tertiary care hospital (Pusan National University Yangsan Hospital, Yangsan, South Korea) between January 2009 and December 2012, 134 (8.1%) patients with unsuccessful biliary cannulation after 5 min trial of conventional methods, together with 5 or more repetitive unintentional pancreatic cannulations, were enrolled in the study. Early NKF and DGT groups were assigned 67 patients each. In the DGT group, NKF was performed for an additional 7 min if successful cannulation was not achieved.

RESULTS: The success rates with early NKF and

the DGT were 79.1% (53/67) and 44.8% (30/67) (P < 0.001), respectively. The incidence of post-ERCP pancreatitis (PEP) was lower in the early NKF group than in the DGT group [4.5% (3/67) *vs* 14.9% (10/67), P = 0.041]. The mean cannulation times in the early NKF and DGT groups after assignment were 257 s and 312 s (P = 0.013), respectively.

CONCLUSION: Our data suggest that early NKF should be considered as the first approach to selective biliary cannulation in patients with repetitive unintentional pancreatic cannulations.

Key words: Endoscopic retrograde cholangiopancreatography; Cannulation; Pancreatitis; Needle knife fistulotomy; Double guidewire technique

Core tip: This retrospective single center analysis of outcomes of early needle-knife fistulotomy (NKF) and double-guidewire technique (DGT) revealed that early NKF has a higher success rate of selective biliary cannulation with a lower incidence of post-endoscopic retrograde cholangiopancreatography pancreatitis and shorter procedural time than DGT in patients with repetitive unintentional pancreatic cannulations.

Kim SJ, Kang DH, Kim HW, Choi CW, Park SB, Song BJ, Hong YM. Needle-knife fistulotomy *vs* double-guidewire technique in patients with repetitive unintentional pancreatic cannulations. *World J Gastroenterol* 2015; 21(19): 5918-5925 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5918.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5918

INTRODUCTION

Successful cannulation of an intended duct is the most important first step for effective biliary and pancreatic procedures during endoscopic retrograde cholangiopancreatography (ERCP)[1]. The success rates of conventional methods for biliary cannulation range from 80% to 95%[1-3]. When conventional methods fail to achieve selective cannulation, various alternative techniques can be used. However, prolonged and repetitive manipulation of the papilla during various procedures increases the risk of post-ERCP pancreatitis (PEP)[4]. Therefore, much effort has been made to develop useful endoscopic techniques in order to perform successful cannulation and reduce PEP. The most commonly used technique in patients with difficult cannulation is precut sphincterotomy, including needle-knife fistulotomy (NKF) and needle-knife papillotomy[5-7]. NKF could be safer than needle-knife papillotomy in terms of PEP because an incision is made a few millimeters apart from the papillary orifice[8,9]. A recent study also reported that early use of NKF in experienced hands is safe and effective[10-12].

The double-guidewire technique (DGT) has also been introduced as a useful method for overcoming difficult biliary cannulation[13-15]. In a previous study comparing DGT with precut sphincterotomy technique, the former required a significantly shorter procedural time but still showed a similar success rate as the latter in biliary cannulation. However, it induced pancreatitis more frequently[16]. A recent study reported that a novel sequential 3-step protocol (traditional cannula with guidewire, DGT, and then NKF) showed an even higher success rate of biliary cannulation (99%)[17]. But 2 prospective randomized studies comparing DGT with using conventional methods in patients with difficult biliary cannulation showed controversial results in terms of selective biliary cannulation and PEP[13,18]. Maeda *et al*[13] reported that DGT showed a higher cannulation rate with no PEP than the conventional technique (93% and 58%, respectively). However, Herreros de Tejada *et al*[18] reported that DGT was not superior to the standard cannulation technique (success rates; 47% and 56%, respectively) and was associated with frequent PEP (17% and 8%, respectively)[18]. Therefore, the present study was performed to evaluate whether early NKF or DGT is useful for overcoming difficulty in patients with repetitive unintentional pancreatic cannulations.

MATERIALS AND METHODS

Patients and study design

Between January 2009 and December 2012, a total of 1650 patients with pancreaticobiliary disorders and naïve papillae who were admitted for diagnostic or therapeutic ERCP at a single tertiary care hospital (Pusan National University Yangsan Hospital, Yangsan, Korea) and who gave written informed consent were included in the study. Patients were excluded from the study if one of the following criteria was present: age younger than 15 years, previous surgical biliary-intestinal operations, tumor in the ampulla of Vater, clinical evidence of acute pancreatitis at the time of procedure, coagulopathy, and pregnancy. In patients for whom we had failed to achieve biliary cannulation after attempting for 5 min, accompanied by repetitive pancreatic duct cannulations, early NKF or DGT was performed to achieve biliary cannulation. The data were collected prospectively, but data analysis was done retrospectively.

In the DGT group, NKF was performed for an additional 7 min if successful cannulation was not achieved during the trial of DGT. In the early NKF group, endoscopists finished the ERCP without an additional procedure after failure of NKF. Of the 1650 patients with naïve ampullae, 1340 (81.2%) underwent selective cannulation without difficulty, within 5 min. The incidence of PEP was 2.5% in the successful cannulation group without difficulty. Of the 310 patients in whom we did not achieve successful

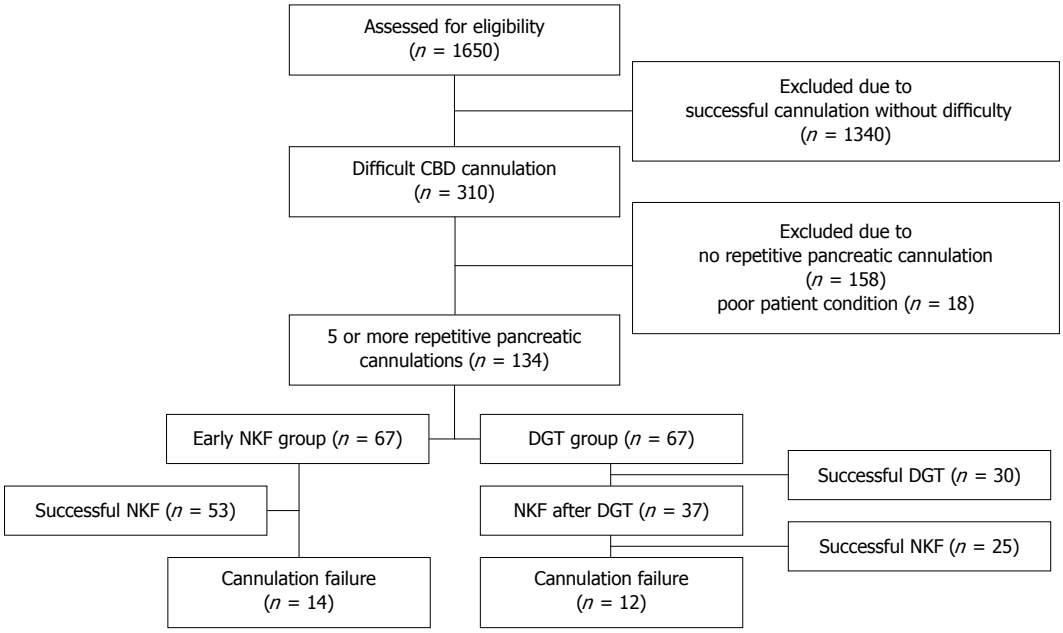

Figure 1 Study flow diagram. CBD: Common bile duct; NKF: Needle-knife fistulotomy; DGT: Double-guidewire technique.

cannulation using the conventional method, 158 patients without repetitive pancreatic cannulation using the conventional method were excluded because of multiple failed attempts to insert the guidewire into the pancreatic duct, which caused excessive edema and papillary trauma and could increase the incidence of PEP. The poor condition/cooperation of 18 patients prevented continued ERCP after conventional method failure. The remaining 134 patients (43.2%) with 5 or more repetitive unintentional pancreatic cannulations were assigned to the early NKF or DGT group in alternating sequence for selective biliary cannulation (Figure 1).

Two endoscopists who had clinical experience with ERCP for 10 to 20 years participated in the present study; both of them also had experience with NKF and DGT in patients with difficult cannulation. The numbers of patients were distributed equally by two experts.

Our hypothesis was that in patients with repetitive unintentional pancreatic cannulation, NKF has a higher success rate to achieve biliary access compared with DGT. Power calculations indicated that a sample size of 140 patients (70 in each group) was needed for an α error of 0.05 and 95% power, based on an expected 80% of cannulation success rate in patients with repetitive unintentional pancreatic cannulations by early NKF and 50% by DGT. This proportion was based on the results of a previous prospective, randomized trial evaluation NKF or DGT[2,16,18]. The study was approved by the Ethics Committee of the Pusan National University Yangsan Hospital (IRB No. 05-2014-036) and written informed consent from all patients was obtained prior to study inclusion.

Definitions

Difficult cannulation was defined as failed selective biliary cannulation within 5 min of using the conventional method, regardless of the number of unintentional pancreatic cannulations. We regarded 5 or more guidewire passings or contrast injections through the pancreatic duct as repetitive pancreatic cannulations. PEP was defined as a new-onset or increased abdominal pain persisting for at least 24 h after the procedure with serum amylase levels 3 times the upper normal limit. The severity of pancreatitis was classified as mild if hospitalization was extended 2 to 3 d after the ERCP, moderate if hospitalization was extended 4 to 10 d, and severe if hospitalization was extended for more than 10 d. Asymptomatic hyperamylasemia after ERCP was defined as 3-fold or greater increase in serum amylase levels at 24 h after the procedure without abdominal pain. Baseline and 24 h post-ERCP serum amylase levels were obtained in all patients. Bleeding was defined as clinically apparent with a decrease in the hemoglobin level higher than 2 g/dL. Failure was defined when biliary cannulation was not achieved within 7 min of trial. The procedural time was defined as the time lapse from assignment to cannulation.

Endoscopic procedure

All patients underwent ERCP using a side-view duodenoscope (JF-240 or TJF-240; Olympus Optical Co, Ltd, Tokyo, Japan). A sphincterotome (Ultratome XL, Boston Scientific, Natick, Mass) or an ERCP catheter (Fluoro Tip, Boston Scientific) with a hydrophilic guidewire (0.025- or 0.035-inch Jagwire, Boston Scientific) was used for initial cannulation. Patients

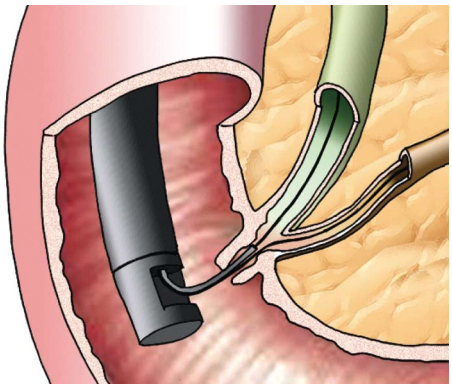

Figure 2 Common bile duct cannulation with a catheter preloaded with another guidewire after previous insertion of a guidewire in pancreatic duct.

Table 1 Baseline characteristics (n = 134) n (%)

	Early NKF group	DGT group	*P* value
Age (yr), mean ± SD	65.5 ± 13.1	65.2 ± 14.0	0.889
Male sex	33 (49.3)	34 (50.7)	0.863
ERCP indications			0.165
Choledocholithiasis	30 (44.8)	38 (56.7)	
Malignant stricture	32 (47.8)	20 (29.9)	
Benign biliary stricture	4 (6.0)	7 (10.4)	
Other	1 (1.5)	2 (3.0)	
Periampullary diverticulum	9 (13.4)	25 (37.3)	0.001
Intradiverticular ampulla	0	2	
Juxtadiverticular ampulla	9	23	
Pancreas duct stent	4 (6.0)	5 (7.5)	1.000

NKF: Needle-knife fistulotomy; DGT: Double-guidewire technique.

with 5 or more repetitive unintentional pancreatic cannulations were assigned to either NKF or DGT. NKF was done with a needle knife (MicroKnife XL, Boston Scientific). A fistulotomy was performed by making a puncture at the most prominent portion of the papillary roof and then cutting downward toward the papillary orifice. After needle puncture of the bile duct, a sphincterotome with guidewire was used to cannulate the common bile duct. After successful cannulation, endoscopic sphincterotomy was performed. DGT was performed with a device preloaded with a guidewire (Figure 2). Insertion of the guidewire into the main pancreatic duct was guided by fluoroscopy. Placement of a guidewire in the pancreatic duct facilitated cannulation of the bile duct with another sphincterotome or catheter *via* the same working channel alongside the first guidewire. In cases of failed selective biliary cannulation using DGT, NKF was attempted as a rescue procedure. We placed a pancreatic plastic stent when patients were considered to be at high-risk of PEP (more than 2 of the following risk factors: suspected dysfunction of the sphincter of Oddi, young women, injection of contrast into the pancreatic duct, or > 12 min of cannulation attempt). If the patient's condition permitted delay of procedure for the therapeutic purpose, we performed repeated ERCP in a short interval (at least 2 d after first ERCP). ERCP was performed under conscious sedation using midazolam and pethidine. All patients received pharmacologic prophylaxis of PEP using nafamostat mesilate (Futhan; SK chemicals Life Science Biz, Seoul, Korea).

Statistical analysis

All statistical analyses were performed using SPSS software version 19.0 (SPSS, Chicago, IL, United States). Continuous data are summarized as mean ± SD. The χ^2 test or *F*-test and *t* test were used for the comparison of categorical variables when appropriate. Statistical significance was set at $P < 0.05$.

The statistical methods of this study were reviewed by Jun Hee Han from Research and Statistical Support, Research Institute of Convergence for Biomedical Science and Technology, Pusan National University Yangsan Hospital.

RESULTS

Of the 310 patients who did not achieve successful cannulation using the conventional method, the success rate using NKF of 158 patients (50.1%) without repetitive pancreatic cannulation was 81.6% (129/158), and the rate of PEP was 7.6% (12/158). For 18 patients (5.8%) with poor condition, an alternative approach could not be permitted after selective cannulation failure and the incidence of PEP was 5.5% (1/18). The remaining 134 patients (43.2%) with 5 or more repetitive unintentional pancreatic cannulations were assigned to the early NKF or DGT group in alternating sequence for selective biliary cannulation (Figure 1).

The patient characteristics of both the early NKF and DGT groups are summarized in Table 1. There were no significant differences in the baseline characteristics except for incidence of periampullary diverticula between the early NKF and DGT groups [9 patients (13.4%) *vs* 25 patients (37.3%), $P = 0.001$]. The most common indication for ERCP was biliary lithiasis (50.7%, 68/134). Pancreatic stents were successfully placed in all patients who were considered to be at high risk of PEP. There was no difference in the percentage of patients regarding the use of a prophylactic pancreatic duct stent (6.0% in early NKF group and 7.5% in DGT group, $P = 1.000$).

Successful cannulation

In the early NKF group, successful cannulation was achieved in 53 patients (79.1%). Of the 14 patients with unsuccessful cannulation after the first ERCP, 3, 2, and 9 patients underwent percutaneous transhepatic biliary drainage (PTBD), magnetic resonance cholangiopancreatography (MRCP), and second ERCP, respectively, within 2 d after the ERCP. Of patients undergoing second ERCPs, 8 patients achieved successful cannulation. The overall success rate of

Table 2 Clinical outcomes of the patients *n* (%)

	Early NKF group	DGT group	*P* value
Success rate of assigned technique	53 (79.1)	30 (44.8)	< 0.001
Total success rate of			
1st ERCP	53 (79.1)	55 (82.1)	0.662
2nd ERCP	61 (91.0)	62 (92.5)	0.753
Cannulation time after assignment, mean ± SD	4 min 17 s ± 115 s	5 min 12 s ± 137 s	0.013
Pancreatitis, total	3 (4.5)	10 (14.9)	0.041
Mild	1	5	
Moderate	2	5	
Hyperamylasemia	20 (29.9)	21 (31.3)	0.851
Perforation[1]	0	1	1.000

[1]A perforation occurred during NKF in the DGT group and was treated conservatively. ERCP: Endoscopic retrograde cholangiopancreatography; NKF: Needle-knife fistulotomy; DGT: Double-guidewire technique.

biliary cannulation was 91.0% in the early NKF group including second ERCPs (61/67).

Successful cannulation was achieved in 30 patients (44.8%) of the DGT group. These included 5 patients who obtained wire-guided direct biliary cannulation without DGT (during trials to place a pancreatic guidewire). The cross-over treatment from DGT to NKF was performed in 37 patients who failed biliary cannulation using DGT. Additional success using NFK was achieved in 25 patients (67.5%, 25/37). The overall success rate of biliary cannulation was 82.1% (55/67) in the DGT group. Of the 12 patients with unsuccessful biliary cannulation during first ERCP, 2, 2, and 8 patients underwent PTBD, endoscopic ultrasonography (EUS), and second ERCP, respectively. Of the patients undergoing second ERCPs, 7 patients had successful biliary cannulation. The overall success rate of biliary cannulation was 92.5% in the DGT group including second ERCPs (62/67) (Table 2).

The initial success rate was higher in the early NKF group than in the DGT group (79.1% *vs* 44.8%, $P < 0.001$). With an additional NKF as a rescue procedure, the DGT group achieved similar success rate as early NKF alone (82.1% *vs* 79.1%, $P = 0.663$). The average procedural time was 4 min 17 s ± 115 s in the early NKF group and 5 min 12 s ± 137 s in the DGT group ($P = 0.013$).

Adverse events

The early NKF group showed a significantly lower incidence of PEP than the DGT group [3 patients (4.5%) *vs* 10 patients (14.9%), $P = 0.041$]. In all patients with prophylactic pancreatic duct stent, PEP was not observed. The severity of pancreatitis was mild ($n = 1$) and moderate ($n = 2$) in the early NKF group, and it was mild ($n = 5$), and moderate ($n = 5$) in the DGT group. No severe PEP occurred in either group. The incidence of hyperamylasemia showed no significant difference between the early NKF and DGT groups [20 patients (29.9%) *vs* 21 patients (31.3%), $P = 0.851$] (Table 2).

No post-NKF bleeding was observed in either group. A single case of perforation occurred during NKF in the DGT group, which was conservatively treated without any eventful outcomes. There was no death related to ERCP in either group.

Patients with periampullary diverticula

In patients with diverticula, the initial success rate of biliary cannulation was higher in the early NKF group than in the DGT group [77.8% (7/9) *vs* 48.0% (12/25), $P = 0.240$], although the number of each group was too small to conclude statistical significance ($P = 0.240$). NKF was performed on 13 patients who failed biliary cannulation using DGT. The success of biliary cannulation using NKF after DGT failure was observed in 8 patients (61.5%; 8/13). The DGT subgroup (80.0%) that had additional NKF achieved similar success rate to the early NKF subgroup (77.8%; $P = 1.000$).

A difference of PEP incidence between the early NKF subgroup (0%; 0 of 9) and DGT subgroup (8.0%; 2 of 25) was observed. Pancreatitis severity was mild ($n = 2$) in all subgroup patients.

DISCUSSION

When conventional methods are unsuccessful in achieving deep biliary cannulation, precut sphincterotomy or DGT can be used[1]. NKF is a needle-knife precutting method which has been commonly used to overcome difficult cannulation[5]. It can reduce the risk of PEP by making an incision a few millimeters from the papillary orifice and preventing a direct mechanical injury to the pancreatic duct orifice. Many meta-analyses advocate that early precutting should be performed to reduce the incidence of PEP by decreasing the number of cannulation attempts and unintentional pancreatic cannulations[19-21]. A recent prospective cohort study concluded that if the endoscopist is experienced in ERCP and precut techniques, an early precut strategy should be the preferred cannulation strategy because of its safety and effectiveness[22].

Since its first description by Dumonceau *et al*[23], DGT has been performed as a promising technique to overcome difficult cannulation and has been widely used in patients with repetitive pancreatic cannulations. Placement of a guidewire deep into the main pancreatic duct facilitates cannulation of the bile duct by providing a variety of benefits, such as opening a stenotic papillary orifice, stabilizing the papilla, lifting the papilla toward the working channel, straightening the pancreatic duct and common channel, or potentially minimizing repetitive injections into the pancreatic duct[1].

In 2009, an algorithm for biliary cannulation suggested that needle-knife sphincterotomy should be performed to overcome difficult cannulation after a

Table 3 The success rate of biliary cannulation and the incidence of post-endoscopic retrograde cholangiopancreatography pancreatitis in double-guidewire technique in 5 randomized studies *n* (%)

Study	Study design	Patients screened	Patients randomized DGT	Timing of DGT	Success rate	Inadvertent success in DGT	PEP	Failure
Maeda *et al*[13], 2003	Single-center randomized study	107	27 (25)	Unsuccessful within 10 min	93%	NA	0%	NA
Herreros de Tejada *et al*[18], 2009	Multicenter randomized study	845	97 (11)	After 5 attempts	47%	18%	17%	10 more attempts
Angsuwatcharakon *et al*[16], 2012	Single-center randomized study	426	23 (5)	Unsuccessful within 10 min	73.9%	NA	21.7%	Another 10 min
Coté *et al*[27], 2012	Two-center randomized study	442	42 (10)	Unsuccessful within 6 min or 3 PD cannulations	54.8%	16.7%	2.4%	Another 6 min
Yoo *et al*[28], 2013	Single-center randomized study	1394	34 (2)	Unsuccessful within 10 min	79.4%	NA	38.2%	10 more attempts

PEP: Post-endoscopic retrograde cholangiopancreatography pancreatitis; DGT: Double-guidewire technique; PD: Pancreatic duct; NA: Not available.

minimum of 4 attempts using conventional methods. DGT or pancreatic duct stent insertion should be considered when repetitive unintentional pancreatic cannulations take place without selective biliary access[24]. However, compared to consistently high success rates of biliary cannulation using NKF (83% to 96%), DGT showed marked variation of successful biliary cannulation rates (47% to 92.6%)[9,11,13,16,18,25]. In addition, a previous study reported that the success rate of biliary cannulation using DGT was just 43.8% (49/112) in patients with repetitive pancreatic duct cannulations[26]. Therefore, there is the need to evaluate the usefulness of early NKF and DGT for selective biliary cannulation in patients with repetitive unintentional pancreatic cannulations.

Our results showed a low success rate of biliary cannulation using DGT (44.8%) compared to early NKF (79.1%). The success rate of biliary cannulation using DGT in our study is similar to those reported by Herreros de Tejada *et al*[18] (47%) and Coté *et al*[27] (54.8%). Considering cases with inadvertent success in those studies, our result is somewhat better. The results from other studies summarized in Table 3 show higher success rates (73.9%-93%) without any description of unintentional biliary cannulations[13,16,28]. Our low success rate of biliary cannulation could be associated with a shorter procedural time and a slightly larger number of patients. Compared to procedural time limits (6-7 min) of studies with low success rates of biliary cannulation using DGT, studies with high success rates had longer procedural times (another 10 min and 10 more attempts without the time limit)[16,27,28]. Although a long procedural time increased the success rate of biliary cannulation using DGT to 73.9% or 79.4%, the incidence of PEP was markedly increased to 21.7% or 38.2% compared to 2.4% or 17% in studies with low success rates[16,18,27,28]. A low success rate of biliary cannulation was also observed in a multicenter study with a relatively large number of patients[18]. In contrast, previous studies that were performed on a small number of patients in a single center and by a single endoscopist showed high success rates of biliary cannulation[16,28]. In addition, a study with the highest success rate of biliary cannulation, shown in Table 3, reported the difficult cannulation rate was as high as 49.5%[13]. Therefore, a selection bias caused by a small sample size and a single endoscopist can lead to a good success rate of biliary cannulation using DGT.

The success rate of biliary cannulation using a stepwise approach (DGT and NKF sequentially) in the DGT group was similar to that in the early NKF group alone (82.1% *vs* 79.2%, $P = 0.665$). This means that DGT itself does not provide additional advantages in achieving selective biliary cannulation. Instead, a long procedural time is required because half of the patients not only failed cannulation using DGT, but also underwent additional NKFs to rescue DGT failure. In addition, the DGT group (14.9%) showed a significantly higher incidence of PEP than the early NKF group (4.2%). Repetitive contact with the papilla during DGT and a longer procedural time may be the reason for the high incidence of PEP. Pancreatic stent placement could be useful for reducing the incidence of PEP[29,30]. However, failure of pancreatic stent placement can cause severe forms of pancreatitis[31]. Therefore, it is appropriate to place a pancreas stent selectively in a high-risk group. Considering these results, DGT is frequently complicated by pancreatitis and requires more time to achieve a similar success rate to NKF.

There was a significant difference in the baseline characteristics of periampullary diverticula between

the 2 groups. The success rate of biliary cannulation using NKF was not significantly different between patients with periampullary diverticula and those without [77.8% (7/9) *vs* 79.5% (46/58)]. As for DGT, there was also no significant difference in the success rate of biliary cannulation whether periampullary diverticula were present or not [48% (12/25) *vs* 42% (18/42)]. The presence of periampullary diverticula did not influence the success rate of biliary cannulation. Despite the small number of patients in the early NKF group, our results are consistent with a previous study which reported that NKF can be performed effectively and safely in patients with periampullary diverticula and difficult biliary cannulation[32].

Considering a low success rate of biliary cannulation, a long procedural time, and a high incidence of PEP in the DGT group, NKF should be performed as early as possible in patients with repetitive unintentional pancreatic duct cannulations. Subsequent options to pursue when NKF fails include repeated ERCP attempts or consideration of alternative approaches, such as percutaneous or EUS duct-access procedures. DGT could be considered in special conditions, such as a very small and flat papilla in which NKF has high risk for perforation, or the location of the papilla in the lower rim or just inside the diverticulum where DGT can evert the papilla to the duodenal lumen. DGT can help less experienced endoscopists to avoid the risks of precut sphincterotomy, such as bleeding and perforation.

This study has several limitations. Firstly, the study was not a randomized prospective controlled study and this could cause uneven distribution of patients with periampullary diverticula among the two groups. Endoscopists knew the assigned method before the allocation; this might have influenced the decision to include patients in this study. Secondly, it has a single-center design with small sample size, although its sample size is relatively large compared to that of previous studies. This might influence the interpretation of the differences in adverse events including PEP. Finally, this study was done by very experienced endoscopists, limiting the generalizability of this study finding. Therefore, large-scale prospective multicenter studies are needed to overcome these limitations. However, we think that a strengthening factor in the current study includes minimizing PEP risk caused by unsuccessful conventional cannulations by using the limit of 5 min before allocation into an assigned method, and it is the first study to compare early NKF and DGT in patients with repetitive unintentional pancreatic cannulations.

In conclusion, in patients with repetitive unintentional pancreatic cannulations, NKF had a higher success rate of selective biliary cannulation with a lower incidence of PEP than DGT. Therefore, these data suggest that early NKF should be considered as the first approach to selective biliary cannulation in such patients.

COMMENTS

Background

Precut sphincterotomy using needle-knife fistulotomy (NKF) or needle-knife papillotomy has been used in patients with failed conventional biliary cannulation. Double-guidewire technique (DGT) has also been reported to be useful for difficult biliary cannulation.

Research frontiers

Most previous studies evaluated the outcomes of DGT compared to precut sphinterotomy or standard cannulation technique to overcome difficult biliary cannulation. There has been no study to compare the usefulness of early NKF and DGT in patients with repetitive unintentional pancreatic cannulations.

Innovations and breakthroughs

An algorithm for biliary cannulation suggested by Bourke *et al* described that DGT or pancreatic duct stent insertion should be considered when repetitive unintentional pancreatic cannulations take place without selective biliary access. The authors measured the usefulness of early NKF and DGT for selective biliary cannulation in patients with repetitive unintentional pancreatic cannulations.

Applications

Early NKF should be considered as the first approach to selective biliary cannulation in patients with repetitive unintentional pancreatic cannulations. DGT can help less experienced endoscopists to avoid the risks of precut sphincterotomy, such as bleeding and perforation.

Terminology

Although there is no universally agreed cut-off for difficult cannulation, the authors defined an unsuccessful biliary cannulation within 5 min of using the conventional method as failure, regardless of the number of unintentional pancreatic cannulations. The authors regarded 5 or more guidewire passings or contrast injections through the pancreatic duct as repetitive pancreatic cannulations.

Peer-review

This article is about selective biliary cannulation techniques for the difficult biliary cannulation cases. The authors especially compared early NKF with DGT, and concluded that early NFK achieved a higher success rate of cannulation with a lower incidence of PEP. We often experience difficult cannulation cases, and we try DGT or some precut techniques. This paper is very important clinically and interesting for many endoscopists who perform ERCP.

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ORIGINAL ARTICLE

Retrospective Study

Risk scoring system and predictor for clinically relevant pancreatic fistula after pancreaticoduodenectomy

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Abstract

AIM: To establish a scoring system to predict clinically relevant postoperative pancreatic fistula (CR-POPF) after pancreaticoduodenectomy (PD).

METHODS: The clinical records of 921 consecutive patients who underwent PD between 2008 and 2013 were reviewed retrospectively. Postoperative pancreatic fistula (POPF) was defined and classified by the international study group of pancreatic fistula (ISGPF). We used a logistic regression model to determine the independent risk factors of CR-POPF and developed a scoring system based on the regression coefficient of the logistic regression model. The optimal cut-off value to divide the risk strata was determined by the Youden index. The patients were divided into two groups (low risk and high risk). The independent sample *t* test was used to detect differences in the means of drain amylase on postoperative day (POD) 1, 2 and 3. The optimal cut-off level of the drain amylase to distinguish CR-POPF from non-clinical POPF in the two risk strata groups was determined using the receiver operating characteristic (ROC) curves.

RESULTS: Grade A POPF occurred in 106 (11.5%) patients, grade B occurred in 57 (6.2%) patients, and grade C occurred in 32 (3.5%) patients. A predictive scoring system for CR-POPF (0-6 points) was constructed using the following four factors: 1 point for each body mass index $\geqslant$ 28 [odds ratio (OR) = 3.86; 95% confidence interval (CI): 1.92-7.75, P = 0.00], soft gland texture (OR = 4.50; 95%CI, 2.53-7.98, P = 0.00), and the difference between the blood loss and transfusion in operation $\geqslant$ 800 mL (OR = 3.45; 95%CI, 1.92-7.75, P = 0.00); and from 0 points for a 5 mm or greater duct diameter to 3 points for a less than 2 mm duct (OR = 8.97; 95%CI: 3.70-21.77, P = 0.00). The ROC curve showed that the area under the curve of this score was 0.812. A score of 3 points was suggested to be the best cut-off value (Youden index = 0.485). In the low risk group, a drain amylase level $\geqslant$ 3600 U/L on POD3 could distinguish CR-POPF from non-clinical

POPF (the sensitivity and specificity were 75% and 85%, respectively). In the high risk group, the best cut-off was a drain amylase level of 1600 (the sensitivity and specificity were 77 and 63%, respectively).

CONCLUSION: A 6-point scoring system accurately predicted the occurrence of CR-POPF. In addition, a drain amylase level on POD3 might be a predictor of this complication.

Key words: Pancreatic fistula; Pancreaticoduodenectomy; Postoperative complication; Risk factor; Logistic model

Core tip: Clinically relevant (CR) postoperative pancreatic fistula (POPF) after pancreaticoduodenectomy (PD) remains a challenge, even at high-volume centres. In our study, we established a novel predictive scoring system for CR-POPF after PD based on a large number of cases in a single centre and discovered that the drain amylase level on postoperative day 3 could distinguish CR-POPF from non-clinical POPF in the early period after PD according to the different risk strata of scores. This tool could help surgeons anticipate, identify and control CR-POPF proactively, with the aim of achieving better outcomes from this daunting postoperative complication.

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INTRODUCTION

Pancreaticoduodenectomy (PD) has been established as a standard surgical operation for malignant and benign diseases in the pancreatic head and periampulary regions[1,2]. With recent advances in surgical techniques and perioperative management, the mortality rate has decreased to less than 2% (in high-volume centres)[3-7]. However, the morbidity rate after PD remains high (30%-65%). In particular, postoperative pancreatic fistula (POPF) remains the most important cause of morbidity; this also contributes significantly to a prolonged hospitalisation course, increased health care costs, and mortality[1-8].

Although attempts have been made to decrease POPF rates by improving reconstruction techniques for the pancreatoenteric anastomosis[9-12], including the placement of pancreatic duct stents[13,14] or the use of somatostatin analogues[15], an effective strategy to prevent POPF has not yet been found. There has been a paradigm shift among pancreatic surgeons in the management of POPF, from a reactive "wait and see" approach that depends on treating fistulas when they become evident, to a proactive strategy that instead relies on early anticipation and timely prevention[8,16-18]. Recent studies have suggested that many factors influence POPF, such as gender, preoperative jaundice, operative time, pancreatic duct diameter and soft pancreatic parenchyma[2-8]. However, the predictive risk factors that can precisely distinguish clinically relevant POPF (CR-POPF) from transient pancreatic fistula in the early postoperative period remain unclear.

The aim of the present study was to construct a new and convenient scoring tool to predict CR-POPF and discover ways to distinguish CR-POPF from non-clinical POPF in the early period after PD. This was done using preoperative and surgical variables in a study group of 921 patients, according to the different risk strata of scores.

MATERIALS AND METHODS

Patients

From January 2008 to December 2013, 921 consecutive patients underwent PD. Various patient factors were analysed at the Institution and Hospital of Hepatobiliary Surgery, PLA General Hospital, China. Informed consent for the surgical procedures was obtained from each patient. The local ethics committee approved this study.

Perioperative management

The standard Whipple type operation was performed in 491 patients (53%), and the remaining 430 patients (47%) underwent a pylorus-preserving PD (PPPD). Pancreatic anastomosis after PD and PPPD was performed by duct-to-mucosa and end-to-side pancreaticojejunostomy in all patients. Biliary drainage was achieved by end-to-side hepaticojejunostomy. None of the patients received radiotherapy or chemotherapy perioperatively. All patients were managed in the intensive care unit for at least one day before transfer to the ward. Prophylactic octreotide was given subcutaneously and continued routinely for three days postoperatively.

Definitions of postoperative complications

POPF was defined and classified by the international study group of pancreatic fistula (ISGPF)[19]. Grade A POPF is a transient and asymptomatic fistula that does not need specific treatment. Grade B is symptomatic, clinically apparent, and requires diagnostic evaluation and specific medical treatment or prolonged drainage for longer than 3 wk. Grade C requires a major change in clinical management or deviation from the normal clinical pathway. Combined grade B + C is defined as CR-POPF. Biliary fistula[20] was defined as the presence of bile in the drainage fluid that persisted to postoperative day (POD) 4. Delayed gastric emptying

was defined as any of the following: output from a nasogastric tube of > 500 mL per day that persisted beyond POD10, the failure to maintain oral intake by POD14 or reinsertion of a nasogastric tube[21].

Data collected

Preoperative variables included patient demographics, past medical history, laboratory tests and preoperative biliary drainage by ERCP or PTBD. Intraoperative variables included pancreatic duct diameter, consistency of the pancreas, operation time, blood loss, blood transfusion, and the difference between the blood loss and transfusion. Postoperative variables included postoperative complications, amylase in the drainage fluid from POD1 to POD7, the day of starting oral feeding, the length of postoperative stay and hospital mortality. All pathological specimens were reviewed to confirm the diagnosis.

Statistical analysis

Statistical computations were performed using Statistical Package for the Social Sciences 16.0 for Windows (SPSS, Inc). For continuous variables, descriptive statistics were calculated and reported as the mean ± standard deviation (SD). Categorical variables were described using frequency distributions. The independent sample *t* test was used to detect differences in the means of continuous variables; the χ^2 test was used in cases with low expected frequencies. A *P* value < 0.05 was considered to be significant. Variables with *P* < 0.1 were entered into a logistic regression model to determine independent risk factors of CR-POPF. We developed a scoring system using each independent risk factor, which was based on the regression coefficient of the logistic regression model. The points of this scoring system were further modified to develop a more utilitarian application. Receiver operating characteristic (ROC) curves and the corresponding area under the curve (AUC) were used to evaluate the performance of the prediction model. The optimal cut-off value to divide the risk strata was determined by the Youden index (sensitivity +, specificity - 1). The 921 patients were divided into two groups (low risk and high risk). The independent sample *t* test was used to detect differences in the means of drain amylase on POD1, 2, and 3. The optimal cut-off level of the drain amylase to distinguish CR-POPF from non-clinical POPF in the two risk strata groups was determined by the ROC curves.

RESULTS

Overview

Nine hundred and twenty one consecutive patients [591 (64%) men and 330 (36%) women] underwent PD; their mean age was 56 ± 12 years (range: 11-82 years). Preoperative biliary stenting was performed in 181 patients (19.7%), 491 patients (53%) underwent classic PD, and the remaining 430 patients (47%) underwent PPPD. Combined portal vein resection was performed in 31 patients (3.4%). Median operative time was 380 min (range: 135-1265 min) and the median operative blood loss was 400 ml (range: 100-5300 mL). Two hundred and twenty three patients (24.2%) received a blood transfusion; the median amount of blood received was 710 mL (range: 280-1700 mL). The mean difference between the blood loss and intra-operative transfusion was 400 mL (range: 50-2100 mL).

Postoperatively, the median hospital stay was 18 d (range: 3-72 d). Regarding postoperative complications, the overall morbidity was 294 (31.9%): 195 patients (21.2%) developed a POPF; 106 patients (11.5%) had an A-type fistula; 57 patients (6.2%) had a B-type fistula; and the remaining 32 patients (3.5%) had a C-type fistula. Other postoperative complications included delayed gastric emptying in 215 patients (23.3%), intra-abdominal infection in 42 patients (4.6%), wound infection in 44 patients (4.8%), biliary leakage in 33 patients (3.6%), pulmonary complication in 38 patients (4.1%) and postoperative haemorrhage in 54 patients (5.9%). The hospital mortality in this series was 29 patients (3.1%). Haemorrhage and secondary multiple organ failure was the main cause of death.

Risk factors for CR-POPF

Univariate and multivariate analyses were used to determine the risk factors of CR-POPF. Table 1 shows the result of 19 parameters that were examined univariately as potential risk factors for the 89 patients with CR-POPF *vs* 832 with no CR-POPF. Body mass index (BMI) ⩾ 28, alcohol use, pancreatic duct size < 3 mm, soft pancreatic parenchyma, ⩾ 800 mL difference between the blood loss and intra-operative transfusion, and non-pancreatic diseases were associated with CR-POPF. However, on multivariate logistic regression analysis, only BMI ⩾ 28, pancreatic duct < 3 mm, soft pancreatic parenchyma, and a difference ⩾ 800 mL between the blood loss and intra-operative blood transfusion were significant factors. Further analysis reflected the effects of narrowing of the pancreatic duct diameter. A pancreatic duct diameter measuring 5 mm was considered a reasonable baseline, because this has been referred to as the normal diameter of the main pancreatic duct[8,22]. Table 2 shows that each 1-mm decrease in the diameter of the pancreatic duct from a baseline of 5 mm resulted in a more than 4-fold increase in the odds of developing CR-POPF (OR = 4.59, 95%CI: 2.47-8.53, *P* = 0.00).

CR-POPF risk score model

We developed a score model using each standardised variable, based on the regression coefficient of the logistic regression model. The equation for the scoring system was developed on the assumption that a patient

Table 1 Univariate analyses of risk factors for clinically relevant postoperative pancreatic fistula *n* (%)

Parameters	Non B/C grade POPF group (*n* = 832)	B/C grade POPF group (*n* = 89)	*P* value
Age (*n* = 921)			
< 65 yr	640 (76.9)	68 (76.4)	0.91
⩾ 65 yr	192 (23.1)	21 (23.6)	
Sex (*n* = 921)			
Male	529 (63.6)	62 (69.7)	0.26
Female	303 (36.4)	27 (30.3)	
BMI (*n* = 921)[3]			
< 28	773 (92.9)	71 (80.8)	0.00
⩾ 28	59 (7.1)	18 (20.2)	
Personal history			
Hypertension (*n* = 921)			
Yes	177 (21.3)	16 (18.0)	0.47
No	655 (78.7)	73 (82.0)	
Diabetes mellitus (*n* = 921)			
Yes	95 (11.4)	11 (12.4)	0.79
No	737 (88.6)	78 (87.6)	
Coronary artery disease (*n* = 921)			
Yes	81 (9.7)	4 (4.5)	0.54
No	781 (93.9)	85 (95.5)	
Smoking (*n* = 921)			
Yes	199 (23.9)	23 (25.8)	0.69
No	633 (76.1)	66 (74.2)	
Drinking (*n* = 921)[3]			
Yes	174 (20.9)	12 (13.5)	0.09
No	658 (79.1)	77 (86.5)	
Abdominal operation history (*n* = 921)			
Yes	111 (13.3)	14 (15.7)	0.53
No	721 (86.7)	75 (84.3)	
Serum albumin (g/L, *n* = 893)			
< 35	660 (81.8)	13 (15.1)	0.48
⩾ 35	147 (18.2)	73 (84.9)	
Serum total bilirubin (μmol/L, *n* = 905)[1]			
< 171	604 (73.9)	61 (69.3)	0.40
⩾ 171	213 (26.1)	27 (30.7)	
Type of resection (*n* = 921)			
PD	446 (53.6)	45 (50.6)	0.58
PPPD	386 (46.4)	44 (49.4)	
Pancreatic duct (mm, *n* = 921)[3]			
< 3	250 (30.0)	62 (69.7)	0.00
⩾ 3	582 (70.0)	27 (30.3)	
Texture of remnant pancreas (*n* = 921)[3]			
Soft	289 (34.7)	68 (76.4)	0.00
Hard	543 (65.3)	21 (23.6)	
Operative time (min, *n* = 913)			
< 360	346 (42.0)	35 (39.3)	0.65
⩾ 360	478 (58.0)	54 (60.7)	
Difference between the blood loss and transfusion in operation (mL, *n* = 920)[3]			
< 800	772 (92.9)	75 (84.3)	0.00
⩾ 800	59 (7.1)	14 (15.7)	
Reconstruction of blood vessels (*n* = 921)			
Yes	29 (3.5)	2 (2.2)	0.76
No	803 (96.5)	87 (97.8)	
Pancreaticoduodenectomy extending to adjacent (*n* = 921)[2]			
Yes	21 (2.5)	0 (-)	0.25
No	786 (94.5)	89 (100.0)	
Pancreatic carcinoma			
Yes	219 (26.3)	15 (16.9)	0.05
No	613 (73.7)	74 (83.1)	

[1]This level of serum total bilirubin was assayed just before the operation (tested within three days before operation); [2]Combined resection of adjacent organs including: liver, colon, kidney and so on; [3]Statistically significant. POPF: Postoperative pancreatic fistula; PD: Pancreaticoduodenectomy; PPPD: Pylorus-preserving pancreaticoduodenectomy.

receives 1 point each for BMI ⩾ 28, soft gland texture, and the difference between the blood loss and intra-operative transfusion ⩾ 800 mL, and from 0 points for a 5 mm or greater duct diameter to 3 points for less than a 2 mm duct (Table 3). The score values for individual patients ranged from 0 to 6. The ROC curve (Figure 1A)

Table 2 Multivariate logistic regression models of independent risk factors for clinically relevant postoperative pancreatic fistula (n = 921)

	P value	OR	95%CI
BMI (≥ 28)	0.00	3.86	1.92-7.75
Pancreatic duct (< 3 mm)			
≥ 5 mm		1.00	
3-5 mm	0.00	4.59	2.47-8.53
2-3 mm	0.00	7.91	4.07-15.39
< 2 mm	0.00	8.97	3.70-21.77
Texture of remnant pancreas (soft)	0.00	4.50	2.53-7.98
Difference between the blood loss and transfusion in operation (≥ 800 mL)	0.00	3.45	1.92-7.75

BMI: Body mass index.

Table 3 Risk scoring system for clinically relevant postoperative pancreatic fistula

Risk factor	Points contributed
BMI (kg/m^2)	
< 28	0 point
≥ 28	1 point
Gland texture	
Firm	0 point
Soft	1 point
The difference between the blood loss and transfusion in operation	
< 800 mL	0 point
≥ 800 mL	1 point
Pancreatic duct diameter	
≥ 5 mm	0 point
3-5 mm	1 point
2-3 mm	2 points
< 2 mm	3 points

BMI: Body mass index.

showed that the AUC of this score was 0.812 (95%CI: 0.766-0.858). A score of 3 points was suggested to be the best cut-off value to divide the risk strata because the Youden index was 0.485. Two risk strata were assigned according to the total score: low risk (0 to 2 points) and high risk (3 to 6 points).

Predictive drain amylase level for CR-POPF

These patients were divided into a low risk and a high risk group. The low risk group comprised 652 patients whose score was less than 3 points; the remaining 269 patients were classified into the high risk group. In the low risk group, there was no significant difference in the drain amylase level on POD1 and 2 between CR-POPF and non-clinical POPF. However, the mean drain amylase levels on POD3 were 26416.6 ± 16865.0 U/L in patients with CR-POPF compared with 2952.9 ± 606.0 U/L in those without complications (P = 0.000). Considering the sensitivity and specificity of the drain amylase on POD3, the AUC was 0.838 (Figure 1B). A drain amylase level ≥ 3600 U/L on POD3 was determined to be the best cut-off value for prediction

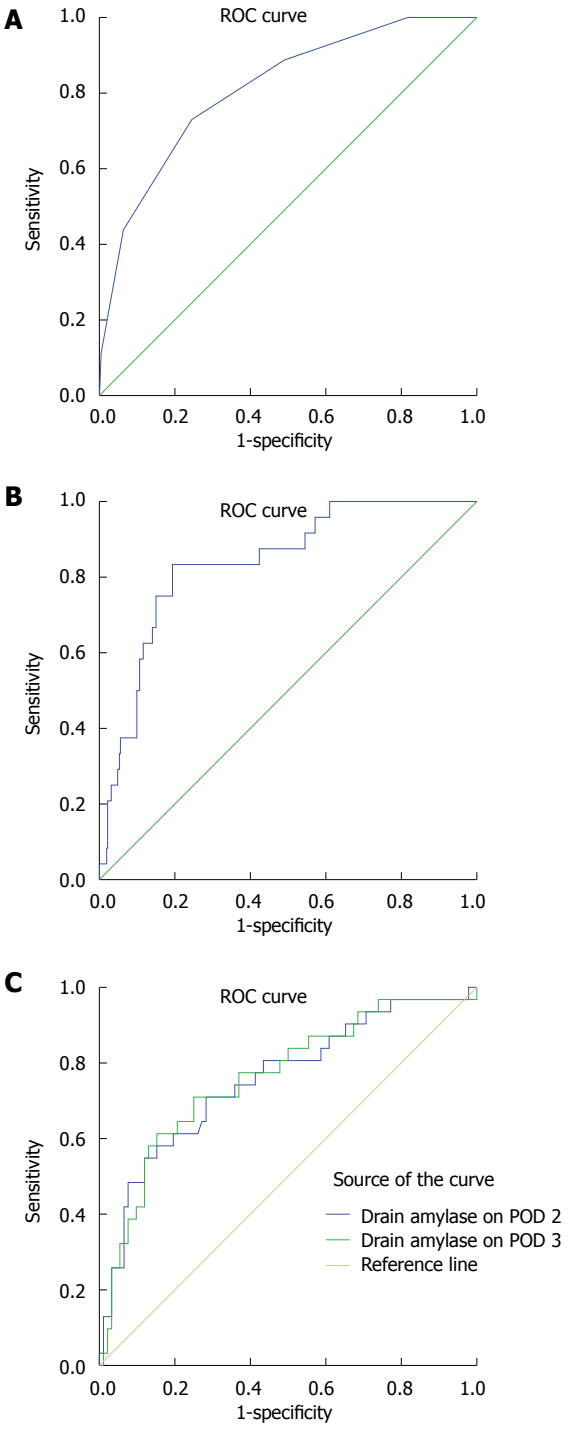

Figure 1 Receiver operating characteristic curve. A: Receiver operating characteristic (ROC) curve for the predictive scoring system; B: Drain amylase on postoperative day 3 in the low risk group. Area under the receiver operator characteristics curve was 0.838. C: Drain amylase on postoperative day 2 and 3 in high risk group. Areas under the receiver operator characteristic curves were 0.756 and 0.761, respectively. POD: Postoperative day.

of CR-POPF (the sensitivity and specificity of cut-off levels were 75% and 85%, respectively).

In the high risk group, there was no significant difference in the drain amylase level on POD1. The mean drain amylase level on POD2 was 22935 ± 8568 U/L in patients with CR-POPF compared with 6227 ±

2540 U/L in those without complications (P = 0.01), and on POD3, the mean levels were 13709 ± 2626 U/L *vs* 5122 ± 1290 U/L for these groups, respectively (P = 0.01). Regarding the sensitivity and specificity of the drain amylase on POD2 and 3, the AUCs were 0.756 and 0.761, respectively (Figure 1C). The drain amylase on POD 3 had a better performance. The drain amylase level ⩾ 1600 U/L on POD 3 was the best cut-off for prediction of CR-POPF (the sensitivity and specificity of the cut-off levels were 77% and 63%, respectively).

DISCUSSION

CR-POPF remains the major cause of morbidity after PD. The ability to make a reliable individual prediction of the risk of CR-POPF may be a step towards more individualised surgical management of patients scheduled for PD[9]. However, the current widely used scoring systems are nonspecific and do not accurately predict CR-POPF, because they mostly focus on the physical status and operation tolerance of patients[8]. Given these drawbacks, we have developed a novel predictive scoring system for CR-POPF after PD using the following four independent perioperative parameters: (1) soft gland texture; (2) the narrowed pancreatic duct diameter; (3) BMI ⩾ 28; and (4) the difference between the blood loss and intra-operative blood transfusion ⩾ 800 mL.

The former two factors are associated with the presence of chronic pancreatitis and are a challenge for reconstruction. The soft gland texture and narrowed pancreatic duct diameter demonstrate that exocrine function is generally preserved, which is more susceptible to ischemia and injury[8], and results in the increased secretion of pancreatic juices[23-25]. These two factors may also increase the difficulty of performing a pancreaticojejunostomy[2,8]. A high BMI is associated with intra-abdominal obesity and fat tissue volume in the pancreas[24-28]. Finally, the difference between the blood loss and intra-operative blood transfusion is associated with rapid volume loss, which causes ischemia and tissue oedema, and may directly affect the healing of the pancreatic duct-to-mucosa anastomosis[8].

The risk factors of POPF have been proposed by recent studies, and some of these studies have also proposed a risk scoring system; the advantage of our risk assessment tool over other models[4,8,27] lies in three factors. First, our research is based on large single centre retrospective cases, where each surgeon performed more than 30 cases of PD, annually. Moreover, the form of pancreaticojejunostomy and perioperative management has a unified standard and thus can avoid the influence caused by the reconstruction techniques for the pancreatoenteric anastomosis. Second, the scoring is based on the independent perioperative factors (accurately determined in the operating room), without the need for information regarding postoperative parameters. Third, this system is different from the other risk assessment tools for POPF after PD[4,27] and provides a good early prediction of the occurrence of CR-POPF. Grade A POPF does not need specific clinical treatment; therefore, distinguishing CR-POPF from transient POPF in the early postoperative period is valuable in the clinical setting.

The role of a surgically placed prophylactic intra-abdominal drain after PD and its effect on the morbidity rate and optimal timing for drain removal, remain controversial[15,29]. However, there is a consensus that a prolonged period of drain placement may increase the rate of infection at the surgical site and may also increase the rate of POPF[17]. For this reason, although a high level of drain amylase indicates POPF, it is important to determine whether the POPF is grade A or grade B/C as soon as possible. In cases of grade A POPF, we could remove the drain, even if the drain amylase level was high. We considered whether CR-POPF could be distinguished from non-clinical POPF using only postoperative factors, such as drain amylase. However, previous studies have reported that measuring daily levels of amylase in drainage fluid may not reflect the severity of POPF, although the increase was significantly greater in cases of POPF than in those without POPF[3,30]. El Nakeeb *et al*[2] used 4000 U/L as a cut-off. A low drain amylase on POD1 excluded a CR-POPF. The sensitivity of this study was only 28.1%, but the specificity was 97.2%. Therefore, we divided patients into two groups according to the different risk strata of scores. We found that there was a relationship between CR-POPF and drain amylase level on POD3 in the two groups. Our study demonstrated that in the low risk group, a drain amylase level ⩾ 3600 U/L on POD 3 was the best cut-off for the prediction of CR-POPF. The sensitivity and specificity for this cut-off level were 75% and 85%, respectively. Using 1600 U/L as a cut-off in the high risk group, a low drain amylase on POD3 excluded CR-POPF with a sensitivity and specificity of 77% and 63%, respectively.

Using the present scoring system and predictive drain amylase level, the following clinical advantages can be expected in the perioperative risk management of PD: (1) the selection of high risk patients for CR-POPF, with the surgeon planning the surgery accordingly; (2) the selection of patients who qualify for early removal of their drain; and (3) the selection of low-risk patients for PD and pancreatic reconstruction by junior trainees.

The current work is not a randomised controlled study and, therefore, is subject to certain limitations secondary to the retrospective nature of the data collection. First, gland texture was measured at the discretion of the operating surgeon and was classified as either firm or soft, rather than on a gradient as others have described[9]. Second, the surgical procedures, such as standard Whipple type operation, pylorus-preserving PD, or use of a pancreatic stent

were not randomised, but depended on the surgeon's preference. Therefore, further studies are necessary to evaluate prospectively the risk scoring system and the predictive drain amylase level for CR-POPF.

In conclusion, despite these limitations, this study has developed a novel predictive scoring system for CR-POPF after PD, with good discriminating ability. In addition, the drain amylase level on POD3 was useful to distinguish CR-POPF from non-clinical POPF in the early postoperative period following PD. The strength of this study lies in its ability to validate this scoring system in a high volume centre hospital. This tool may help surgeons anticipate, identify and control CR-POPF proactively, with the aim of achieving better outcomes from this complication.

COMMENTS

Background

Pancreaticoduodenectomy (PD) has been established as a standard surgical operation for malignant and benign diseases in the pancreatic head and periampullary regions. However, the morbidity rate after PD remains high (30%-65%). In particular, postoperative pancreatic fistula (POPF) remains the most important cause of morbidity. The ability to make a reliable individual prediction of the risk of clinically relevant- POPF (CR-POPF) may be a step towards more individualised surgical management of patients scheduled for PD.

Research frontiers

There has been a paradigm shift among pancreatic surgeons in the management of POPF, from a reactive "wait and see" approach to a proactive strategy. Recent studies have suggested that many factors influence POPF, such as gender, preoperative jaundice, operative time, pancreatic duct diameter and soft pancreatic parenchyma. However, the predictive risk factors that can precisely distinguish CR-POPF from transient pancreatic fistula in the early postoperative period remain unclear.

Innovations and breakthroughs

In this study, a novel predictive scoring system for CR-POPF after PD was established. The ROC curve showed that the area under the curve of this score was 0.812 (95%CI: 0.766-0.858), which confirmed that this system provides a good early prediction of the occurrence of CR-POPF. Moreover, the authors discovered that the drain amylase level on postoperative day 3 could distinguish CR-POPF from non-clinical POPF in the early period after PD, according to the different risk strata of scores. In the low risk group, the sensitivity and specificity were 75% and 85%, and in the high risk group, the sensitivity and specificity were 77% and 63%, respectively.

Applications

This scoring system may help surgeons anticipate, identify, and control CR-POPF proactively, with the aim of achieving better outcomes from this complication. Further studies are necessary to evaluate prospectively the risk scoring system and the predictive drain amylase level for CR-POPF.

Terminology

PD is a surgical procedure that includes resection of pancreatic head, stomachus pyloricus, duodenum, the lower part of common bile duct and regional lymph node, and then reconstruction, including pancreaticojejunostomy, hepaticojejunostomy and gastrojejunostomy. POPF: Any measurable volume of fluid on or after postoperative day 3 with an amylase content greater than three times the serum amylase activity.

Peer-review

This is an interesting article about some 900 patients that underwent PD. It is clever to construct a predictive scoring system to predict CR-POPF following PD. This scoring system may help surgeons anticipate, identify and control CR-POPF proactively and may be a step towards more individualised surgical management of patients scheduled for PD.

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ORIGINAL ARTICLE

Retrospective Study

Factors associated with early recurrence after curative surgery for gastric cancer

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Abstract

AIM: To characterize patterns of gastric cancer recurrence and patient survival and to identify predictors of early recurrence after surgery.

METHODS: Clinicopathological data for 417 consecutive patients who underwent curative resection for gastric cancer were retrospectively analyzed. Tumor and node status was reclassified according to the 7th edition of the American Joint Committee on Cancer tumor-node-metastasis classification for carcinoma of the stomach. Survival data came from both the patients' follow-up records and telephone follow-ups. Recurrent gastric cancer was diagnosed based on clinical imaging, gastroscopy with biopsy, and/or cytological examination of ascites, or intraoperative findings in patients who underwent reoperation. Predictors of early recurrence were compared in patients with pT1 and pT2-4a stage tumors. Pearson's χ^2 test and Fisher's exact test were used to compare differences between categorical variables. Survival curves were constructed using the Kaplan-Meier method and compared *via* the log-rank test. Variables identified as potentially important for early recurrence using univariate analysis were determined by multivariate logistic regression analysis.

RESULTS: Of 417 gastric cancer patients, 80 (19.2%) were diagnosed with early gastric cancer and the remaining 337 (80.8%) were diagnosed with locally advanced gastric cancer. After a median follow-up period of 56 mo, 194 patients (46.5%) experienced

recurrence. The mean time from curative surgery to recurrence in these 194 patients was 24 ± 18 mo (range, 1-84 mo). Additionally, of these 194 patients, 129 (66.5%) experienced recurrence within 2 years after surgery. There was no significant difference in recurrence patterns between early and late recurrence (P < 0.05 each). For pT1 stage gastric cancer, tumor size (P = 0.011) and pN stage (P = 0.048) were associated with early recurrence of gastric tumors. Patient age, pT stage, pN stage, Lauren histotype, lymphovascular invasion, intraoperative chemotherapy, and postoperative chemotherapy were independent predictors of early recurrence in patients with pT2-4a stage gastric cancer (P < 0.05 each).

CONCLUSION: Age, pT stage, pN stage, Lauren histotype, lymphovascular invasion, intraoperative chemotherapy, and postoperative chemotherapy are independent factors influencing early recurrence of pT2-4a stage gastric cancer.

Key words: Stomach neoplasms; Gastrectomy; D2 lymphadenectomy; Recurrence; Chemotherapy

Core tip: Few studies have assessed recurrence patterns or predictors of early recurrence after curative surgery in Chinese patients with gastric carcinoma. This study found that survival after gastric cancer recurrence was poor. Large tumor size and advanced pN stage were associated with early recurrence of tumor pT1 stage tumors. Age, pT stage, pN stage, Lauren histotype, lymphovascular invasion, intraoperative chemotherapy, and postoperative chemotherapy were independent predictors of early recurrence of pT2-4a stage tumors.

Kang WM, Meng QB, Yu JC, Ma ZQ, Li ZT. Factors associated with early recurrence after curative surgery for gastric cancer. *World J Gastroenterol* 2015; 21(19): 5934-5940 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5934.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5934

INTRODUCTION

Although it has declined somewhat, the overall age-standardized incidence of gastric cancer in China in 2009 was 17.85 per 100000 persons[1,2]. Despite improvements in diagnostic procedures and the introduction of multimodal treatment strategies, patient survival remains dismal owing to early recurrence originating from minimal residual disease[3-8]. More than 70% of recurrences and tumor-related deaths occur within 2 years after surgery[9-11], with tumor recurrence being the leading cause of death in patients who undergo curative surgery for gastric cancer.

Although several studies have sought to identify clinicopathological factors that predict early recurrence, their methodologies and definitions of early gastric cancer vary[11-13]. Few studies to date have focused on patterns and timing of recurrence, or on predictors of early recurrence, after curative surgery in Chinese patients.

Multimodal treatments, including intraoperative and/or postoperative chemotherapy, have been used for some patients undergoing D2 gastrectomy for locally advanced gastric cancer[14]. However, little is known about the effect of intraoperative and postoperative chemotherapy on early recurrence of gastric cancer. This study therefore retrospectively analyzed patterns and timing of recurrence in patients who underwent curative surgery for gastric cancer. Clinicopathological factors and therapeutic modalities significantly associated with early recurrence were identified to develop appropriate treatments and follow-up programs.

MATERIALS AND METHODS

Patients

Between January 2002 and February 2008, 516 patients with gastric adenocarcinoma underwent radical gastrectomy and D2 lymphadenectomy in the Department of General Surgery, Peking Union Medical College Hospital (PUMCH), Chinese Academy of Medical Science and Peking Union Medical College. Patients with gastric stump cancer (n = 7), those who received neoadjuvant chemotherapy (n = 4) or postoperative radiotherapy (n = 2), patients with incomplete or inaccurate medical records (n = 10), patients lost to follow-up within 2 years after surgery (n = 68) and those who died of disease other than gastric cancer within 2 years after curative surgery (n = 8 cases) were excluded. The study therefore included a total of 417 patients. None of these patients had distant or peritoneal metastasis at the time of resection, as shown by chest X-ray or chest computed tomography (CT) scan and abdominal pelvic CT scan before surgery. Tumor (T) and node (N) status was reclassified according to the 7th edition of the American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) classification for carcinoma of the stomach[15,16].

Variables

Clinicopathological features and therapeutic modalities reviewed included sex, age at diagnosis, tumor size, Lauren histotype (intestinal or diffuse-mixed type)[17,18], lymphovascular invasion, AJCC pT stage of the primary tumor, AJCC pN stage, intraoperative chemotherapy and postoperative chemotherapy.

Treatments

All patients in this study underwent curative (R0)

resection and D2 lymphadenectomy as the primary treatment[19,20]. Of the 80 patients with early gastric cancer (pT1), 20 (25%) received intraoperative chemotherapy, and 3 (2 pT1N1M0 and 1 pT1N2M0) received six cycles of postoperative adjuvant chemotherapy. Of the 337 patients with locally advanced gastric cancer (pT2-4a), 190 (56.4%) received intraoperative chemotherapy, and 246 (73%) received postoperative adjuvant chemotherapy, with 200 (81.3%) of the latter completing at least six cycles.

Intraoperative chemotherapy consisted of intravenous administration of epirubicin 20 mg/m^2, leucovorin 200 mg, 5-fluorouracil (5-FU) 600 mg/m^2 (maximum $\leqslant$ 1000 mg), and mitomycin 5 mg/m^2 (maximum $\leqslant$ 10 mg).

Three main postoperative adjuvant chemotherapy regimens were used: XELOX[21] (2-h intravenous infusion of oxaliplatin 130 mg/m^2 on day 1 and oral capecitabine 1000 mg/m^2 twice daily on days 1-14, with cycles every 21 d); FOLFOX4[22] (intravenous infusion of oxaliplatin 85 mg/m^2 on day 1, leucovorin 200 mg/m^2 as a 2-h infusion followed by bolus injection of 5-FU 400 mg/m^2 on days 1, 2, and 22-h continuous intravenous infusion of 5-FU 600 mg/m^2 on days 1, 2, every 2 wk for at least six cycles); and FOLFOX6[23] (intravenous infusion of oxaliplatin 100 mg/m^2 on day 1, leucovorin 200 mg/m^2 as a 2-h infusion followed by bolus injection of 5-FU 400 mg/m^2 on day 1, and 46-h continuous intravenous infusion of 5-FU 3000 mg/m^2 starting on day 1, every 2 wk for at least six cycles).

Follow-up

All patients were followed regularly from the date of surgery to death, emigration, or February 10, 2012, whichever came first. Survival data were obtained from both patients' follow-up records and telephone follow-up. History and physical examinations were routinely performed every 3-6 mo for the first 3 years and every 6-12 mo thereafter. CT examinations were performed at least twice a year for the first 2 years, and annually thereafter. Gastroscopy with or without biopsy was performed every 1-2 years.

Recurrent gastric cancer was diagnosed based on clinical imaging, gastroscopy with biopsy, and/or cytological examination of ascites, or intraoperative findings in patients who underwent reoperation. Recurrences were classified as locoregional, hematogenous, peritoneal or distant lymphatic, according to the sites of relapse.

Statistical analysis

Categorical variables were compared using Pearson's χ^2 tests and Fisher's exact tests. Variables differing significantly on univariate analysis were included in multivariate models of logistic regression analysis. Survival curves were constructed using the Kaplan-Meier method and compared by the log-rank test. Multicollinearity, defined as a tolerance < 0.1, was diagnosed by the linear regression model. All analyses were performed using SPSS 12.0 (SPSS, Chicago, Illinois, United States). The prognostic powers of covariates were expressed by calculating odds ratios (ORs) and 95% confidence internals (CIs). All *P* values were two-sided and *P* values < 0.05 were considered statistically significant.

The study was approved by the Ethics Committee of Chinese Academy of Medical Science and Peking Union Medical College, Beijing, China.

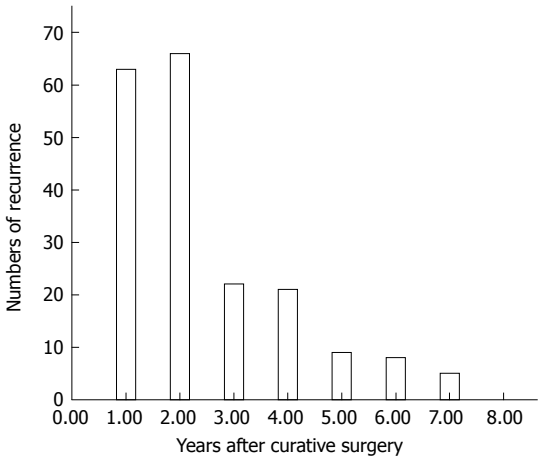

Figure 1 Number of patients in this study dying of recurrent gastric cancer per year. Most recurrences (129/194, 66.5%) occurred within 2 years (early recurrence).

RESULTS

Of the 417 patients included in the current study, 80 (19.2%) were diagnosed with early gastric cancer and 337 (80.8%) with locally advanced gastric cancer. The median follow-up time of all 417 patients was 56 mo (range, 3-117 mo), during which gastric cancer recurrence was detected in 194 (46.5%) patients, with 184 patients dying of gastric cancer recurrence. Ten patients with recurrence remained alive after the end of follow-up. In contrast, 9 of 223 patients without recurrence died of other diseases within 2 years after surgery, with the other 214 patients remaining alive without recurrence during follow-up.

The mean time from curative surgery to recurrence in the 194 patients with recurrence was 24 ± 18 mo (range, 1-84 mo), with 129 (66.5%) of these patients experiencing recurrence within 2 years (Figure 1). Those 129 patients were classified as the early recurrence group, whereas the late/no recurrence group was defined as patients who lived without recurrence for more than 2 years after surgery.

Patterns of initial recurrence

Of the 194 patients with recurrence, 99 (51.0%) experienced locoregional recurrence, 86 had peritoneal

Table 1 Initial recurrence patterns in the 194 patients with recurrence *n* (%)

Pattern	Total recurrence	Early recurrence	Late recurrence	*P* value
	n = 194 (100.0)	*n* = 129 (66.5)	*n* = 65 (33.5)	
Hematogenous recurrence	77 (39.7)	56 (43.4)	21 (32.3)	0.136
Liver	55 (28.4)	42 (32.6)	13 (20.0)	0.067
Lung	16 (8.2)	10 (7.8)	6 (9.2)	0.724
Bone	14 (7.2)	8 (6.2)	6 (9.2)	0.442
Brain	3 (1.5)	2 (1.6)	1 (1.5)	0.995
Locoregional recurrence	99 (51.0)	68 (52.7)	31 (47.7)	0.509
Remnant stomach	10 (5.2)	6 (4.7)	4 (6.2)	0.734
Anastomosis	32 (16.5)	25 (19.4)	7 (10.8)	0.127
Perigastric area	40 (20.6)	27 (20.9)	13 (20.0)	0.880
Peripancreatic area	13 (6.7)	10 (7.8)	3 (4.6)	0.410
Abdominal wall	2 (1.6)	2 (1.9)	0 (0.0)	0.313
Local lymph node	34 (17.5)	27 (20.9)	7 (10.8)	0.079
Peritoneal recurrence	86 (44.3)	59 (45.7)	27 (41.5)	0.579
Distant lymphatic recurrence	11 (5.7)	7 (5.4)	4 (6.2)	1.000
Virchow's node	6 (3.1)	4 (3.1)	2 (3.1)	1.000
Inguinal lymph node	1 (0.5)	0 (0.0)	1 (1.5)	0.335
Mediastinal lymph node	3 (1.5)	2 (1.6)	1 (1.5)	1.000
Para-aortic lymph node	2 (1.0)	2 (1.6)	0 (0.0)	0.552

P values were evaluated by Pearson's χ^2 test or Fisher's exact test, as appropriate. Some patients experienced initial recurrence at multiple sites.

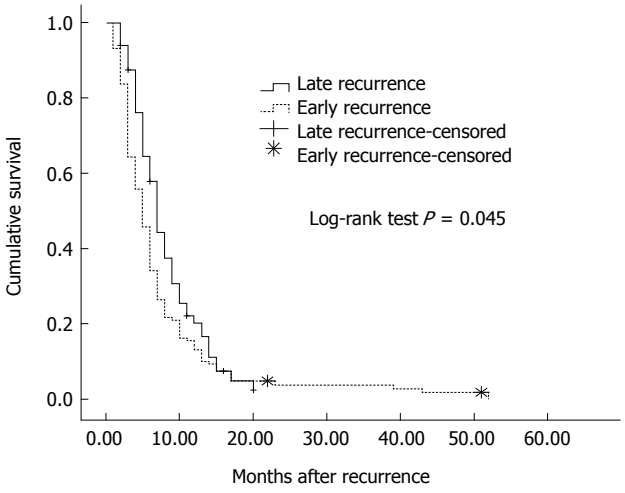

Figure 2 Survival after recurrence. Survival was significantly poorer in patients with early than late recurrence of gastric cancer (*P* = 0.045).

dissemination (44.3%), 77 (39.7%) showed hematogenous metastases, and 11 (5.7%) had distant lymphatic recurrence (Table 1). Of the 129 patients with early recurrence, 56 (43.4%) had hematogenous recurrence, 59 (45.7%) had peritoneal recurrence, and 68 (52.7%) had locoregional recurrence. Recurrence patterns did not differ significantly in patients with early and late recurrence (Table 1).

Table 2 Univariate analysis of factors predicting early recurrence in patients with early gastric cancer *n* (%)

Variable	Early recurrence	Late/no recurrence	Univariate analysis
	n = 4	*n* = 76	*P* value
Gender			0.623
Women	1 (25.0)	35 (46.1)	
Men	3 (75.0)	41 (53.9)	
Age at diagnosis (yr)			0.646
≤ 60	3 (75.0)	45 (59.2)	
60	1 (25.0)	31 (40.8)	
Tumor size (cm)			0.011
≤ 5.0	2 (50.0)	74 (97.4)	
>5.0	2 (50.0)	2 (2.6)	
Lauren histotype			0.646
Intestinal	3 (75.0)	45 (59.2)	
Diffuse-mixed	1 (25.0)	31 (40.8)	
Lymphovascular invasion			< 0.144
Present	1 (25.0)	2 (2.6)	
Absent	3 (75.0)	74 (97.4)	
pT stage			< 0.639
pT1a	2 (50.0)	47 (61.8)	
pT1b	2 (50.0)	29 (38.2)	
pN stage			0.048
N0	2 (50.0)	70 (92.1)	
N1, N2	2 (50.0)	6 (7.9)	
Intraoperative chemotherapy			1.000
Yes	1 (25.0)	19 (25.0)	
No	3 (75.0)	57 (75.0)	

P values were evaluated by Fisher's exact test.

Survival time after recurrence

Median survival after recurrence in all patients with recurrence was 6 mo (95%CI: 5.32-6.68 mo), 5 mo (95%CI: 6.01-7.92 mo) in patients with early recurrence and 7 mo (95%CI: 4.21-5.80 mo) in patients with late recurrence (log rank test, *P* = 0.045) (Figure 2).

Factors predictive of early recurrence

Of the 80 patients with early gastric cancer, 4 (5.0%) experienced early recurrence, as did 125 of the 337 patients (37.1%) with advanced gastric cancer. Analysis of predictors of early recurrence in patients with early gastric cancer (pT1) showed that larger tumor size (*P* = 0.011) and advanced AJCC pN stage (*P* = 0.048) were significantly associated with early recurrence (Table 2). Univariate analysis of clinicopathological factors predictive of recurrence in patients with locally advanced gastric cancer showed that age at diagnosis, tumor size, Lauren histotype, lymphovascular invasion, AJCC pT stage, AJCC pN stage, intraoperative systemic chemotherapy, and postoperative chemotherapy were significantly associated with early recurrences (Table 3). Multivariate analysis showed that age at diagnosis (*P* = 0.033), AJCC pT stage (*P* < 0.001), AJCC pN stage (*P* < 0.001), Lauren histotype (*P* < 0.001), lymphovascular invasion (*P* = 0.011), intraoperative chemotherapy (*P* < 0.001),

Table 3 Univariate analysis of factors predicting early recurrence in patients with locally advanced gastric cancer *n* (%)

Variable	Early recurrence	Late/no recurrence	Univariate analysis
	n = 125	*n* = 212	*P* value
Gender			0.683
Women	41 (32.8)	65 (30.7)	
Men	84 (67.2)	147 (69.3)	
Age at diagnosis (yr)			0.016
≤ 60	52 (41.6)	117 (55.2)	
> 60	73 (58.4)	95 (44.8)	
Tumor size (cm)			0.019
≤ 5.0	76 (60.8)	155 (73.1)	
> 5.0	49 (392)	57 (26.9)	
Lauren histotype			0.016
Intestinal	85 (68.0)	169 (79.7)	
Diffuse-mixed	40 (32.0)	43 (20.3)	
Lymphovascular invasion			< 0.001
Present	21 (16.8)	9 (4.2)	
Absent	104 (83.2)	203 (95.8)	
pT stage			< 0.001
pT2, pT3	30 (24.0)	105 (49.5)	
pT4a	95 (76.0)	107 (50.5)	
pN stage			< 0.001
N0	13 (10.4)	82 (38.7)	
N1	25 (20.0)	52 (24.5)	
N2	30 (24.0)	46 (21.7)	
N3	57 (45.6)	32 (15.1)	
Intraoperative chemotherapy			< 0.001
Yes	54 (43.2)	136 (64.2)	
No	71 (56.8)	76 (35.8)	
Postoperative chemotherapy			0.036
No	42 (33.6)	49 (23.1)	
5-FU-based regimen	83 (66.4)	163 (76.9)	

P values were evaluated by Pearson's χ^2 test.

and postoperative chemotherapy (P = 0.007) were independent predictors of early recurrence, whereas gender was not (Table 4). All tolerance values of significant factors were greater than 0.1.

DISCUSSION

Many studies have reported that approximately 70% of patients with gastric cancer experience early tumor recurrence, defined as within 2 years after surgery[9,11,24]. Less is known, however, about patterns of recurrence and predictors of early recurrence, especially in Chinese patients. This study therefore investigated recurrence patterns and factors predicting early recurrence in patients undergoing curative surgery for gastric cancer.

In agreement with earlier findings[9,11], we found that most recurrences (129/194, 66.5%) were early, occurring within 2 years after curative surgery. The three main types of early recurrence were hematogenous (56/129, 43.4%), peritoneal (59/129, 45.7%), and locoregional (68/129, 52.7%). Patients with early recurrence had more liver metastases than

Table 4 Multivariate analysis of factors predicting early recurrence by binary logistic regression model

Factor	*P* value	OR	95%CI
A Age at diagnosis	0.033	1.813	1.050-3.131
pT pT stage	< 0.001	2.865	1.603-5.123
pN stage	< 0.001		
N0		1.00 (reference)	
N1	0.001	4.029	1.708-9.500
N2	0.001	4.425	1.889-10.365
N3	< 0.001	9.860	4.314-22.536
Lauren histotype	< 0.001	3.492	1.810-6.736
Lymphovascular invasion	0.011	3.460	1.335-8.969
Intraoperative chemotherapy	< 0.001	0.327	0.190-0.564
Postoperative chemotherapy	0.007	0.423	0.225-0.793

The constant of the model was -2.087 (standard error = 0.468).

patients with late recurrence (32.6% *vs* 20.0%), although the difference was not significant (P = 0.067). No other differences in recurrence patterns were observed between the early and late recurrence groups. As previously reported[12], survival after recurrence was poor, especially in patients with early recurrence.

Because 66.5% of recurrences occurred within 2 years after surgery and this study focused on factors associated with early recurrence, clinicopathologic characteristics were compared in patients with and without early recurrence within 2 years after surgery.

Age, lymphatic metastasis and submucosal invasion have been reported to be significantly associated with recurrence of gastric cancer[25]. In the current study, only 5% (4/80) of patients with early gastric cancer experienced early recurrence. Although larger tumor size and advanced AJCC pN stage were found to be significantly associated with early recurrence in this group, the number of patients was small, indicating that these results require further validation.

The early recurrence rate was significantly higher in patients with locally advanced than early gastric cancer. Age at diagnosis, AJCC pT stage, AJCC pN stage, Lauren histotype, lymphovascular invasion, intraoperative chemotherapy, and postoperative chemotherapy were found to be independent factors influencing timing of recurrence in patients who underwent curative surgery for locally advanced gastric cancer. No obvious multicollinearity was observed among these factors.

Interestingly, we found that more patients in the late- and non-recurrence groups received intraoperative chemotherapy than did patients in the early recurrence group, with intraoperative chemotherapy found to be an independent predictor of early recurrence [hazard ratio (HR) = 0.327, P < 0.001]. Most previous studies of intraoperative chemotherapy assessed only patients who received hyperthermic intraperitoneal chemotherapy, not

intravenous chemotherapy, although intravenous 5-FU during surgery for advanced gastric cancer was first administered in 1987[26,27]. A randomized controlled clinical trial is required to confirm the benefits of intraoperative chemotherapy.

The current study also found that postoperative chemotherapy was an independent predictor of early recurrence (HR = 0.423, $P < 0.007$). Results of both the Capecitabine and Oxaliplatin Adjuvant Study in Stomach Cancer and the Adjuvant Chemotherapy Trial of TS-1 for Gastric Cancer trials suggested that FU-based adjuvant therapy improves survival of patients with gastric cancer following gastrectomy combined with D2 lymphadenectomy[28-31]. However, another study found that adjuvant chemotherapy was not a significant predictor of the timing of recurrence[12]. Further evaluation is necessary to assess the effect of adjuvant chemotherapy on early recurrence.

The main limitations of this study were its retrospective nature and relatively small sample size.

In summary, this retrospective study found that larger tumor size and advanced AJCC pN stage were significantly associated with early recurrence of early gastric cancer; whereas age at diagnosis, AJCC pT stage, AJCC pN stage, Lauren histotype, and lymphovascular invasion were all independent predictors of early recurrence in patients undergoing curative surgery for locally advanced gastric cancer. Chemotherapy, both intraoperative and postoperative, was associated with early recurrence of gastric carcinoma. Patients with gastric cancer should be closely monitored and actively followed for at least 2 years after surgery.

COMMENTS

Background

Tumor recurrence is the leading cause of death in patients who undergo curative surgery for gastric cancer. Few studies have assessed the patterns and timing of recurrence or predictors of early recurrence following surgery in Chinese patients. This retrospective study analyzed patterns and timing of recurrence in patients who underwent curative surgery for gastric cancer, and identified clinicopathological factors and therapeutic modalities, especially intraoperative chemotherapy, significantly associated with early recurrence, in order to develop appropriate treatments and follow-up programs.

Research frontiers

This study was based on the experience of a large single center with intraoperative chemotherapy, curative surgery and postoperative chemotherapy for gastric cancer. Factors correlated with early recurrence were analyzed.

Innovations and breakthroughs

Intraoperative chemotherapy, as well as postoperative chemotherapy, age, pT stage, pN stage, Lauren histotype, and lymphovascular invasion were found to be independent predictors of early recurrence of pT2-4a stage gastric cancer.

Applications

Intraoperative chemotherapy combined with postoperative chemotherapy may reduce rates of early recurrence in patients with locally advanced gastric cancer.

Peer-review

This manuscript identified predictors of early recurrence after curative surgery and characterized patterns of recurrence and survival after gastric cancer recurrence. The study was very well designed and interesting.

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ORIGINAL ARTICLE

Retrospective Study

Laparoscopic *vs* computerized tomography-guided radiofrequency ablation for large hepatic hemangiomas abutting the diaphragm

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Abstract

AIM: To compare safety and therapeutic efficacy of laparoscopic radiofrequency (RF) ablation *vs* computed tomography (CT)-guided RF ablation for large hepatic hemangiomas abutting the diaphragm.

METHODS: We retrospectively reviewed our sequential experience of treating 51 large hepatic hemangiomas abutting the diaphragm in 51 patients by CT-guided or laparoscopic RF ablation due to either the presence of symptoms and/or the enlargement of hemangioma. Altogether, 24 hemangiomas were ablated *via* a CT-guided percutaneous approach (CT-guided ablation group), and 27 hemangiomas were treated *via* a laparoscopic approach (laparoscopic ablation group).

RESULTS: The mean diameter of the 51 hemangiomas was 9.6 ± 1.8 cm (range, 6.0-12.0 cm). There was no

difference in the diameter of hemangiomas between the two groups ($P > 0.05$). RF ablation was performed successfully in all patients. There was no difference in ablation times between groups ($P > 0.05$). There were 23 thoracic complications in 17 patients: 15 (62.5%, 15/24) in the CT-guided ablation group and 2 (7.4%, 2/27) in the laparoscopic ablation group ($P < 0.05$). According to the Dindo-Clavien classification, two complications (pleural effusion and diaphragmatic rupture grade Ⅲ) were major in two patients. All others were minor (grade Ⅰ). Both major complications occurred in the CT-guided ablation group. The minor complications were treated successfully with conservative measures, and the two major complications underwent treatment by chest tube drainage and thoracoscopic surgery, respectively. Complete ablation was achieved in 91.7% (22/24) and 96.3% (26/27) in the CT-guided and the laparoscopic ablation groups, respectively ($P > 0.05$).

CONCLUSION: Laparoscopic RF ablation therapy should be used as the first-line treatment option for large hepatic hemangiomas abutting the diaphragm. It avoids thermal injury to the diaphragm and reduces thoracic complications.

Key words: Hepatic hemangioma; Radiofrequency ablation; Diaphragm; Computed tomography; Laparoscopy

Core tip: Radiofrequency (RF) ablation is an accepted non-surgical treatment for hepatic hemangiomas. If a tumor is located in the hepatic dome which abuts the diaphragm, complete tumor ablation without injury to the diaphragm or lung is challenging under percutaneous approach. The study preliminarily proved that laparoscopic RF ablation therapy should be used as the first-line treatment option for hepatic hemangiomas abutting the diaphragm, which can avoid thermal injury to the diaphragm effectively and reduce the thoracic complications obviously.

Gao J, Kong J, Ding XM, Ke S, Niu HG, Xin ZH, Ning CM, Guo SG, Li XL, Zhang L, Dong YH, Sun WB. Laparoscopic *vs* computerized tomography-guided radiofrequency ablation for large hepatic hemangiomas abutting the diaphragm. *World J Gastroenterol* 2015; 21(19): 5941-5949 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5941.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5941

INTRODUCTION

Hepatic hemangiomas are the most common benign tumors affecting the liver. They occur in the general population at an incidence ranging from 0.4% to 20%. In most cases, they are discovered incidentally on abdominal imaging studies[1,2]. As most hepatic hemangiomas are < 5 cm in diameter and asymptomatic, medical or surgical intervention is not necessary. Treatment is required, however, if the tumor is growing and manifests abdominal symptoms or if rupture has occurred (or may occur in the future)[2-5]. Although surgical resection is the most effective treatment for symptomatic, enlarging hepatic hemangiomas, it is a highly invasive procedure associated with morbidity and mortality rates of up to 27% and 3%, respectively[6-9]. Alternatively, minimally invasive procedures of transcatheter arterial embolization or radiation therapy may be used, but these treatments are not curative[10-13].

Radiofrequency (RF) ablation is an effective, minimally invasive, safe treatment for hepatic hemangiomas > 5 cm[14-20]. On the basis of the accumulated experience with treating hepatic hemangiomas by RF ablation and the development of RF equipment for this procedure, this therapy has also been performed successfully for those larger than > 10 cm[21-23].

The RF technique can be performed percutaneously, laparoscopically, or as an open procedure. Each approach has theoretical and proven advantages and disadvantages, which can lead to confusion and inefficiencies in the referral process for the patients. The percutaneous approach can be performed under general or local anesthesia as an outpatient procedure, which has the advantage of not requiring a surgical procedure. However, lesions located in the dome of the liver abutting the diaphragm or close to the stomach or colon are not always accessible by a percutaneous approach because of the risk of injuring adjacent organs. The laparoscopic approach has the advantage of being minimally invasive while providing access for intraoperative ultrasonography (US) examination of the liver for better lesion detection and more accurate targeting, although it requires a high level of skill. The laparoscopic approach is also indicated when the tumor is adherent to structures that may be damaged by thermal ablation such as the colon, stomach, or duodenum[24].

If a tumor is located in the hepatic dome, which abuts the diaphragm, complete tumor ablation without injury to the diaphragm or lung is challenging using the percutaneous approach[25-29]. In contrast, using the laparoscopic approach, establishment of pneumoperitoneum causes elevation of the diaphragm, which increases the operative space and avoids injuring the diaphragm. It also facilitates needle placement. We therefore hypothesized that the risk of thoracic complications would be reduced during RF ablation for large hepatic hemangiomas abutting the diaphragm under the laparoscopic approach.

To our knowledge, there are no published comparative controlled studies evaluating the protective effect on the diaphragm or therapeutic efficacy

of various approaches to RF ablation for large hepatic hemangiomas abutting the diaphragm. This study aimed to evaluate the protective effect on the diaphragm and the therapeutic efficacy of a laparoscopic *vs* a computed tomography (CT)-guided percutaneous approach to RF ablation for large hepatic hemangiomas.

MATERIALS AND METHODS

Patients

We retrospectively reviewed the records of consecutive patients with hepatic hemangiomas abutting the diaphragm whom we had treated by CT-guided or laparoscopic RF ablation from October 2011 to May 2014. The following hospitals in China participated in the study: Beijing Chaoyang Hospital Affiliated to Capital Medical University, Beijing, China; Affiliated Hospital of Chifeng University, Neimenggu, China; Chaoyang Central Hospital, Liaoning, China; Shanxi Provincial People's Hospital, Shanxi, China; Fenyang Hospital, Shanxi, China; and Zhanhua People's Hospital, Shandong, China.

From October 2011 to May 2014, a total of 9978 patients were diagnosed with hepatic hemangioma in the outpatient clinics of the six hospitals. Among them, 1025 patients suffered from large hepatic hemangiomas (⩾ 5 cm). All were initially managed by clinical observation. Over time, 187 of these patients were considered candidates for active treatment because of the following indications[20]: (1) the patient complained of persistent abdominal pain or discomfort related to the hemangioma. Upper gastrointestinal endoscopy and colonoscopy were performed to rule out any potential gastrointestinal diseases that might be causing these symptoms; and (2) the lesion increased in size by > 1 cm, which was confirmed on regular follow-up imaging studies during a 2-year observation period. On the basis of the accumulated experience of treating large hepatic hemangiomas by RF ablation[20,22], we treated these patients with hepatic hemangiomas using RF ablation as the first-line treatment.

Of the 187 patients, 51 patients with 51 hepatic hemangiomas abutting the diaphragm were included in the study. We defined tumors abutting the diaphragm when they were located near the diaphragm (< 5 mm) on axial CT scans[27,28]. All tumors in this study were mainly located in liver segment 4, 5, 7, or 8.

The staff team consisted of hepatobiliary surgeons, anesthesiologists, and radiologists. For the fact that intraoperative US guidance was available in Beijing Chao-yang Hospital, the patients in this hospital were treated under laparoscopic approach. Whereas, intraoperative US guidance was not available in the other 5 hospitals, so the patients there were treated under CT-guided percutaneous approach. The same experienced operator (Sun WB) performed all of the procedures, using internally cooled cluster electrodes, Cool-tip ACTC 2025 or ACTC 1525 electrodes, and an RF generator (Covidien Healthcare, Dublin, Ireland). Out of 51 hemangiomas, 27 were treated by laparoscopic RF ablation (laparoscopic ablation group), and 24 were treated by CT-guided percutaneous RF ablation (CT-guided ablation group).

Local review boards approved the study. Written informed consent was obtained from each patient before the treatment.

Laparoscopic RF procedure

For laparoscopic RF ablation, after induction of general anesthesia, patients were placed in a supine position. Two 10-mm trocars were placed in the abdomen, and initial laparoscopic exploration of the peritoneal cavity was performed. Under US guidance, the RF probe was introduced into the peritoneal cavity through the subcostal abdominal wall with laparoscopic visualization and deployed into the tumor. The ablation strategies were described in our previously published article[22]. The RF process was monitored by intraoperative US. The ablated lesion became hyperechoic because of outgassing from heated tissues.

CT-guided RF procedure

For the CT-guided RF procedure, a 35F or 37F left-side double-lumen endobronchial tube was intubated under general anesthesia. The tube position was checked and confirmed by auscultation or by fiberoptic bronchoscopy. The right lung was permitted to collapse, with selective left lung ventilation. The skin entrance point of the RF probe was chosen in the CT scanning plane containing the tumor. With CT monitoring, the RF probe was inserted through the chest wall and then through the empty pleural space and the diaphragm to the liver, finally reaching the targeted tumor. After the position of the probe was confirmed to be appropriate by CT, RF procedures were performed in a manner similar to that used for the laparoscopic procedure.

Postoperative evaluation

One day after ablation, all patients were evaluated on lung CT scans, which were repeated on the following day if necessary. All patients were followed by enhanced CT or magnetic resonance imaging (MRI) 1 mo after ablation. Complete ablation was defined as no nodular or irregular enhancement adjacent to the ablated zone, as shown on enhanced CT or MRI scans. Incomplete ablation was defined as irregular, peripheral-enhanced foci in the ablated zone. In the case of complete ablation, subsequent CT or MRI examinations were repeated at 6-mo intervals. In the case of incomplete ablation, repeat RF ablation was not performed unless the residual tumor had progressed during follow-up at 6-mo intervals.

Study endpoints

Primary endpoints of the study were technical success,

Table 1 Demographic characteristic of patients in the study *n* (%)

Group	CT-guided ablation (*n* = 24)	Laparoscopic ablation (*n* = 27)	*P* value
Age (yr), mean (SD)	50.0 (14.5)	49.5 (8.27)	0.748
Gender (male: female)	10:14	11:16	0.947
Co-morbidities			
Gallbladder stones	0 (0)	3 (11.1)	0.238
Type 2 diabetes mellitus	2 (8.3)	3 (10.3)	1.000
History of open cholecystectomy	1 (4.2)	0 (0)	0.471
Chronic hepatitis B	1 (4.2)	0 (0)	0.471
History of previous liver surgery	0 (0)	1 (3.7)	1.000
Hepatic cysts	0 (0)	1 (3.7)	1.000
Reasons for radiofrequency ablation			
Abdominal discomfort only	3 (12.5)	3 (11.1)	1.000
Enlargement of hemangioma only	9 (37.5)	11 (40.7)	0.813
Abdominal discomfort and enlargement	12 (50.0)	13 (48.2)	0.895
Maximal size of hemangioma, (cm), mean (SD)	9.6 (2.5)	9.4 (1.8)	0.686
Min	6.0	6.5	
Max	11.5	12.0	

Table 2 Outcome of radiofrequency ablation for hepatic hemangiomas *n* (%)

Group	CT-guided ablation (*n* = 24)	Laparoscopic ablation (*n* = 27)	*P* value
Technical success rate	24 (100)	27 (100)	
Complete ablation	22 (91.7)	26 (96.3)	0.595
Time of ablation per lesion (min), mean (SD)	94.2 (20.4)	95.4 (18.3)	0.875
Diameter of ablated zone 1 mo after ablation (cm), mean (SD)	6.4 (1.3)	4.4 (1.4)	0.457
Diameter of ablated zone 6 mo after ablation (cm), mean (SD)	5.5 (1.4)	3.7 (1.5)	0.387

safety (no thoracic complications related to RF ablation), mean hospital stay, and confirmed complete ablation. Secondary endpoints were alleviation of symptoms, change in the size of the ablation zone, recurrence of the residual tumor, and quality of life. The endpoints of the study were defined at 6 mo after the RF ablation treatment.

Statistical analysis

Values are expressed as mean ± SD. Continuous variables were compared between groups using Student's *t*-test and analysis of variance. Differences in the categorical data were analyzed by use of the χ^2 test or Fisher's exact test. Two-tailed *P* values < 0.05 were deemed significant. Statistical analyses were performed using SPSS version 15.0 for Windows (SPSS, Chicago, IL, United States). The statistical methods of this study were reviewed by Chunmin Ning and Shigang Guo from Department of General Surgery, Chaoyang Central Hospital

Table 3 Thoracic complications of radiofrequency ablation *n* (%)

Group	CT-guided ablation (*n* = 24)	Laparoscopic ablation (*n* = 27)	*P* value
Total No. of patients with complication	15 (62.5)	2 (7.4)	0.000
Incidence of complication			
Right shoulder pain	11 (45.8)	2 (7.4)	0.003
Transient lung injury	3 (12.5)	0 (0)	0.097
Plural effusion	5 (20.8)	0 (0)	0.018
Diaphragmatic perforation	1 (4.2)	0 (0)	0.471
Hemothorax	1 (4.2)	0 (0)	0.471
Hospital stay, mean (SD)	9.3 (6.5)	3.7 (0.9)	0.032

RESULTS

RF ablation procedure

Of the 51 patients, 21 (41.2%) were male and 30 (58.8%) female. The mean diameter of the 51 hemangiomas was 9.6 ± 1.8 cm (range, 6.0-12.0 cm). The patients' demographic characteristics are given in Table 1.

Outcome data for the RF ablation treatments are given in Table 2. Ablation treatment was conducted according to predefined protocols. There were no technical failures. There was no difference in ablation times between the two groups ($P > 0.05$) (Table 2).

In the laparoscopic ablation group, laparoscopic cholecystectomy or deroofing was performed during the ablation procedure in patients with gallstones ($n = 3$) or hepatic cysts ($n = 1$).

Safety of RF ablation: thoracic complications

There were 23 thoracic complications in 17 patients, including 15 (62.5%, 15/24) in the CT-guided ablation group and 2 (7.4%, 2/27) in the laparoscopic ablation group ($P < 0.05$) (Table 3). According to the Dindo-Clavien classification[30], two complications (pleural effusion and diaphragmatic rupture, grade Ⅲ) were major in two patients. All others were minor (grade Ⅰ).

Eleven patients who underwent CT-guided ablation and two who underwent laparoscopic ablation had right shoulder pain after ablation. All of these patients required a pethidine injection for relief. The mean duration of the postprocedural pain was 2.5 d in the CT-guided ablation group and 1 d in the laparoscopic ablation group.

Three cases of transient lung injury without respiratory symptoms were detected on CT scans in the CT-guided ablation group 1 d after ablation. The injuries appeared as an abnormal round shape of the lung parenchymal density on the CT scans. These lesions resolved spontaneously without specific treatment as detected on follow-up CT scans.

Five patients in the CT-guided ablation group developed pleural effusion. Four of the five patients

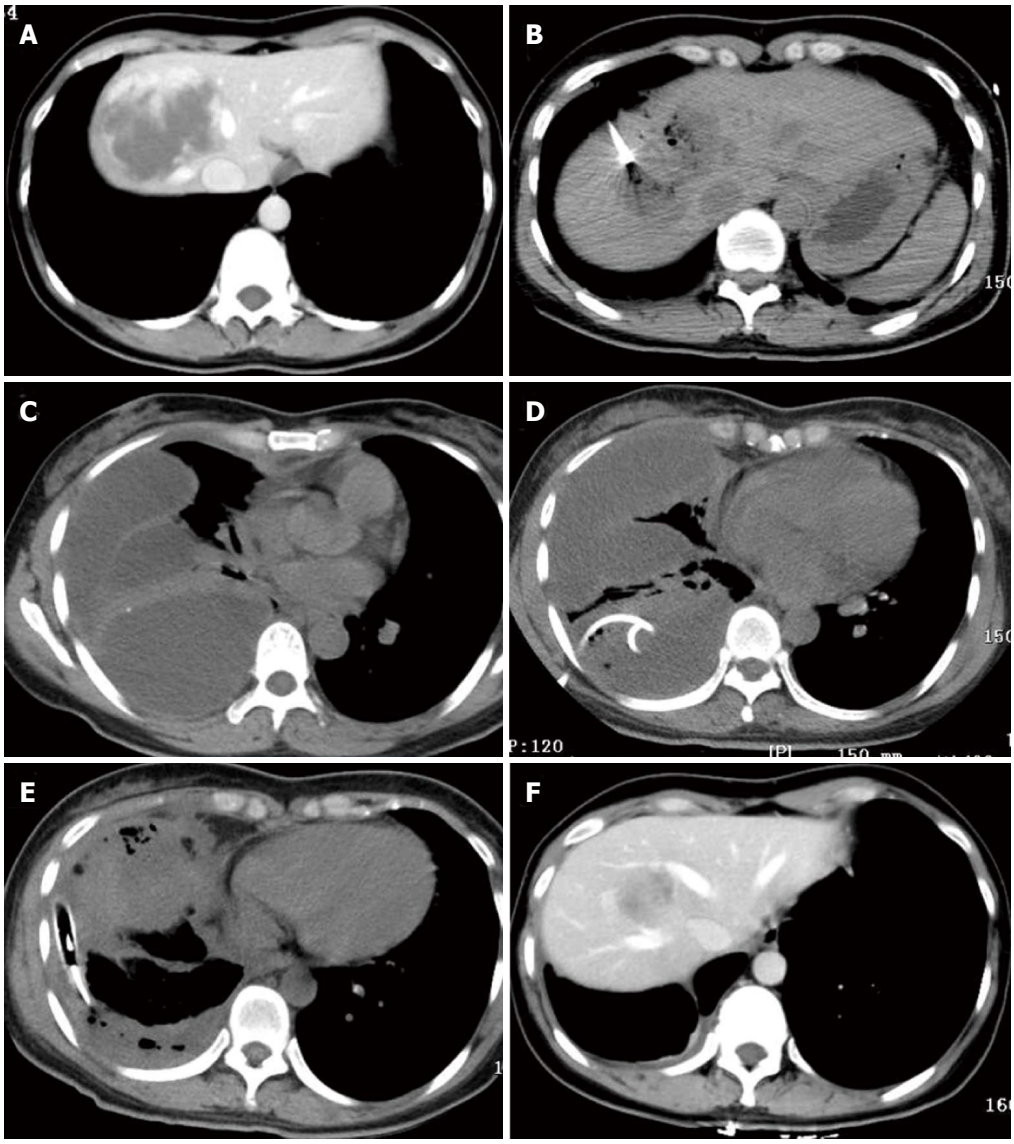

Figure 1 One patient with a 7.5-cm hemangioma mainly in segment 8 in the computed tomography-guided ablation group developed a diaphragmatic rupture after ablation. A: A 40-year-old woman in the computed tomography (CT)-guided radiofrequency (RF) ablation group had a 7.5-cm hemangioma in segment 8, as illustrated on an abdominal CT scan; B: During CT-guided ablation, the lesion became depressed and commenced outgassing; C: Four days after ablation, CT scan of the chest showed a multiloculated pleural effusion occupying the right hemithorax with collapse of the right lung; D: Drainage *via* a chest tube was unsuccessful. She subsequently underwent thoracotomy; E: Two chest tubes were inserted into the pleural space; F: At 6 mo after ablation, CT scans showed that the hemangioma was completely ablated and markedly smaller without development of a diaphragmatic hernia.

recovered after conservative treatment. The other patient underwent drainage *via* a chest tube (grade Ⅲ complication).

One patient with a 7.5-cm hemangioma mainly in segment 8 in the CT-guided ablation group developed a diaphragmatic rupture after ablation (Figure 1A and B). She was clinically normal for the first 2 d after ablation but then developed right shoulder pain and a high fever (39.0 ℃). CT scans of the chest (Figure 1C) showed a multiloculated pleural effusion occupying the right hemithorax with collapse of the right lung. An attempt at drainage *via* a chest tube yielded only 100 mL of bloody fluid (Figure 1D). She subsequently underwent thoracoscopic surgery, which showed multiple, fluid-filled loculations mostly around the right lower lobe with significant adhesions to the diaphragm and along the major and minor fissures. The loculations were dissected along with decortication of a thick pleural rind, after which a diaphragmatic defect of 1.0 cm diameter was found. The diaphragm was not repaired because of the tissue edema surrounding the defect. Two chest tubes were inserted into the pleural space and were removed 1 wk later (Figure 1E). The patient was discharged 23 d after ablation. Six months after ablation, CT scans in the arterial phase showed that the hemangioma had been completely ablated without formation of a diaphragmatic hernia (Figure 1F).

The hospital stay was significantly shorter in the laparoscopic ablation group than in the CT-guided

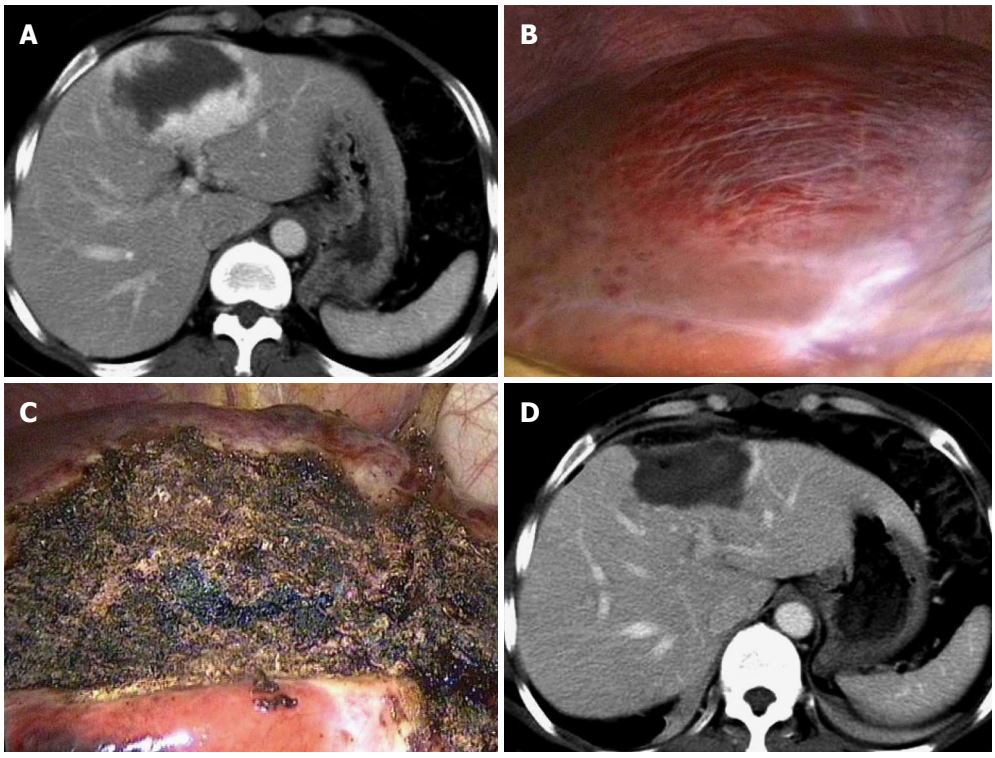

Figure 2 Complete ablation is achieved in a patient in the laparoscopic ablation group. A: A 45-year-old woman in the laparoscopic ablation group had an 8.0-cm hemangioma in segment 5, as seen on an abdominal computerized tomography (CT) scan; B: On laparoscopic views, the tumor is evident on the superior surface; C: The lesion became a depressed mass of hard texture after RF ablation; D: The hemangioma disappeared completely 1 mo after ablation, as illustrated on an abdominal CT scan.

ablation group (3.7 ± 0.9 d *vs* 9.3 ± 6.5 d, $P < 0.05$). This difference was directly related to thoracic complications in the CT-guided ablation group.

Efficacy of RF ablation

Complete ablation was achieved in 91.7% (22/24) and 96.3% (26/27) of the CT-guided and laparoscopic ablation groups, respectively ($P > 0.05$) (Table 3 and Figure 2). Three hemangiomas were incompletely ablated, showing subtle enhancement on the peripheral rim of the ablated tumors on follow-up CT or MRI.

At 1 mo, the mean diameter of the ablation zone was reduced from 9.6 ± 2.5 cm to 6.4 ± 1.3 cm in the CT-guided ablation group and from 9.4 ± 1.8 cm to 4.4 ± 1.4 cm in the laparoscopic ablation group - an insignificant difference between the groups ($P > 0.05$). At 6 mo after ablation, the mean diameter of the ablation zone had decreased further in both groups (to 5.5 ± 1.4 cm in the CT-guided ablation group and to 3.7 ± 1.5 cm in the laparoscopic ablation group), also an insignificant difference.

Follow-up results

After RF ablation, there was no perioperative mortality or delayed complications, such as local tumor progression, destructive biliary damages, or liver abscess. The three residual lesions shrunk somewhat during the follow-up period and necessitated no further treatment. Of the 31 patients who had pretreatment symptoms related to their hemangiomas, 28 had complete resolution of symptoms and three experienced symptom amelioration without further therapy after ablation. At the 6-mo follow-up, no patient had developed new symptoms attributable to the hemangiomas. The subjective health status and quality of life were rated as good to excellent in 100% of patients at follow-up[3]. After RF ablation of the hemangiomas, all patients were able to perform full-time or part-time work.

DISCUSSION

The study aimed to compare the safety and therapeutic efficacy for patients who underwent CT-guided percutaneous or laparoscopic RF ablation for large hepatic hemangiomas abutting the diaphragm. Our data suggest that the laparoscopic approach for RF ablation of large hepatic hemangiomas abutting the diaphragm was successful. It was associated with fewer thoracic complications than the CT-guided percutaneous approach (7.4% *vs* 62.5%). We also found that the hospital stay was significantly shorter for patients treated with the laparoscopic approach because of their lower incidence of complications. A high frequency of complete ablation of these tumors was attained with both treatment approaches. The immediate and sustained reduction in the size of the tumors was also similar in the two groups.

The hepatic tumors situated in the hepatic dome area abut the diaphragm. Thus, collateral thermal damage to the diaphragm easily occurs during RF treatment under the percutaneous approach. Several thoracic complications have been reported, including diaphragmatic perforation, right shoulder pain, pleural effusion, and other problems following RF ablation of subcapsular malignant tumors abutting the diaphragm[25-29].

The diaphragm is innervated by phrenic nerves arising from nerve roots C3, C4, and C5, which represent the same dermatome as that in shoulder skin. Thus, diaphragmatic irritation is referred to the shoulder and can cause right shoulder pain. Hence, right shoulder pain (referred pain) was reportedly the representative indicator of diaphragmatic thermal injury[25-29]. Diaphragmatic perforation and herniation were reported as major complications of RF ablation for hepatic tumors abutting the diaphragm in nine cases[29]. Kang *et al*[27] retrospectively assessed 80 patients who underwent percutaneous RF ablation for a single nodular (< 4 cm) hepatocellular carcinoma. They divided the patients into two groups based on whether the index tumor was abutting the diaphragm: group A (abutting; $n = 31$) *vs* group B (nonabutting; $n = 49$). They found that the percutaneous RF ablation of hepatocellular carcinoma abutting the diaphragm seemed safe without major complications, although it was associated with right shoulder pain (26%) and transient lung injury (23%). It was less effective, however, with regard to technical success (84% *vs* 98%) and local tumor progression (29% *vs* 6%). The operator's concern about thermal injury to the adjacent diaphragm may lead to incomplete ablation.

In theory, it is more difficult and risky for percutaneous RF ablation of large hepatic hemangiomas abutting the diaphragm than hepatic malignant tumors abutting the diaphragm. This is because the hemangiomas to be treated are usually larger than the malignant tumors and have wider contact area with the diaphragm. Theoretically, this difficulty and the risk can be maximally solved with a preference for the laparoscopic approach. Laparoscopic RF ablation is increasingly being used for malignant tumors because of its several advantages[31]. It allows accurate identification of patients with extrahepatic disease (*i.e.*, peritoneal metastases) who would otherwise be undetectable by CT. Moreover, intraoperative ultrasonography was used routinely in conjunction with the laparoscopic approach to increase the ability to determine real-time RF electrode placement and evaluate the efficacy of ablation[20,31]. For the large hepatic hemangiomas abutting the diaphragm, establishment of pneumoperitoneum causes elevation of the diaphragm, which increases the operative space, thereby avoiding diaphragmatic injury and facilitating needle placement during laparoscopic RF ablation. Also, a single well-done ablation of a hepatic hemangioma can lead to obvious collapse of tumor tissue around the ablation zone, increasing the operative space further, making the following ablation easier.

During this era of evidence-based medical practice, determining the best approach for each patient is increasingly important. To our best knowledge, the present study is the first comparative controlled study to evaluate the protective effect on the diaphragm or therapeutic efficacy of different approaches of RF ablation for large hepatic hemangiomas abutting the diaphragm. The study shows that the incidence of thoracic complications was significantly lower in patients undergoing laparoscopic ablation than in those subjected to CT-guided ablation. Most of the thoracic complications were trivial, although two were of major significance. In the current study, a high frequency of complete ablation of these tumors was attained with both treatment approaches. Interestingly, the remnant tumor tissues were situated within the liver in the laparoscopic ablation group, whereas in the CT-guided ablation group they were on the surface of the lesions. We therefore suggest that the laparoscopic approach of RF ablation should be the first-line treatment for large hepatic hemangiomas abutting the diaphragm. If a second ablation session is needed[22], the repeat RF ablation would be performed percutaneously.

The major limitations of our study include its retrospective nature, the nonrandomized selection of patients, the short follow-up period, and the relatively small number of patients evaluated. We believe it is important that all of our RF procedures were performed by a single surgeon, thus minimizing the chance of bias that might have occurred had multiple surgeons been involved. Also, the patients in our study were managed from a surgical perspective and by a team of surgeons and making the results less applicable for nonsurgical institutions.

In conclusion, laparoscopic RF ablation therapy should be used as the first-line treatment for large hepatic hemangiomas abutting the diaphragm. Its use avoids thermal injury to the diaphragm and reduces the occurrence of thoracic complications.

COMMENTS

Background

Radiofrequency (RF) ablation is an accepted non-surgical treatment for hepatic hemangiomas. If a tumor is located in the hepatic dome which abuts the diaphragm, complete tumor ablation without injury to the diaphragm or lung is challenging under percutaneous approach. In contrast, using the laparoscopic approach, establishment of pneumoperitoneum causes elevation of the diaphragm, which increases the operative space and avoids injuring the diaphragm. In theory, it is more difficult and risky for percutaneous RF ablation of large hepatic hemangiomas abutting the diaphragm than hepatic malignant tumors abutting the diaphragm. This is because the hemangiomas to be treated are usually larger than the malignant tumors and have wider contact area with the diaphragm.

Research frontiers

The present study is the first comparative controlled study to evaluate the protective effect on the diaphragm or therapeutic efficacy of different approaches of RF ablation for large hepatic hemangiomas abutting the

diaphragm.

Innovations and breakthroughs

The study shows that the incidence of thoracic complications was significantly lower in patients undergoing laparoscopic ablation than in those subjected to computerized tomography (CT)-guided ablation.

Applications

The study results provide evidence that laparoscopic RF ablation therapy should be used as the first-line treatment for large hepatic hemangiomas abutting the diaphragm. Its use avoids thermal injury to the diaphragm and reduces the occurrence of thoracic complications.

Terminology

The tumor abutting the diaphragm was defined as the lesion that was located near the diaphragm (< 5 mm) on axial CT scans.

Peer-review

This is an excellent retrospective study in which the authors used data of 6 institutions to evaluate the protective effect on the diaphragm or therapeutic efficacy of various approaches to RF ablation for large hepatic hemangiomas abutting the diaphragm. The results are interesting and suggest that laparoscopic RF ablation therapy should be used as the first-line treatment option for hepatic hemagiomas abutting the diaphragm, which can avoid thermal injury to the diaphragm effectively and reduce the thoracic complications obviously.

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ORIGINAL ARTICLE

Retrospective Study

Endoscopic treatment for pancreatic diseases: Needle-knife-guided cannulation *via* the minor papilla

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Abstract

AIM: To determine the efficacy and safety of meticulous cannulation by needle-knife.

METHODS: Three needle-knife procedures were used to facilitate cannulation in cases when standard cannulation techniques failed. A total of 104 cannulations *via* the minor papilla attempted in 74 patients at our center between January 2008 and June 2014 were retrospectively reviewed.

RESULTS: Standard methods were successful in 79 cannulations. Of the 25 cannulations that could not be performed by standard methods, 19 were performed by needle-knife, while 17 (89.5%) were successful. Needle-knife use improved the success rate of cannulation [76.0%, 79/104 *vs* 92.3%, (79 + 17)/104; $P = 0.001$]. When the 6 cases not appropriate for needle-knife cannulation were excluded, the success rate was improved further (80.6%, 79/98 *vs* 98.0%, 96/98; $P = 0.000$). There were no significant differences in the rates of post-endoscopic retrograde cholangiopancreatography adverse events between the group using standard methods alone and the group using needle-knife after failure of standard methods (4.7% *vs* 10.5%, $P = 0.301$).

CONCLUSION: The needle-knife procedure may be an alternative method for improving the success rate of cannulation *via* the minor papilla, particularly when standard cannulation has failed.

Key words: Needle-knife; Minor papilla; Cannulation; Meticulous procedure; Endoscopic retrograde cholangiopancreatography

Core tip: Cannulation of the minor papilla into the duct of Santorini can be difficult to achieve *via* standard procedures because of the tiny minor papilla orifice. Although various methods have been advocated to facilitate cannulation, some procedures still fail. The needle-knife has a slim tip, which is a unique advantage for insertion into the minor papilla orifice. After failure of standard cannulation techniques, we used three

needle-knife procedures to facilitate cannulation *via* the minor papilla, which significantly improved the success rates of cannulation. In this series, we describe the meticulous procedures involved in this technique.

Wang W, Gong B, Jiang WS, Liu L, Bielike K, Xv B, Wu YL. Endoscopic treatment for pancreatic diseases: Needle-knife-guided cannulation *via* the minor papilla. *World J Gastroenterol* 2015; 21(19): 5950-5960 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5950.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5950

INTRODUCTION

Endoscopic treatment *via* the minor papilla may be the only option for some patients, including those with pancreatic divisum, recurrent acute pancreatitis, chronic pancreatitis (CP) with severe abdominal pain, and pancreatographic changes[1-9]. The most important step in endoscopic treatment of these diseases is insertion of the guidewire into the duct of Santorini[10-12]. Standard procedures involve wire-guided cannulation with a standard sphincterotome (5-7F cannula) that has a straight or tapered tip that can accept a 0.035-inch guidewire[11,13]. However, it is often difficult to cannulate the minor papilla.

Experts have advocated various procedures to facilitate minor papilla cannulation, including spraying the minor papilla with methylene blue and indigo carmine to help identify the orifice[11,14], administering intravenous secretin to induce flow of pancreatic secretions and to make the minor papilla orifice more evident[5,10,15,16], using a needle-knife to perform minor papilla fistulotomy[4], using tapered catheters or sphincterotomes with or without a small-caliber wire[1-5,7-10,14-21], and performing rendezvous techniques, wire-guided cannulation, or physician-controlled wire-guided cannulation[4,16,21,22]. These procedures produced successful cannulation in 73% to 100% of documented cases (*i.e.*, 0% to 27% of procedures failed; Table 1)[1-5,7-11,14-22].

The main reason for cannulation failure is that the minor papilla orifice can be inconspicuous or appear as a tiny dimple or papule, with almost no elevation or apparent orifice, and so guidewires and sphincterotome cannulas are larger than the minor papilla orifice. Although smaller-caliber guidewires (*e.g.*, 0.018- to 0.025-inch) are available, they are soft and difficult to control. Furthermore, intravenous secretin is contraindicated in children/adolescents, cases of acute pancreatitis, patients who have used anticholinergic medications within one week of endoscopic retrograde cholangiopancreatography (ERCP), patients with known hypersensitivity or adverse reactions to secretin, and patients who are pregnant or breastfeeding[15]. Thus, further innovations are needed to improve success rates and minimize the adverse events of these techniques.

The needle-knife has a slim tip, which is a unique advantage for insertion into the minor papilla orifice. Many centers have used needle-knife-guided cannulation *via* the minor papilla. However, some centers have discontinued using the technique, which has mainly been assessed for therapeutic potential[5,9,13,16,20]. Since 2008, our center has used needle-knives for cannulation when the minor papilla orifice is too small to allow a catheter or guidewire to advance deeply. The objective of this study was to describe the efficacy and safety of this meticulous procedure.

MATERIALS AND METHODS

Patients

The ERCP database was retrospectively reviewed to find all patients who underwent cannulation *via* the minor papilla at the Digestive Endoscopy Center at Ruijin Hospital from January 2008 to June 2014. Patients who did not receive cannulation *via* the minor papilla were excluded. After approval by the Institutional Review Board, medical records were accessed and the following data were acquired for each patient: age, sex, presenting problem, number and success of procedures, diagnostic findings, therapeutic measures, cannulation time, adverse events, patient management, and follow-up visits and management.

Definition

"Successful cannulation" was defined as deep placement of the guidewire into the duct of Santorini (*i.e.*, the dorsal duct), which was assessed by the injection of contrast medium[13]. "Cannulation time" was defined as the elapsed time from the commencement of cannulation attempts to the successful cannulation of the duct of Santorini[15]. "Cannulation attempt" was defined as contact between the cannulating device and the papilla for at least 5 s[23]. Because few centers use a needle-knife to introduce a guidewire *via* the minor papilla during cannulation, we referred to previous studies that used the needle-knife for difficult biliary cannulation[13,24-27] and our past experiences. We arbitrarily defined "unsuccessful cannulation" as "a skilled endoscopist not achieving cannulation after 5 min or more than 15 attempts".

Pancreatic divisum was diagnosed *via* the presence or absence of ductal abnormalities by ERCP[10]. Intraductal papillary mucinous neoplasm (IPMN) was diagnosed from ERCP data or histologic examination[28]. Similar to Li and Wang *et al*[29], Wang currently at Ruijin Hospital North, CP was diagnosed on the basis of abdominal pain and the presence of any of the following findings: (1) ductal changes on ERCP, (2) pancreatic calcification (including ductal stones) revealed by imaging, and (3) histological analyses (when surgical intervention was performed).

Major adverse events of ERCP were defined

Table 1 Published data on cannulation *via* the minor papilla

Ref.	Year	No.	Cannulation procedure	Success (%)	Complication (%)
18	1984	6	Blunt tipped needle catheter	83.3	0
1	1986	6	Flexible Seldinger wire with a dilator and papillotome	100	0
19	1987	18	Needle-tipped catheter or 0.018 inch guidewire	72.9	4.2
11	1990	136	Tapered or needle-tipped catheter + secretin (35% patients)	91	1.5
20	1992	19	Tapered catheter with 0.018 inch guidewire	83	NA
2	2000	25	Tapered catheter with 0.018 or 0.02 inch guidewire	73.5	0
3	2002	24	Tapered catheter with 0.018 inch guidewire	NA	38
23	2003	6	Contour catheter with 0.025 or 0.035 inch wire (rendezvous technique)	100	0
15	2003	14	Methylene blue + needle tipped catheter with 0.018 inch guidewire	85.7	7.1
16	2003	28	Synthetic porcine secretin	89.3	0
4	2004	11	Catheter with 0.025 inch guidewire (including rendezvous technique), needle-knife to minor papilla fistulotomy	90.9	0
21	2006	184	Tapered or metal tip catheter with 0.018 or 0.025 inch guidewire	NA	8.2
6	2008	57	Tapered cannula with a guidewire + secretin (10% patients)	86	11.7
17	2009	64	Pull-sphincterotome with 0.018-0.035 inch guidewire (wire-guided cannulation) + secretin (17% patients)	85	26.5
22	2010	25	Tip sphincterotome with a 0.025 inch guidewire (physician-controlled wire-guided cannulation)	96	12
8	2013	34	Tapered catheter with or without 0.025 inch guidewire	80	4.5
9	2013	48	Tapered cannula and a 0.025 or 0.035 inch guidewire	97.9	2.0
10	2013	45	Tapered-tip or needle-tip catheters, short-nose pull-sphincterotomes, and 0.018-0.035 inch guidewires	91.9	16.1

PD: Pancreas divisum; NA: Not available; No.: Number of patients.

according to consensus criteria. Post-ERCP pancreatitis was defined as new or worsened abdominal pain for more than 24 h after endoscopy, with amylase levels more than threefold the normal upper limit, which required hospitalization or prolonged planned hospitalization for at least one night. Mild infection (cholangitis) was defined as a temperature of more than 38 ℃ for 24 to 48 h after the procedure, accompanied by colicky pain and cholestasis/jaundice without evidence of unrelated infections. "Moderate bleeding" was defined as bleeding requiring the maximum number of transfusions (4 units of packed cells) in the absence of angiographic or surgical intervention[29,30].

Cannulation via minor papilla

The risks and benefits of the procedure and alternative treatments were explained. Written informed consent was obtained from adult patients and parents/guardians of children before the procedure. Patients were mildly sedated (with diazepam, meperidine, and scopolamine butylbromide) or deeply sedated (with propofol injection, fentanyl, and vecuronium bromide) by anesthetists. Side-viewing duodenoscopes (JF-240 for children and adolescents, JF-260 for adult patients; Olympus Optical Co. Ltd., Tokyo, Japan) were used, and cannulation was performed with standard pull-sphincterotomes (ENDO-FLEX, Germany) with or without a guidewire (Jagwire; 0.025- or 0.035-inch in diameter, 450-cm in length; Boston Scientific).

When endoscopists detected pancreatic divisum, distortion of the duct of Wirsung, a tight stricture, or an impacted stone downstream from the junction of the main and accessory ducts, cannulation *via* the minor papilla was attempted and a two-step sequence guideline was followed. The direct approach *via* the minor papilla was attempted by wire-guided cannulation with a standard sphincterotome (physician-controlled wire-guided cannulation) or directly using the tapered tip of the sphincterotome (Step 1). When the main pancreatic duct was distorted and a guidewire introduced *via* the major papilla entered the duodenum *via* the minor papilla, the "rendezvous method" was performed (Step 1.1). A guidewire was inserted into the duct of Wirsung *via* the major papilla, and then into the duodenum *via* the duct of Santorini and minor papilla. The guidewire was grasped and removed through the biopsy channel of the endoscope[5,22].

A needle-knife (MicroKnife TM XL, Boston Scientific, USA) was used for cannulation when the pull-sphincterotome failed, when the minor papilla orifice was too small to receive the pull-sphincterotome or guidewire, or when the expert (Gong B) deemed it necessary (Step 2). For needle-knife cannulation, the needle tip was extended 5 to 7 mm beyond the cannula tip and passed in the direction of the minor papilla orifice. If the orifice was visible, then a small incision was made by the needle-knife (precut papillotomy; Step 2.1). The incision was started at the top of the papillary orifice (12 o'clock position) and extended in the cephalad direction until the papillary mound was divided or the incision was sufficiently large to receive the tip of the guidewire or pull-sphincterotome. The cut size was determined by the size of the minor papilla, and generally ranged from 3 to 6 mm. After the incision was made, the needle-knife was exchanged for a pull-sphincterotome with a guidewire, which was carefully advanced into the duct of Santorini. This procedure was named "needle-knife papillotomy with introduction of a guidewire and pull-sphincterotome" (NPI-GS; Figure 1). If the

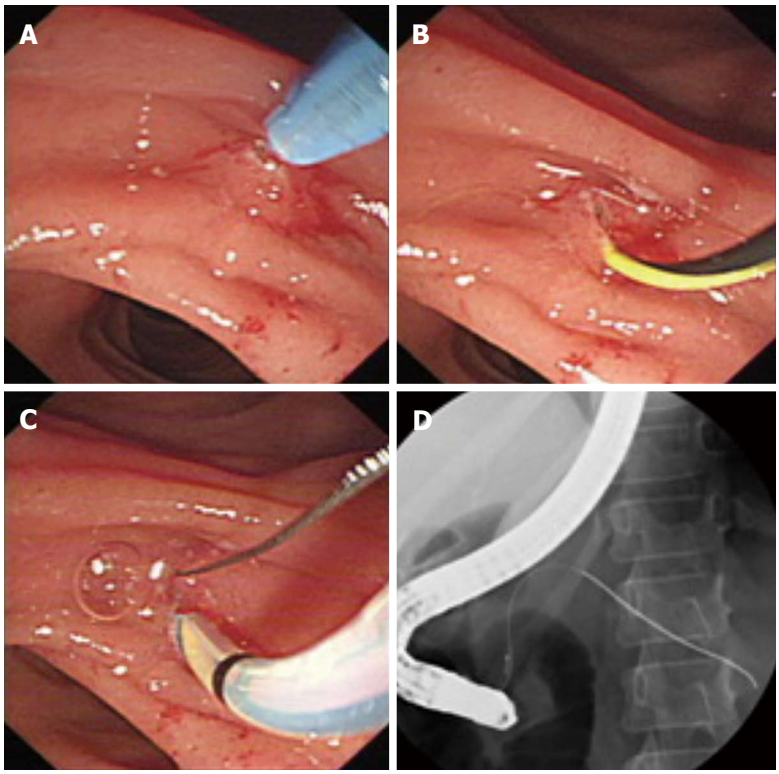

Figure 1 "Needle-knife papillotomy with introduction of a guidewire and pull-sphincterotome" procedure. A: A small incision was made with the needle-knife (precut papillotomy); B: The needle-knife was exchanged for a pull-sphincterotome with a guidewire, which was carefully advanced into the duct of Santorini through the sphincterotome; C: The sphincterotome advanced along the guidewire; D: Guidewire located in the duct of Santorini.

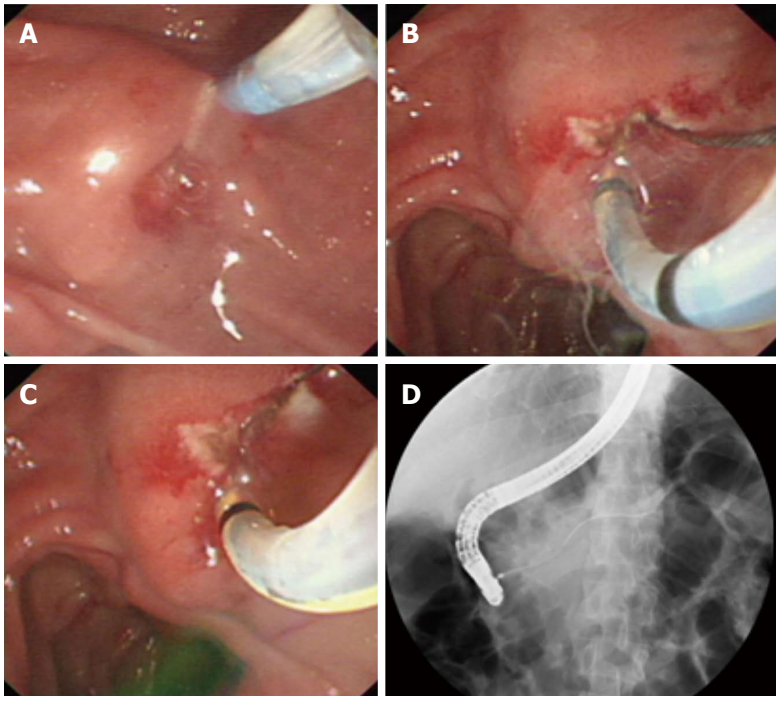

Figure 2 "Needle-knife papillotomy with introduction of a guidewire, pull-sphincterotome, and guidewire" procedure. A: A small incision was made with the needle-knife (precut papillotomy); B: The needle-knife was exchanged for a pull-sphincterotome, which was used to enlarge the incision; C: A guidewire was carefully advanced into the duct of Santorini through the sphincterotome; D: Guidewire located in the duct of Santorini.

incision in the minor papilla orif ce was too small to receive a guidewire, then the sphincterotome was used to perform a small papillotomy (Step 2.1.1). This procedure was named "needle-knife papillotomy with introduction of a guidewire, pull-sphincterotome, and guidewire" (NPI-GSG) (Figure 2).

When the minor papilla orifice was invisible, we used the "needle-knife introduction of a guidewire" (NI-G) procedure (Step 2.2). The needle tip was aimed at and carefully inserted into the orifice. The needle-knife cannula was pushed forward, while the needle-knife tip was pulled back until the cannula anastomosed with the minor papilla orifice. The needle-knife cannula was lightly pressed on or adjacent to the minor papilla orifice while the needle-knife tip was in the orifice. A guidewire (Jagwire; 0.025- or 0.035-inch in diameter, 450-cm in length; Boston Scientific) was carefully advanced approximately 25 mm into the duct of Santorini through the needle-knife cannula and the minor papilla orifice until it passed the cross-point of

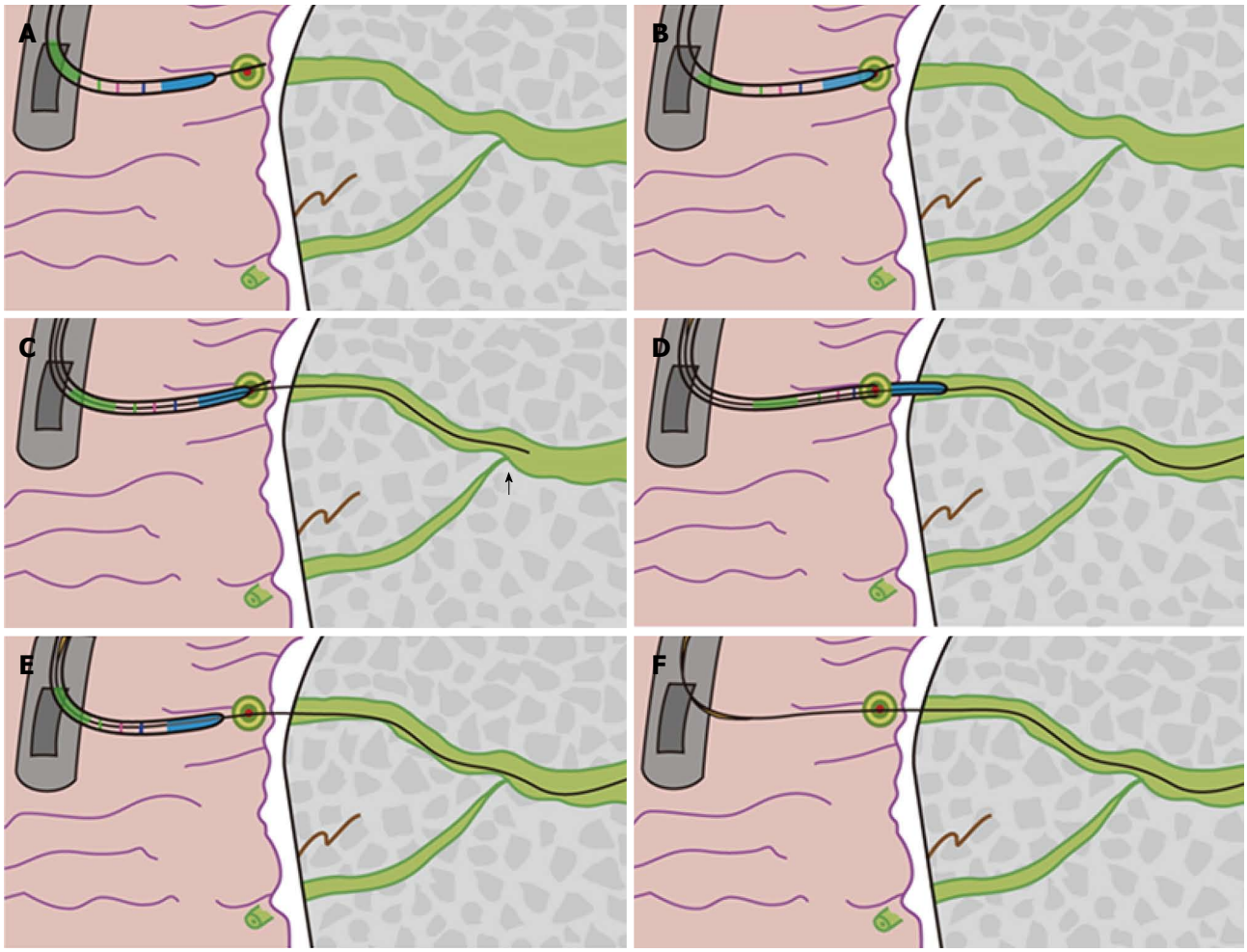

Figure 3 Schematic for the "needle-knife introduction of a guidewire" procedure. A: The needle tip was inserted into the orifice; B: The needle-knife cannula was placed on the minor papilla orifice; C: The guidewire was carefully advanced through the cannula until it passed the cross-point (black arrow) of the ducts of Wirsung and Santorini; D: The cannula was inserted into the capitular head of the duct of Santorini along the guidewire and advanced; E: The needle-knife was removed; F: The guidewire was left in place.

the ducts of Wirsung and Santorini. The needle-knife cannula was inserted into the capitular head of the duct of Santorini along the guidewire and contrast medium was injected. After the course of the duct of Santorini was confirmed, the guidewire was advanced deeply into the duct for therapeutic intervention, while the needle-knife cannula was pulled back until it was completely withdrawn. Finally, the needle-knife was removed, leaving the guidewire in place (Figures 3-5).

After successful cannulation *via* the minor papilla and the duct of Santorini, endoscopic interventions were performed. These interventions included papillotomy, stone extraction, nasopancreatic drainage, stricture dilation, stent insertion, and stent retrieval.

Follow-up

Eligible patients were contacted, with follow-up information obtained by Jiang WS. A questionnaire was completed *via* telephone interviews or face-to-face meetings with patients. The scripted telephone questionnaire included questions regarding the present condition of the patient. Answers were provided on a 5-point Likert scale: 1, cured; 2, better; 3, same; 4, worse; and 5, much worse. Data regarding any repeat ERCP, surgery, and narcotic requirements were collected[6]. The follow-up period was defined as the period between the date of the first endoscopic intervention at our hospital to the last follow-up[30], date of death, or date of surgical intervention.

Statistical analysis

Quantitative data were summarized by mean ± SD or median (range). Data were analyzed by *t*-tests (for normal distributions) or by Wilcoxon rank-sum tests (for non-normal distributions). Categorical data were presented as frequencies (percentages) and analyzed by χ^2 tests with or without the Yates correction for continuity or the Fisher exact test, where appropriate. SPSS 17.7 software for Windows (SPSS, Chicago, IL) was used for statistical analyses. A P value < 0.05 was considered statistically significant[30].

RESULTS

A total of 5385 ERCPs were performed, and 104 cannulations (1.9%) *via* the minor papilla were

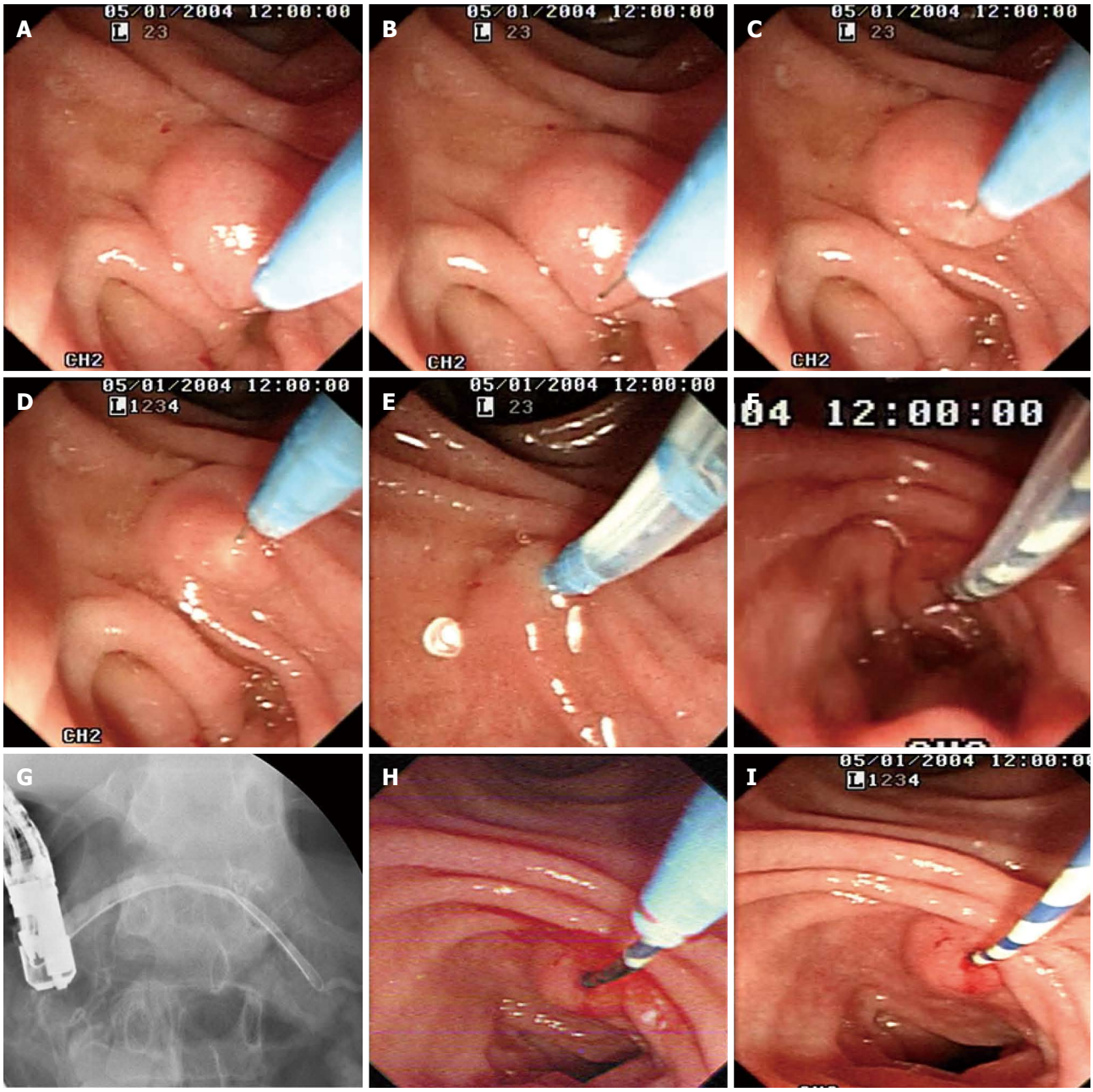

Figure 4 "Needle-knife introduction of a guidewire" procedure. A: The cannula of the needle-knife approaching the minor papilla orifice; B: The needle tip was extended 3 to 5 mm beyond the cannula tip and aimed at the orifice; C, D: The needle tip was inserted into the orifice; E: The cannula of the needle-knife was placed on the minor papilla orifice and a guidewire was advanced through the cannula; F: The cannula was inserted into the capitular head of the duct of Santorini along the guidewire and advanced continually; G: Contrast material was injected and the location within the duct of Santorini was confirmed; H: The needle-knife was removed; I: The guidewire was left in place.

attempted in 74 patients (mean: 1.4 ERCPs/patient; range: 1-5 ERCPs/patient). Diagnoses and clinical indications for the procedures are presented in Table 2. Three patients with pancreatic divisum and CP also received surgical interventions (post-pancreaticojejunostomy, n = 1; Billroth Ⅰ reconstructi on after gastrectomy, n = 1; Billroth Ⅱ reconstruction after gastrectomy, n = 1).

Cannulations using sphincterotomes with or without guidewires were successful in 79/104 ERCPs (76.0%). Among the 25 failed cannulations performed with sphincterotomes, 19 cannulations with needle-knives were performed. Seventeen of these procedures (89.5%, 17/19) were successful. One of the 2 patients who underwent failed procedures with needle-knife (*i.e.*, only performed precut papillotomy) reported improvement; the other patient reported unchanged symptoms. In the remaining 6 cases, the needle-knife was not used because other diagnoses were discovered that dictated the use of other procedures. Thus, the success rate of minor papilla cannulation improved significantly with the needle-knife procedure [76.0%, 79/104 *vs* 92.3%, (79 + 17)/104; P = 0.001]. Excluding the 6 cases that were not appropriate

Table 2 Patient data

Basic clinical data	*n*
Total number of patients	74
Patients that received therapeutic ERCP	70
Patients that received diagnostic ERCP	4
Total cannulation procedures *via* minor papilla	104
Only using standard method[1]	79 (56 cases)
Using needle-knife after failure of standard method	14 (14 cases)
Using needle-knife at start and standard methods later[2]	11 (4 cases)[3]
Age (yr)	40.5 ± 21.8
Children and adolescents (age < 18 yr) (female)	16 (11)
Adults (female)	58 (22)
Clinical indications for cannulation procedures	
Pancreatitis	33
Chronic recurrent pancreatic-type pain without enzyme elevation	37
Biliary disease	2
Definite or suspected pancreatic mass	2
Diagnoses	
Chronic pancreatitis	13
Pancreas divisum	17
Chronic pancreatitis and pancreas divisum	40
Intraductal papillary mucinous neoplasms	4
Follow-up	
Patients who received therapeutic ERCP	70
Patients who received therapeutic ERCP and were followed up	67 (95.7%)
Follow-up period (months)	29.0 ± 22.2
Follow-up results[4]	
Improved	49
Cured	3
Same	7
Worse or much worse	5

[1]Using a tapered tip catheter with or without a guidewire; [2]Using needle-knife after failure of the standard method in the first and/or second cannulation and only using standard methods in later cannulations; [3]Five cannulations using the needle-knife after failure of the standard methods in the first and second cannulations in 4 patients and 6 cannulations using only standard methods in the 4 later cannulations for these cases; [4]Three patients died prior to follow-up. ERCP: Endoscopic retrograde cholangiopancreatography.

for needle-knife cannulation, the success rate was improved even further (80.6%; 79/98 *vs* 98.0%; 96/98, *P* = 0.000; Figure 5).

The overall incidence of post-ERCP adverse events was 5.8% (6/104). Adverse events included post-ERCP pancreatitis (mild, *n* = 1; moderate, *n* = 2; severe, *n* = 1), mild infection (*n* = 1), and moderate bleeding (*n* = 1). There were no ERCP-related perforations or deaths. There were no significant differences in the rates of post-ERCP adverse events between the group using a sphincterotome alone and the group using a needle-knife after sphincterotome failure (4.7% *vs* 10.5%, *P* = 0.301; Table 3).

The needle-knife was used 5 times in 4 children/adolescents (25.0%, 4/16) and 14 times in 14 adult cases (24.1%, 14/58) (*P* = 1.000). There were no significant differences in the rates of stent insertions, cannulation failures, post-ERCP adverse events, and needle-knife use between children/adolescents and adults (Table 4). Needle-knife cannulations were performed in 10 female patients (52.6%, 10/19), while sphincterotome cannulation was performed in 23 female patients (42.6%, 23/55) (*P* = 0.450). Cannulation procedures with the needle-knife after failed cannulation with the sphincterotome were slightly more common in female patients (*P* = 0.105; Table 3). However, the incidence of post-ERCP adverse events (4.9%; 3/61) in male cases was similar to female cases (7.0%; 3/43) (*P* = 0.689).

Only 3 of the 70 patients who received therapeutic ERCPs were lost to follow-up. Among the 67 (95.7%) patients with follow-up information, 3 patients with CP (*n* = 1) or CP and pancreas divisum (*n* = 2) developed pancreatic cancer and died. Improvements (better and cured) were documented in 52 (77.6%) patients. Excluding the 3 patients with pancreatic cancer, the overall rate of improvement was 81.3% (52/64) (Table 2, Figure 5).

DISCUSSION

For cannulation, one size does not fit all[12]. The duration of attempted cannulation varies, and procedures and instruments should be tailored to the risk profile and papillary/ductal anatomy of each patient[12]. Five different cannulation procedures were performed in this study, including 2 procedures with the pull-sphincterotome (physician-controlled wire-guided cannulation or directly using the tapered tip of the sphincterotome and rendezvous method) and 3 procedures with the needle-knife (NI-G, NPI-GS, and NPI-GSG procedures). Use of the needle-knife for cannulation was determined in each case without regard for age or gender.

To the best of our knowledge, the NI-G procedure may be a novel needle-knife procedure. The needle tip was only used to grasp the minor papilla orifice and, subsequently, a guidewire was carefully advanced into the duct of Santorini through the needle-knife cannula. Papillotomy was not performed during the NI-G procedure. Literature searches revealed one study somewhat similar to ours, in which Song *et al*[4] used needle-knives to perform minor papilla fistulotomies in 3 patients with CP. Another study, reported by Wilcox *et al*[31], performed a precut papillotomy when a tapered-tip catheter could not be passed into the dorsal duct over a guidewire (*i.e.*, partly successful cannulation).

Given that the orientation of the sphincterotome to the desired duct is favorable and adjustable compared to a catheter, we preferred to use a tapered tip catheter of a standard pull-sphincterotome with or without a guidewire to cannulate *via* the minor papilla, consistent with many endoscopists[4,5,7-10,14-22]. The rate (76.0%) of successful cannulations when standard procedures were used was slightly lower than the results presented in previous studies. This difference may be because our study included some children and adolescents, some patients with definite or suspected pancreatic masses, and some patients who had

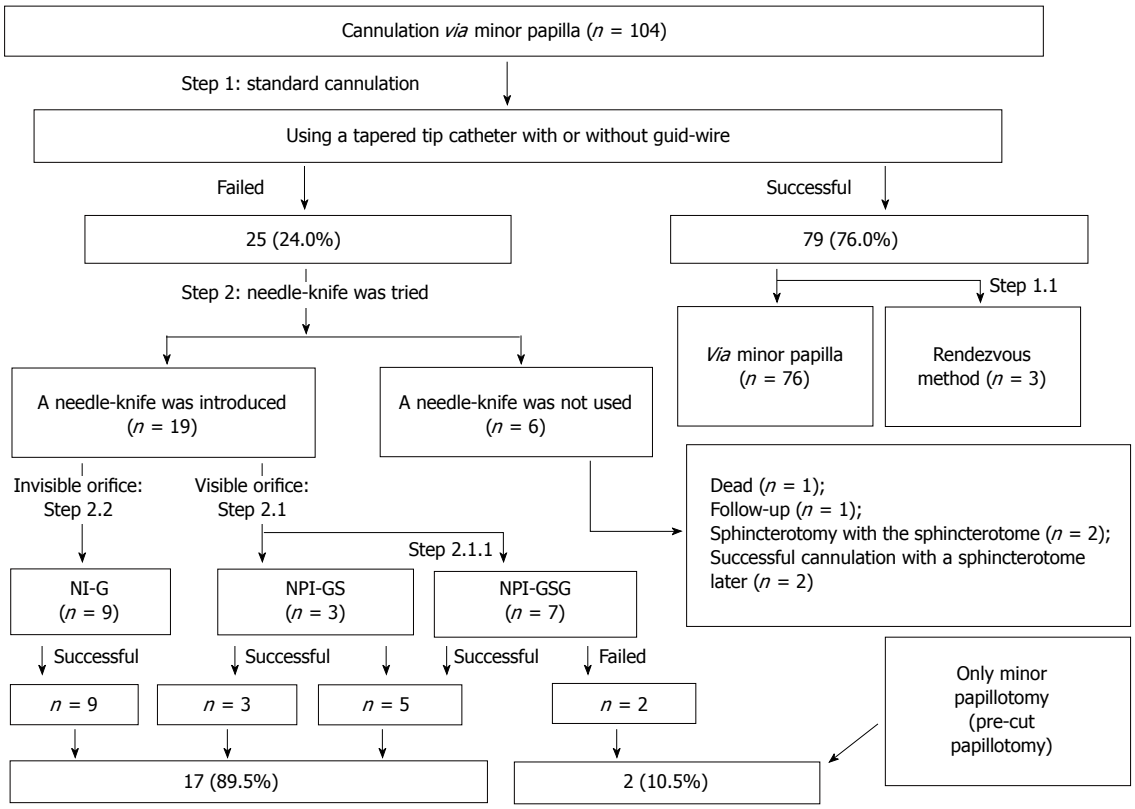

Figure 5 Cannulation procedures *via* the minor papilla. NI-G: Needle-knife introduction of a guidewire; NPI-GS: Needle-knife papillotomy with introduction of a guidewire and pull-sphincterotome; NPI-GSG: Needle-knife papillotomy with introduction of a guidewire, pull-sphincterotome, and guidewire.

Table 3 Endoscopic retrograde cholangiopancreatography interventions and complications *n* (%)

	Sphincterotome[1] (*n* = 85)	Needle-knife[2] (*n* = 19)	*P* value
ERCP procedures in female cases	32 (37.7)	11 (57.9)	0.105
Minor papilla sphincterotomy	49[3] (57.7)	10 (52.6)	0.690
Dilation	25 (30.5)	4 (21.1)	0.578
Stents	53 (61.0)	11 (57.9)	0.796
ENPD tubes	21 (24.7)	4 (21.5)	1.000
Stents + ENPD tubes	74 (87.1)	15 (79.0)	0.468
Stone extraction and clearance of Santorini's duct	18 (21.2)	2 (10.5)	0.355
Retrieving of migrated duct stents	1	1	-
Recorded cannulation time (min)[4]	5.5 ± 4.0	7.3 ± 5.1	0.505[3]
Post-ERCP complications	4 (4.7)	2 (10.5)	0.301

[1]Cannulation using a sphincterotome with or without a guidewire; [2]Cannulation with a needle-knife after failure with a sphincterotome; [3]Two minor papillotomy procedures using the needle-knife were performed along a guidewire after successful cannulation with the sphincterotome; [4]Times were recorded in 17 procedures using a sphincterotome and 5 procedures using a needle-knife. ERCP: Endoscopic retrograde cholangiopancreatography; ENPD: Endoscopic naso-pancreatic drainage.

received surgical interventions. Cannulations in these cases are generally considered to be more difficult.

The total success rate for cannulation in our study, including cannulation by needle-knife, was 92.3%. When we excluded the 6 patients who were not indicated for cannulation by needle-knife, the success rate reached 98.0% (P = 0.000). The success rate of cannulation procedures performed by needle-knife after failure with the sphincterotome still reached 89.5% (17/19). Importantly, our success rates and patient numbers are higher, and the incidence (5.8%) of post-ERCP adverse events was lower, compared to previous studies. Cannulation with the needle-knife, use of stents, age, and gender did not correlate with adverse events. At the end of follow-up, 81.3% of patients reported improvement, similar to previously reported results (range: 58%-96%).

The small or inconspicuous minor papilla orifice often makes it difficult to use a pull-type sphincterotome, guidewire, or catheter[1,11]. As seen through a duodenoscope, the small minor papilla orifice appears like a "point", whereas the cross-sections of the guidewire, sphincterotome cannula, and catheter tip are like "faces". Because of the size differences and peristalsis of the duodenum, it is difficult to produce anastomoses between these "faces" and "points",

Table 4 Cannulation procedures in adolescents and adults *n* (%)

	Adolescents[1] (*n* = 30)	Adults (*n* = 74)	*P* value
Cannulation with needle-knife	5 (16.7)	14 (18.9)	0.788
Stents	20 (66.7)	44 (59.5)	0.494
Cannulation failure	2 (6.7)	6 (8.1)	1.000
Post-ERCP complication	2 (6.7)	4 (5.4)	1.000

[1]Patients < 18 years old. ERCP: Endoscopic retrograde cholangiopancreatography.

especially when the neck or waist behind the "face" is very soft. However, the cross-section of the tiny needle-knife tip is similar to a point, enabling aligning of the point of the needle-knife tip with the "point" of the minor papilla orifice. Thus, using a needle-knife to guide cannulation *via* the minor papilla may be a suitable choice.

When cannulation with a pull-sphincterotome fails, the needle-knife can be used in 2 distinct ways. First, it can be directly inserted into the small orifice of the minor papilla. This procedure is relatively easy to perform, because the orifice and needle are similar in size, and a "point-to-point" connection can be made. Alternatively, if the orifice is visible, then the needle-knife can be used to introduce a small incision or perform a papillotomy (precut papillotomy), thereby turning the minor papilla orifice into a "face". This procedure expands the minor papilla orifice and enables insertion of a guidewire, whose cross-section is also like a "face" (*i.e.*, the NPI-GS procedure). This procedure is also easy to perform, because a "face-to-face" connection can be made with the expanded orifice. If the orifice is still too small to receive a guidewire smoothly, then a small papillotomy can be performed by using the pull-sphincterotome to expand the orifice further (*i.e.*, the NPI-GSG procedure). When the orifice is too small to make an incision with the needle-knife, the NI-G procedure can be used.

Some details of the NI-G cannulation procedure must be emphasized: (1) "point-to-point" indicates that the tip of needle should be aimed at and inserted or aligned adjacent to the minor papilla orifice. When the orifice was too small to distinguish, we tried to insert the needle in the general direction of the orifice; (2) duodenal peristalsis often made it very difficult to cannulate and aim the tip of the pull-sphincterotome at the orifice. During the NI-G procedure, the sphincter was inserted into the needle tip to provide structural support and anchor the needle-knife in the orifice. This fixation step facilitated the aiming of the needle-knife cannula at the minor papilla orifice, resulting in a smooth passage that directed the guidewire into the duct of Santorini; (3) the center of effort is located at the orifice. After the needle-knife tip was inserted into the minor papilla orifice, the needle-knife was anchored in the orifice to increase control over cannulation, similar to standard sphincterotomes; (4) after the needle-knife tip was inserted into the orifice, the needle-knife cannula was pushed forward while the needle-knife tip was pulled back until the cannula anastomosed with the orifice. When the needle-knife cannula is pushed forward, the needle must be pulled back at the same rate, so that the inserting needle tip remains in place and does not follow the cannula to be completely withdrawn; and (5) if the NI-GSG procedure fails, then the NI-G procedure does not need to be performed. Failure of the NI-GSG procedure often indicates that the duct of Santorini is too narrow to receive a guidewire.

Safety is a major concern with needle-knife procedures. Adverse events, including bleeding, retroperitoneal perforation, and (especially) pancreatitis have been reported. In this study, needle-knife cannulation was performed through meticulous procedures with the following considerations: (1) the needle-knife procedures did not always involve minor papillotomy, which are associated with an increased risk of post-ERCP adverse events, especially bleeding and perforation. Of the 19 cannulations performed by needle-knife, 9 (47.4%) NI-G procedures did not involve minor papillotomy; (2) minor papillotomy (precut papillotomy; NPI-GS and NPI-GSG procedures) was used to expand the minor papilla orifice to facilitate guidewire or sphincterotome insertion into the duct of Santorini. Although the maximum extent of each cut was determined by the minor papilla size, the cut was stopped as soon as the incision received the tip of the guidewire or pull-sphincterotome, especially if the practitioner was concerned about bleeding or perforation. If an incision created by the needle-knife was insufficiently large, then the pull-sphincterotome was used to enlarge it; (3) the needle-knife was used before insertion of a guidewire or stent for cannulation. Minor papillotomy was not performed along guidewires or stents, as has been the case in other studies[5,16,20]. The needle-knife was used to accomplish cannulation *via* the minor papilla, and was not used as a therapeutic procedure; (4) seven or more transpapillary cannulation attempts or more than five cannulations of the pancreatic duct have been identified as independent risk factors for post-ERCP adverse events. Compared to late precuts, early precut implementation reduces the risk of pancreatitis. Many endoscopists consider the number of attempts at the papilla (*i.e.*, prolonged cannulation attempts, varying from 5 to 20 min) as an independent risk factor for pancreatitis. Therefore, using the needle-knife to cannulate quickly may be safer than repeated attempts with a sphincterotome, which are likely to fail[13,24-28]; and (5) a skilled assistant worked alongside the endoscopist during these procedures, helping the endoscopist to maintain control over the endoscope and needle-knife. The cannulation times were similar between the needle-knife and the sphincterotome (*P* = 0.505).

Several limitations of this study should be noted. First, the data used in this study were retrospective and nonrandom, which may have introduced unintentional bias in group selection. The search strategies employed were diligent, although some patient information was not directly collected in a standardized manner in the clinic[21]. However, study subjects were identified from clinical information recorded in a computerized database system, and some patient follow-ups were conducted by telephone after hospital discharge. Thus, the data were as complete and reliable as possible. Second, the number of recorded cannulation times was small compared to the total number of procedures performed, with most measurements being made after 2010. All cannulations using the needle-knife were performed or supervised by one expert (Gong B), but not all cannulations using the sphincterotome were supervised. These factors may introduce bias in comparing cannulation times, but the use of the needle-knife for cannulation *via* the minor papilla was exploratory. Cannulation times including procedural consultations may only approximate the true time of cannulation. The data showed that cannulation time using a needle-knife was no longer than when using a sphincterotome. Finally, the small number of patients included in this study may overestimate or underestimate the rates of success and adverse events. Studies with larger numbers of patients are needed to verify our results.

In summary, needle-knife cannulation *via* the minor papilla confers important technical advantages when compared to procedures using sphincterotomes with or without guidewires. Needle-knife cannulation is a safe procedure, but should be performed meticulously. Needle-knife procedures should be included in routine practice to improve the success rates of cannulation *via* the minor papilla, particularly when standard cannulation procedures have failed.

COMMENTS

Background

Endoscopic treatment *via* the minor papilla may be the only possible treatment option for some patients. However, owing to its tiny size, the minor papilla orifice is often difficult to cannulate.

Research frontiers

Despite the development of various procedures to facilitate minor papilla cannulation, some cannulation procedures still fail. The slim tip of the needle-knife offers a unique advantage for its insertion into the minor papilla orifice. However, the needle-knife has been utilized in many centers only as a therapeutic procedure, and its use has been discontinued in cannulation procedures *via* the minor papilla.

Innovations and breakthroughs

Three procedures with the needle-knife were performed to cannulate the minor papilla after standard cannulation techniques had failed. These procedures significantly improved the success rate of cannulation. The study demonstrates the first use of the "needle-knife introduction of a guidewire (NI-G)" procedure, in which the needle tip was only used to grasp the minor papilla orifice and papillotomy was not performed.

Applications

Needle-knife procedures should be included in routine practice to improve the success rate of cannulation *via* the minor papilla, particularly when standard cannulation procedures have failed.

Terminology

NI-G: Needle-knife introduction of a guidewire. NPI-GS: Needle-knife papillotomy with introduction of a guidewire and pull-sphincterotome. NPI-GSG: Needle-knife papillotomy with introduction of a guidewire, pull-sphincterotome, and guidewire

Peer-review

The authors describe three meticulous procedures with the needle-knife to cannulate the minor papilla. This study describes the procedures appropriately and is relevant to clinicians.

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ORIGINAL ARTICLE

Retrospective Study

Novel immunological and nutritional-based prognostic index for gastric cancer

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Data sharing: Technical appendix and statistical code available from the corresponding author at ylh@medmail.com.cn.

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Abstract

AIM: To assess the prognostic significance of immunological and nutritional-based indices, including the prognostic nutritional index (PNI), neutrophil-lymphocyte ratio (NLR), and platelet-lymphocyte ratio in gastric cancer.

METHODS: We retrospectively reviewed 632 gastric cancer patients who underwent gastrectomy between 1998 and 2008. Areas under the receiver operating characteristic curve were calculated to compare the predictive ability of the indices, together with estimating the sensitivity, specificity and agreement rate. Univariate and multivariate analyses were performed to identify risk factors for overall survival (OS). Propensity score analysis was performed to adjust variables to control for selection bias.

RESULTS: Each index could predict OS in gastric cancer patients in univariate analysis, but only PNI had independent prognostic significance in multivariate analysis before and after adjustment with propensity scoring (hazard ratio, 1.668; 95% confidence interval: 1.368-2.035). In subgroup analysis, a low PNI predicted a significantly shorter OS in patients with stage Ⅱ-

Ⅲ disease (P = 0.019, P < 0.001), T3-T4 tumors (P < 0.001), or lymph node metastasis (P < 0.001). Canton score, a combination of PNI, NLR, and platelet, was a better indicator for OS than PNI, with the largest area under the curve for 12-, 36-, 60-mo OS and overall OS (P = 0.022, P = 0.030, P < 0.001, and P = 0.024, respectively). The maximum sensitivity, specificity, and agreement rate of Canton score for predicting prognosis were 84.6%, 34.9%, and 70.1%, respectively.

CONCLUSION: PNI is an independent prognostic factor for OS in gastric cancer. Canton score can be a novel preoperative prognostic index in gastric cancer.

Key words: Gastric cancer; Prognostic nutritional index; Canton score; Prognosis; Neutrophil-lymphocyte ratio; Platelet-lymphocyte ratio

Core tip: This is the first study to compare the prognostic significance of different immuno-nutritional indices including prognostic nutritional index (PNI), neutrophil-lymphocyte ratio (NLR) and platelet-lymphocyte ratio (PLR) in gastric cancer. We found that PNI was an independent prognostic factor for overall survival in gastric cancer before and after the propensity score analysis, especially in patients with advanced disease, deep tumors, or lymph node metastasis. We also proposed that a new index-Canton score (a combination of PNI, NLR and PLT) is a superior prognostic factor compared to PNI, NLR, or PLR alone, as it better represents the relative contribution of each of these indices.

Sun KY, Xu JB, Chen SL, Yuan YJ, Wu H, Peng JJ, Chen CQ, Guo P, Hao YT, He YL. Novel immunological and nutritional-based prognostic index for gastric cancer. *World J Gastroenterol* 2015; 21(19): 5961-5971 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5961.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5961

INTRODUCTION

Gastric cancer continues to be a major cause of morbidity and mortality worldwide, especially in developing countries. Despite an improvement in survival over recent years due to the development of better endoscopic and imaging techniques, surgical skills, and medical treatments, its prognosis remains unfavorable[1]. Surgery continues to be the most effective therapy for gastric cancer. Pathological results after surgery are widely used to evaluate the long-term postoperative prognosis. However, the indices used to evaluate the optimal timing of surgery or to predict survival preoperatively are still limited.

Many researchers have reported that the postoperative prognosis of gastric cancer is associated not only with tumor behavior, but also with the general condition of patients, especially their immunological and nutritional status[2-4]. The prognostic nutritional index (PNI), which is simple to calculate and easy to interpret, has been widely used to assess the preoperative immunological and nutritional status of patients undergoing gastrointestinal and cardiac surgery[5,6]. Its application as a prognostic marker was recently suggested by some researchers, and it was recently used to predict prognosis in a number of malignancies, including pancreatic, hepatocellular, and colorectal carcinoma. However, its prognostic significance in gastric cancer has not been fully studied, and the mechanisms that link PNI to outcome remain unclear[7-9]. In addition to PNI, markers of systematic inflammation, such as the number of white blood cells, neutrophils, platelets, and lymphocytes, and the indices derived from these, including the neutrophil-lymphocyte ratio (NLR)[10,11] and platelet-lymphocyte ratio (PLR), have also been used as prognostic markers. NLR was found to be associated with survival in lung and ovarian cancers, while PLR was found to be associated with prognosis in pancreatic cancer[12-15].

For gastric cancer, it remains unclear whether these parameters are independent prognostic factors in different disease stages, which of them have the highest prognostic value, and whether there is an advantage to combining them. We therefore retrospectively investigated the associations between PNI and clinicopathological features, as well as the predictive significance of PNI, NLR, and PLR, either alone or in combination, for overall survival (OS) in gastric cancer patients. A reliable prognostic index could help in making key clinical decisions such as the timing of surgery and the correct postoperative medical treatment.

MATERIALS AND METHODS

Patients

We enrolled 632 patients with histologically proven gastric cancer who underwent gastrectomy between January 1998 and December 2008 at the First Affiliated Hospital of Sun Yat-sen University. They were all aged over 18 years, and complete clinical and laboratory data were available in each case. Preoperative data were collected within 7 d before surgery and the blood samples were obtained from the first or the second day of patients' admission when they did not receive any treatment. Gastrectomy was performed for all patients for whom this was indicated. Patients were routinely followed, every 3 mo in the first year, every 3-6 mo in the second and third year, and at least once a year thereafter. The latest follow-up was December 2013, and the average follow-up duration was 55.75 mo (range, 0.8-186 mo). Patients with a history of inflammatory disease, active concomitant infection, other malignancies or synchronous immune disease

(*e.g.*, syphilis or hyperthyroidism) that might have interfered with the results of baseline immunological and nutritional status were excluded, as were patients who underwent preoperative chemotherapy. Thus, a total of 632 patients (413 men and 219 women) with a mean age of 57 years were finally eligible and analyzed. The follow-up rate reached 93.2%. Written informed consent was obtained from all patients.

Data

We retrospectively reviewed these patients' medical records to retrieve specific data, such as general clinical information (age, sex, height, and body weight), coexisting comorbidities, surgical data (types of gastrectomy, bleeding, and durations of surgery), tumor depth, lymph node metastasis, distant metastasis, histopathological analysis of the resected specimen, resectability of the tumor, postoperative surgical and medical complications, postoperative chemotherapy, and survival. Gastric cancer stage was classified according to the 7th edition of the American Joint Committee on Cancer TNM classification system[16]. The degree of resectability was classified as R0, R1, or R2 [R0, radical resection (the tumor was cleared macroscopically and histologically); R1, remaining microscopic disease; and R2, remaining macroscopic disease]. Events occurring within 30 d after surgery were classified as postoperative complications or mortality. The Clavien-Dindo classification was applied to rate the severity of each postoperative complication[17]. The presence of postoperative complications in this study was defined as Clavien classification grade Ⅱ or higher and the serious complications were defined as grade Ⅲ or Ⅳ, as there were no grade Ⅴ complications. Immunological and nutritional indices were generated from the data of preoperative blood tests, including the level of serum albumin (ALB) and carcinoembryonic antigen (CEA), the total lymphocyte count (TLC), white blood cell count, neutrophil count, platelet count, and monocyte count. The earliest set of measurements was used if there were more than one set for a given patient. PNI, NLR, and PLR were calculated as ALB (g/L) + 5 × TLC (10^9/L), neutrophil count/lymphocyte count, and platelet count/lymphocyte count, respectively[18].

Statistical analysis

PNI stratification according to nutritional significance has previously been suggested, whereby a value higher than 50 was normal, a value higher than 45 was considered mild malnutrition, a value higher than 40 was considered moderate malnutrition, and a value lower than 40 was considered severe malnutrition. However, there is no validated cut-off value for PNI, NLR, or PLR, and therefore, receiver operating characteristic (ROC) curve analysis was performed with 5-year OS as the outcome, and the Youden index was then estimated[19]. The optimal cut-off value is that which allows the prediction of 5-year OS with the best sensitivity and specificity[20]. According to the defined cut-off values for these three indices, patients were stratified into a PNI low or high group, a NLR low or high group, and a PLR low or high group (Table 1)[10,21]. Events occurring within 30 d after surgery were classified as postoperative complications or mortality.

Table 1 The list of multiple immunological and nutritional-based prognostic indices for gastric cancer

Combined marker	Score
Prognostic Nutritional Index	
⩾ 48.2	1
< 48.2	2
Neutrophil-lymphocyte ratio	
⩽ 1.83	1
> 1.83	2
Platelet-lymphocyte ratio	
⩽ 140	1
> 140	2
Canton score	
PNI ⩾ 48, NLR ⩽ 1.83 and PLT ⩽ 3×10^{11}/L	0
PNI ⩾ 48, NLR ⩽ 1.83 and PLT > 3×10^{11}/L	1
PNI ⩾ 48, NLR > 1.83 and PLT ⩽ 3×10^{11}/L	1
PNI < 48, NLR ⩽ 1.83 and PLT ⩽ 3×10^{11}/L	1
PNI ⩾ 48, NLR > 1.83 and PLT > 3×10^{11}/L	2
PNI < 48, NLR ⩽ 1.83 and PLT > 3×10^{11}/L	2
PNI < 48, NLR > 1.83 and PLT ⩽ 3×10^{11}/L	2
PNI < 48, NLR > 1.83 and PLT > 3×10^{11}/L	3

PNI: Prognostic Nutritional Index; NLR: Neutrophil-lymphocyte ratio; PLR: Platelet-lymphocyte ratio.

The categorical variables are presented as numbers and percentages and the differences between groups were determined using the χ^2 test. The survival curves were calculated by the Kaplan-Meier method and compared using the log-rank test. The areas under the ROC curve (AUC) of all the derived indices (any combination of PNI, NLR, PLR and PLT, n = 15) were calculated to compare the predictive ability of each index at different time points (the end of follow-up, 12 mo, 36 mo and 60 mo), respectively. Then, we further compared the predictive value of the index with the largest AUC to that of PNI by comparing their AUC using the Z-test. OS was calculated from the date of surgery to the date of death or the last follow-up. Mantel-Cox regression methodology was used for univariate analysis of the potential factors related to survival. Factors that showed significant prognostic value in univariate analysis were further analyzed in the final multivariate Cox proportional hazards model adjusted for a propensity score in four strata.

Propensity score analysis was performed to adjust variables to control for selection bias due to the non-randomization of patients allocated in two groups according to the corresponding cut-off value[22,23]. A propensity score that represents the probability of being allocated into different groups was estimated with a logistic regression model for all patients. Any potential factors involved in both group selection

Table 2 Relationship between clinicopathological factors, postoperative complications, and prognostic nutritional index

Variable	*n* (%)	PNI-H	PNI-L	*P* value
Age (yr)				0.407
≤ 65	445 (70.4)	235	210	
> 65	187 (29.6)	92	95	
Sex				0.958
Male	413 (65.3)	214	199	
Female	219 (34.7)	113	106	
Resectability				< 0.001
0	509 (80.5)	285	224	
1, 2	123 (19.5)	42	81	
Tumor depth				0.006
T1, T2	126 (19.9)	79	47	
T3, T4	506 (80.1)	248	258	
Lymph node				0.015
N0	193 (30.5)	114	79	
N1-3	439 (69.5)	213	226	
Distant metastasis				< 0.001
M0	474 (75.0)	266	208	
M1	158 (25.0)	61	97	
Pathological stage				0.002
I, Ⅱ	207 (32.8)	125	82	
Ⅲ, Ⅳ	425 (67.2)	202	223	
WBC				0.279
≤ 11 × 10^9/L	597 (94.5)	312	285	
> 11 × 10^9/L	35 (5.5)	15	20	
ALB				< 0.001
≥ 35 g/L	507 (80.2)	325	182	
< 35 g/L	125 (19.8)	2	123	
PLT				0.072
≤ 300 × 10^9/L	484 (76.6)	260	224	
> 300 × 10^9/L	148 (23.4)	67	81	
CEA				0.353
≤ 5 ng/mL	547 (86.6)	287	260	
> 5 ng/mL	85 (13.4)	40	45	
Histological type				0.958
Well	190 (30.1)	98	92	
Poor	442 (69.9)	229	213	
Blood loss				0.034
≤ 400 mL	423 (66.9)	233	190	
> 400 mL	209 (33.1)	94	115	
Operative time				0.883
≤ 4 h	253 (40.0)	130	123	
> 4 h	379 (60.0)	197	182	
Postoperative chemotherapy				0.666
Absent	237 (37.5)	120	117	
Present	395 (62.5)	207	188	
Postoperative complications				0.488
Absent	551 (87.2)	288	263	
Present	81 (12.8)	39	42	
Severe complication				0.045
Absent	580 (91.8)	307	273	
Present	52 (8.2)	20	32	

WBC: White blood cell; ALB: Albumin; PLT: Platelet; CEA: Carcinoembryonic antigen.

and survival in the univariate analysis were entered in the model. The percentage of correctly classified patients reached 60.1 in this model. According to the propensity score, patients were stratified in four strata with 25% of patients in each. All the calculations were performed using the SPSS statistical package (version 20.0; SPSS Inc., Chicago, IL, United States). A *P* value less than 0.05 was considered statistically significant.

RESULTS

Patient characteristics

The baseline patient characteristics are summarized in Table 2. There were 413 male and 219 female patients (65.3% *vs* 34.7%), with a mean age of 57 years (range, 19-89 years), and 187 patients were older than 65 years. Five hundred and twenty-five patients underwent radical gastrectomy: total gastrectomy, partial gastrectomy, and palliative resection in 262, 263 and 107 cases, respectively. Ninety-five, 112, 267, and 158 patients had stages Ⅰ, Ⅱ, Ⅲ, and Ⅳ disease, respectively. Eighty-one (12.8%) patients had postoperative complications. Decreased serum ALB levels (< 35 g/L) and elevated platelet counts (> 300 × 10^9/L) were noted in 125 (19.8%) and 148 (23.4%) patients, respectively.

ROC curve analysis

We performed ROC curve analysis to determine the optimal cut-off value with 5-year OS as an endpoint. The AUCs were 0.642, 0.636, and 0.614 for PNI, NLR, and PLR, respectively. Cut-off values of 48.2, 1.83, and 140 provided the maximal Youden index with sensitivities of 70.1%, 49.5%, and 73.2%, and specificities of 55.8%, 73.2%, and 60.3% for PNI, NLR, and PLR, respectively. Therefore, 327 (51.7%) and 305 (48.3%) patients were stratified into PNI high and low groups; 421 (66.6%) and 311 (34.4%) patients, into NLR high and low groups; and 340 (53.8%) and 292 (46.2%) patients, into PLR high and low groups, respectively.

Survival

Four-hundred and forty-eight (70.9%) patients died during follow up, with 1-, 3-, and 5-year OS rates of 72.9%, 47.6% and 39.1%, respectively. A decreased PNI and elevated NLR and PLR were associated with a reduced OS (*P* < 0.001 for all; Figure 1). The 1-, 3-, and 5-year OS rates were 80.7%, 59.3%, and 49.5% in patients with a PNI > 48, and 64.6%, 35.1%, and 28.9% in patients with a PNI ≤ 48 (*P* < 0.001 for all groups; Figure 1A).

Predictive factors for OS

In univariate analysis, multiple factors, including PNI, NLR, and PLR, were associated with a shorter OS (Table 3). Of these, a multivariate analysis adjusted for propensity score revealed that PNI, resectability, CEA levels, distant metastasis, pathological stage, postoperative complications, and age were independent prognostic factors for OS (Table 3).

PNI and clinicopathological characteristics

Clinicopathological features such as resectability, tumor depth, lymph node metastasis, distant metastasis, pathological stage, ALB, blood loss, and severe postoperative complications differed significantly

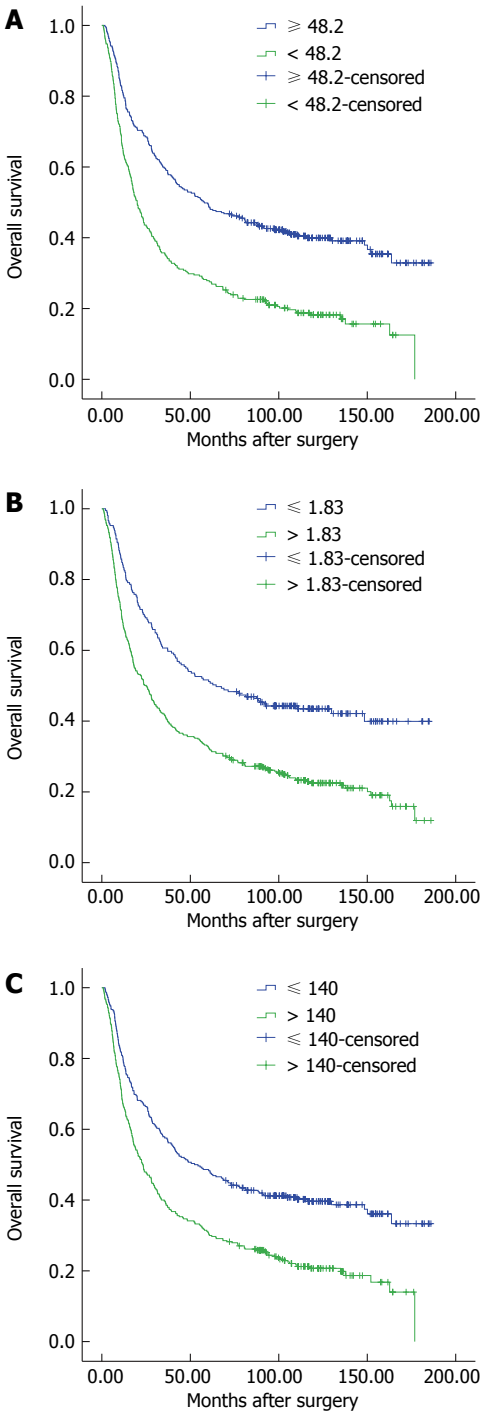

Figure 1 Overall survival according to prognostic nutritional index (A), neutrophil-lymphocyte ratio (B), and platelet-lymphocyte ratio (C). The prognosis of patients with prognostic nutritional index (PNI) ⩾ 48.2, neutrophil-lymphocyte ratio (NLR) ⩽ 1.83, or platelet-lymphocyte ratio (PLR) ⩽ 140 was more favorable than that of patients with PNI < 48.2, NLR > 1.83, or PLR > 140 (P < 0.001 for all).

between the PNI low and high groups (Table 2).

Subgroup analysis by pathological stage

Immunological and nutritional status might vary according to disease stage, and we therefore classified patients into four groups according to pathological

Table 3 Univariate and multivariate analyses of prognostic factors in gastric cancer patients

Variable	Univariate *P* value	Multivariate		
		HR	95%CI	*P* value
Age (yr)	0.041			0.004
⩽ 65		1		
> 65		2.323	1.299-4.156	
Sex	0.299			
Male				
Female				
Resectability	< 0.001			0.018
R0		1		
R1, R2		2.062	1.133-3.759	
Tumor depth	< 0.001			
T1, T2				
T3, T4				
Lymph node	< 0.001			
N0				
N1-3				
Distant metastasis	< 0.001			< 0.001
M0		1		
M1		10.505	6.540-16.874	
Pathological stage				
I		1		
Ⅱ	< 0.001	2.552	1.591-4.095	< 0.001
Ⅲ	< 0.001	4.695	3.053-7.220	< 0.001
Ⅳ	< 0.001	10.505	6.540-16.874	< 0.001
WBC	0.002			
⩽ 11 × 10^9/L				
> 11 × 10^9/L				
ALB	< 0.001			
⩾ 35 g/L				
< 35 g/L				
PLT	< 0.001			
⩽ 300 × 10^9/L				
> 300 × 10^9/L				
CEA	< 0.001			0.004
⩽ 5 ng/mL		1		
> 5 ng/mL		1.457	1.126-1.883	
Histological type	< 0.001			
Well				
Poor				
Blood loss	< 0.001			
⩽ 400 mL				
> 400 mL				
Operative time	0.067			
⩽ 4 h				
> 4 h				
Postoperative chemotherapy	0.479			
Absent				
Present				
Postoperative complications	< 0.001			0.002
Absent		1		
Present		1.516	1.164-1.974	
PNI	< 0.001			< 0.001
1		1		
2		1.668	1.368-2.035	
NLR	< 0.001			0.656
1		1		
2		1.056	0.830-1.343	
PLR	< 0.001			0.113
1		1		
2		1.190	0.960-1.475	
Propensity score				0.398

WBC: White blood cell; ALB: Albumin; PLT: Platelet; CEA: Carcinoembryonic antigen; PNI: Prognostic nutritional index; NLR: Neutrophil-lymphocyte ratio; PLR: Platelet-lymphocyte ratio; HR: Hazard ratio.

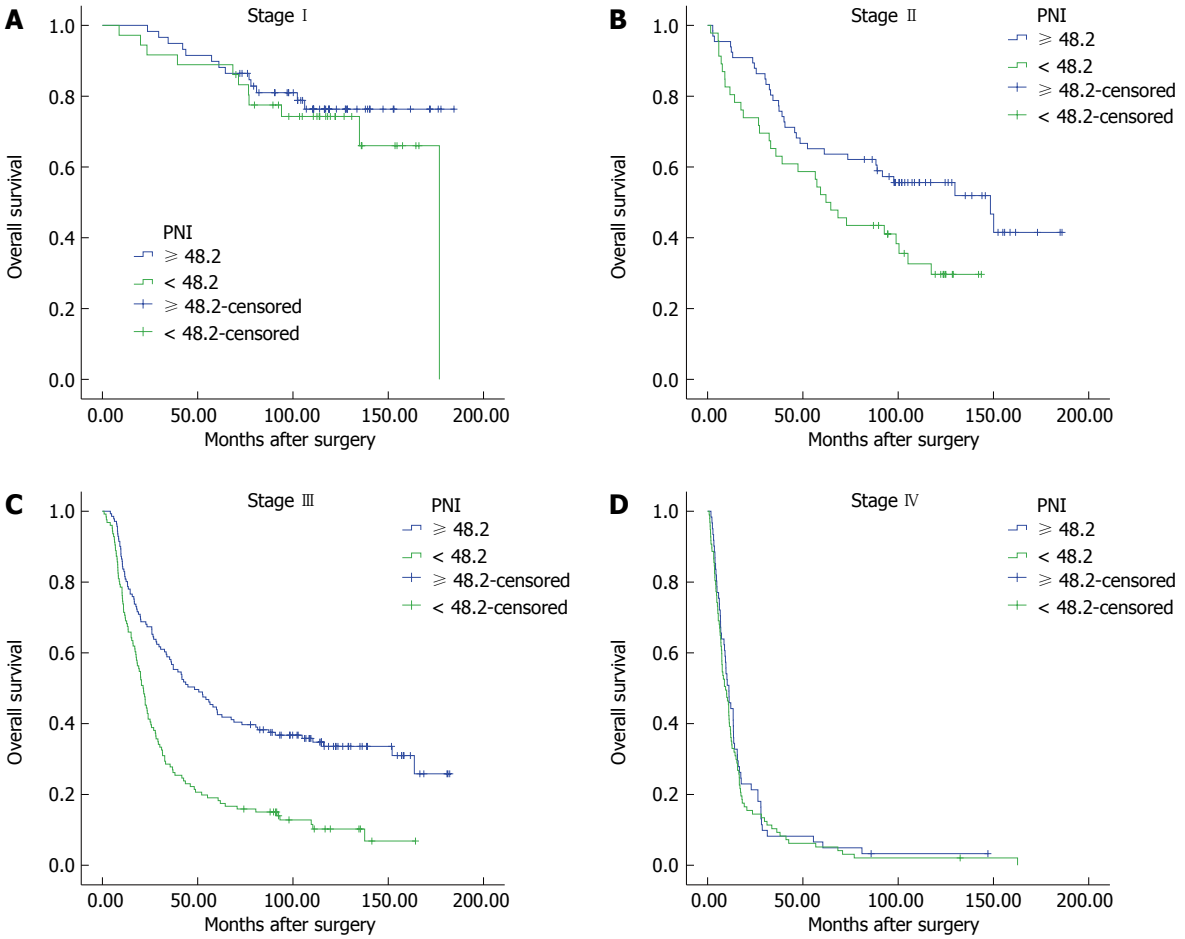

Figure 2 Overall survival of patients with different disease stages according to prognostic nutritional index (A-D). A high prognostic nutritional index (PNI) was significantly associated with a longer overall survival in patients with stage Ⅱ or stage Ⅲ disease ($P = 0.019$ and $P < 0.001$, respectively).

stage (Figure 2). The OS of patients with a low PNI was significantly shorter only if they had stage Ⅱ or Ⅲ disease (stage Ⅱ: PNI-H *vs* PNI-L, 65.15% *vs* 52.17%, $P = 0.019$; stage Ⅲ: 43.97% *vs* 19.05%, $P < 0.001$), but not stage Ⅰ or Ⅳ disease (stage Ⅰ: 89.83% *vs* 88.88%, $P = 0.377$; stage Ⅳ: 6.56% *vs* 5.15%, $P = 0.471$). Patients with a high NLR had a significantly shorter OS only if they had stage Ⅰ disease ($P = 0.007$). However, in patients with stage Ⅲ or Ⅳ disease, a high PLR was significantly associated with a shorter OS ($P = 0.011$, 0.031 *vs* $P = 0.132$, 0.556, respectively).

Subgroup analysis according to tumor depth and lymph node metastasis

To further explore the association between PNI and gastric cancer progression, we performed subgroup analysis according to tumor depth and lymph node metastasis**.** Among patients with T1 or T2 tumors ($n = 126$ in total), those with a high PNI tended to have a longer (but not significantly longer) OS ($P = 0.134$), although those with a high PNI and a T3 or T4 tumor did have a significantly longer OS ($n = 506$, $P < 0.001$). Similarly, OS in the PNI high group was only significantly longer amongst those with lymph node metastasis ($P < 0.001$).

New prognostic index - Canton score (a combination of PNI, NLR and PLT)

As one indicator might have limited predictive value, we combined a number of factors to generate a new preoperative prognostic index. After combining PNI, NLR, PLR, ALB, and PLT to generate several new indices and comparing them, we found two indices with the greatest prognostic significance (Table 4). They were the combination of PNI, NLR, and PLT and the combination of PNI, NLR, PLR and PLT. With the advantage of convenience, the combination of PNI, NLR and PLT, which we referred to Canton score, was chosen as the novel prognostic index considering there was no significant difference between these two derived indices. Canton score is defined as the number of the following prognostic indexes (PNI $\geqslant$ 48, NLR $\leqslant$ 1.83 and PLT $\leqslant 3 \times 10^{11}$/L) and thus has a value of 0, 1, 2, or 3. Detailed definition of the value of Canton score is shown in Table 1. The AUC for Canton score with 5-year OS as an outcome was 0.684, with an obvious difference compared to that of PNI ($P = 0.024$). We then compared the AUCs of Canton score and PNI with 12-, 36-, 60-mo and overall OS as endpoints. The AUC of Canton score in each case was higher than that of PNI ($P = 0.022$, $P = 0.030$, $P < 0.001$, and $P =$

Table 4 Areas under the receiver operating characteristic curve for survival of gastric patients based on all the derived prognostic scores at the end of follow-up, or after 12, 36, or 60 mo

Item	All survival		12 mo		36 mo		60 mo	
	AUC	*P* value	AUC	*P* value	AUC	*P* value	AUC	*P* value
PNI	0.630	< 0.001	0.602	< 0.001	0.621	< 0.001	0.614	< 0.001
NLR	0.613	< 0.001	0.593	< 0.001	0.587	< 0.001	0.588	< 0.001
PLR	0.611	< 0.001	0.580	0.002	0.592	< 0.001	0.593	< 0.001
PLT	0.573	0.004	0.564	0.013	0.562	0.007	0.566	0.005
PNI + NLR	0.535	0.493	0.502	0.968	0.476	0.570	0.487	0.776
PNI + PLR	0.477	0.656	0.480	0.644	0.435	0.123	0.455	0.309
PNI + PLT	0.498	0.970	0.493	0.867	0.469	0.468	0.449	0.251
NLR + PLR	0.477	0.647	0.447	0.229	0.459	0.329	0.472	0.522
NLR + PLT	0.487	0.796	0.466	0.437	0.488	0.772	0.461	0.382
PLR + PLT	0.440	0.240	0.448	0.237	0.448	0.219	0.442	0.190
PNI + NLR + PLR	0.679	< 0.001	0.638	< 0.001	0.651	< 0.001	0.647	< 0.001
PNI + NLR + PLT	0.684	< 0.001	0.655	< 0.001	0.657	< 0.001	0.654	< 0.001
PNI + PLR + PLT	0.668	< 0.001	0.634	< 0.001	0.647	< 0.001	0.646	< 0.001
NLR + PLR + PLT	0.660	< 0.001	0.627	< 0.001	0.629	< 0.001	0.632	< 0.001
PNI + NLR + PLR + PLT	0.685	< 0.001	0.647	< 0.001	0.657	< 0.001	0.655	< 0.001

AUC: Area under the curve; PNI: Prognostic nutritional index; NLR: Neutrophil-lymphocyte ratio; PLR: Platelet-lymphocyte ratio.

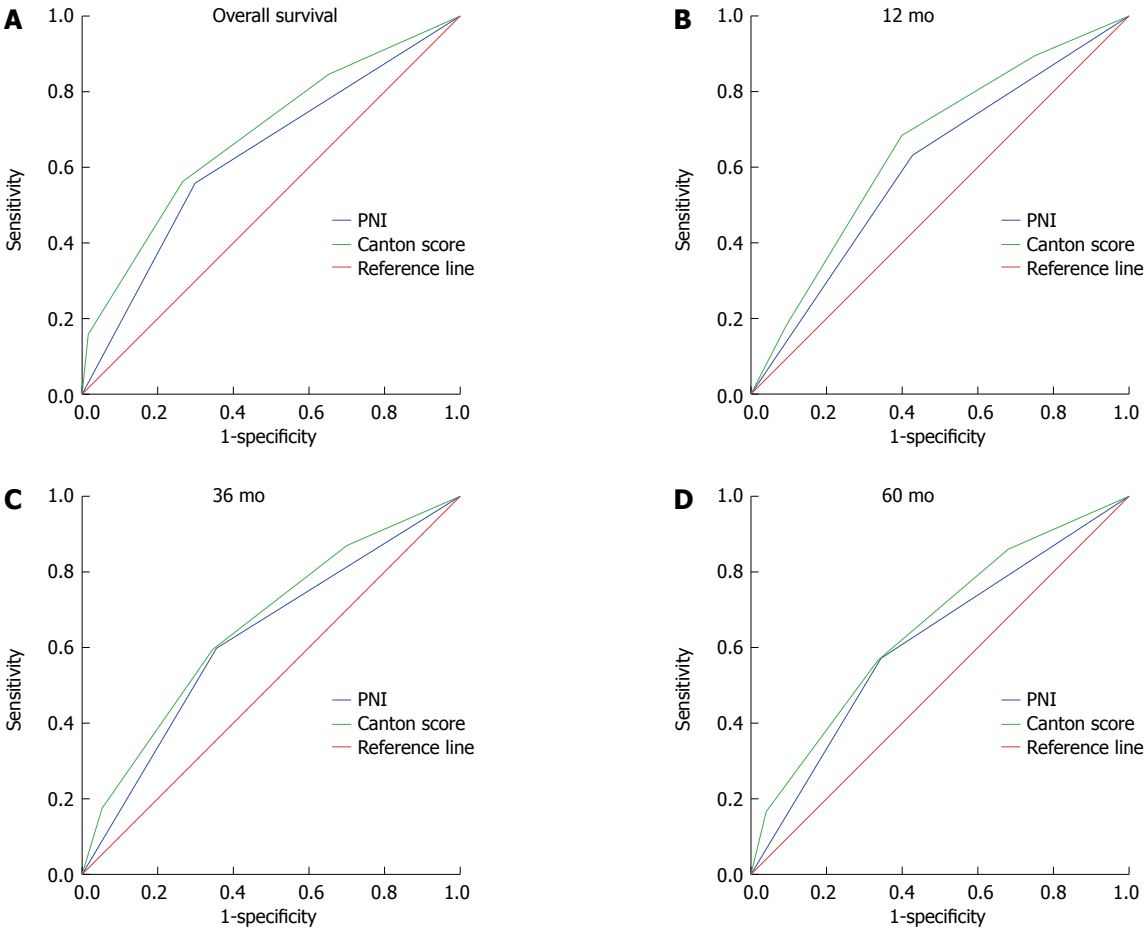

Figure 3 Comparison of the areas under the receiver operating characteristic curve for survival of gastric cancer patients based on prognostic nutritional index and Canton score at the end of follow-up (A), or after 12 mo (B), 36 mo (C), or 60 mo (D). The areas under the receiver operating characteristic curve at these four points were significantly greater for Canton score than for prognostic nutritional index (PNI) ($P = 0.024$, $P = 0.022$, $P = 0.030$ and $P < 0.001$, respectively).

0.024, respectively) (Figure 3). Moreover, in univariate analysis, hazard ratios (HRs) for death were 1.414 [95% confidence interval (95%CI): 1.054-1.895), 2.341 (95%CI: 1.772-3.091), and 3.555 (95%CI: 2.545-4.966)] for patients with a Canton score of 1, 2, and 3, respectively, compared to those with a Canton score of 0 (Table 5). Multivariate analysis also revealed an independent prognostic role for Canton score

Table 5 Univariate and multivariate analyses of prognostic factors including Canton score

Variable	Univariate *P* value	Multivariate		
		HR	95%CI	*P* value
Age (yr)	0.041			0.051
≤ 65		1		
> 65		1.238	0.999-1.536	
Sex	0.299			
Male				
Female				
Resectability	< 0.001			0.002
R0		1		
R1, R2		1.567	1.204-2.040	
Tumor depth	< 0.001			
T1, T2				
T3, T4				
Lymph node	< 0.001			
N0				
N1-3				
Distant metastasis	< 0.001			
M0				
M1				
Pathological stage				0.234
Ⅰ, Ⅱ				
Ⅲ, Ⅳ	< 0.001			
WBC	0.002			
≤ 11× 10^9/L				
> 11× 10^9/L				
ALB	< 0.001			
≥ 35 g/L				
< 35 g/L				
PLT	< 0.001			0.795
≤ 300 × 10^9/L				
> 300 × 10^9/L				
CEA	< 0.001			
≤ 5 ng/mL				
> 5 ng/mL				
Histological type	< 0.001			
Well				
Poor				
Blood loss	< 0.001			0.407
≤ 400 mL				
> 400 mL				
Operative time	0.067			
≤ 4 h				
> 4 h				
Postoperative chemotherapy	0.479			
Absent				
Present				
Postoperative complications	< 0.001			
Absent				
Present				
PLR	< 0.001			0.524
PLR-L				
PLR-H				
Canton score				
0		1		
1	0.021	1.076	0.796-1.454	0.633
2	< 0.001	1.554	1.151-2.097	0.004
3	< 0.001	1.643	1.142-2.364	0.007

WBC: White blood cell; ALB: Albumin; PLT: Platelet; CEA: Carcinoembryonic antigen; PLR: Platelet-lymphocyte ratio.

(Canton score = 1: HR = 1.076; 95%CI, 0.796-1.454; Canton score = 2: HR = 1.554; 95%CI: 1.151-2.097; Canton score = 3: HR = 1.643; 95%CI: 1.142-2.364)

Table 6 Univariate and multivariate analyses of prognostic factors including Canton score

Variable	Univariate *P* value	Multivariate		
		HR	95%CI	*P* value
Resectability	< 0.001			0.002
R0		1		
R1, R2		1.567	1.204-2.040	
Canton score				
0		1		
1	0.021	1.076	0.796-1.454	0.633
2	< 0.001	1.554	1.151-2.097	0.004
3	< 0.001	1.643	1.142-2.364	0.007

HR: Hazard ratio.

(Table 5). The maximum sensitivity, specificity, and agreement rate of Canton score in predicting prognosis were 84.6%, 34.9%, and 70.1%, respectively, superior to these values for PNI, suggesting that Canton score is a novel and effective preoperative prognostic index (Table 6).

DISCUSSION

In our study, we found that PNI, NLR, and PLR were associated with the OS of gastric cancer patients in univariate analysis, but only PNI was an independent prognostic factor in multivariate analysis before and after propensity score adjustment, together with resectability, postoperative complication, distant metastasis, pathological stage, and CEA levels. In addition, subgroup analysis showed that a low PNI predicted a significantly shorter OS in patients with stage Ⅱ or Ⅲ disease, T3 or T4 tumors, or lymph node metastasis. We developed a new index, Canton score, which is a better prognostic indicator for OS than PNI.

Preoperative immunological and nutritional conditions are associated with both the postoperative and long-term outcomes of malignant tumors[1,24]. Identifying prognostic factors before surgery to help determine the optimal preoperative therapy and timing of surgery is important. Previous attempts have focused on a number of immuno-nutritional indices, including PNI, NLR, PLR, PI, and GPS[4,6,7,9,15,21,25-27]. However, their reported prognostic value can vary between studies of the same cancer type; for example, in hepatocellular carcinoma, PLR was found to be a prognostic marker in one study, but another study suggested that only PNI was a prognostic marker[15]. It is therefore crucial to compare different indices to identify a more effective and convenient scoring system. However, to date, these three indices have not been analyzed together, nor has their association with OS been compared in gastric cancer. In this study, we found that PNI had the best predictive value, with the highest AUC for 5-year OS, and it was the only independent prognostic factor for OS. An additional

consideration is the heterogeneity of patient cohorts among studies, which might account for their different findings. To reduce this bias, we performed propensity score analysis and found that PNI was an independent prognostic factor before and after adjustment.

The assessment of different prognostic factors depended on the pathological stage of gastric cancer; for example, PNI had greatest prognostic significance in advanced gastric cancer patients (stages Ⅱ and Ⅲ). Thus, error due to different proportions of patients with a given pathological stage among different studies cannot be excluded. For example, one study with 327 stage Ⅰ patients found a significant difference in OS between stage Ⅰ patients with low or high PNI, but our study with only 95 stage Ⅰ patients found no significant association between PNI and OS[27]. A larger cohort of stage Ⅰ patients may give a different result.

It remains unclear how the association between PNI and OS varied according to pathological stage. We found that for patients in stage Ⅰ or Ⅲ, 5-year OS was significantly shorter in the group of low PNI, however, for patients in stage Ⅰ or Ⅳ, no significant association was found although the 5-year OS of patients with a low PNI was slightly shorter. This implies that the predictive significance of PNI might be greater in advanced gastric cancer, which is supported by the finding that PNI was only significantly associated with OS in patients with deeper tumor invasion and lymph node metastasis. This result is consistent with that of our meta-analysis and the findings of other studies[4,28]. This dependence on tumor invasion and spread might reflect the relatively good immunological and nutritional status of gastric cancer patients with stage Ⅰ disease, and that gastric cancer was not always considered to be the major cause of death in these patients. Indeed, one study found that more than 50% of patients with stage Ⅰ disease died from non-cancer causes, regardless of PNI[27]. Similarly, we found that age and postoperative complications were the two main factors influencing prognosis in stage Ⅰ patients. Advanced age was also an independent factor for poor prognosis, but only after propensity score adjustment, and several other studies did not find age to be an independent factor. This suggests that the non-randomization of patients and the consequent compounding factors need to be accounted for; hence, our propensity score analysis made our results more reliable. Older patients with a decline in both biological and physiologic functions of the digestive system and accompanying disorders such as chronic diseases, malignancies, and psychological illness are often in a poor immunological and nutritional condition[29], and events such as respiratory failure consequent to pneumonia were common non-malignant causes of death in the elderly[30].

The mechanism by which low PNI may impact survival is not fully understood. Serum ALB is required for a number of key physiological functions, including the maintenance of serum osmolality, tissue repair, transport of extrinsic and intrinsic compounds such as drugs and nutrients, and modulating systematic inflammation[31]. Thus, hypoalbuminemia can result in postoperative complications, including anastomotic edema and fistula, delayed tissue repair, reduced therapeutic efficacy of drugs and nutrients, and more importantly, the activation of systematic inflammation and influencing host immunity. Consequently, low ALB levels could promote tumor growth and invasion and trigger infections, which worsen prognosis. In addition to ALB levels, the lymphocyte count reflects immunological status and the degree of systematic inflammation to some extent[10]. Yang *et al*[32] convincingly showed that the impairment of lymphocyte mediated antitumor response is an immunological determinant of prognosis in hepatocellular carcinoma. It has also been reported that a cascade of inflammatory mediators during systematic inflammation can lead to tumor progression. This results from the recruitment of inflammatory cells including lymphocytes by the activation of transcription factors and inflammatory mediators after activation of the extrinsic (pre-existing inflammation) or intrinsic pathway (oncogene activation)[33]. There is also an interaction between nutritional status and systematic inflammation response. Moreover, both malnutrition (hypoalbuminemia) and an inflammatory response (based on the TLC) may affect therapeutic compliance and in turn affect prognosis[34].

Subsequent prospective clinical studies failed to find any benefit for serum ALB supplementation and its preoperative use for cancer patients remains controversial although correcting the serum ALB level before surgery was found to improve survival in early studies. However, anti-inflammatory therapy has been shown to extend survival of gastric cancer patients in a recent trial[35]. Based on our findings, it might be possible to improve survival by boosting immunity and nutritional status. Indeed, previous studies have also found that improved preoperative immunological and nutritional status could reduce the length of hospital stay and improve prognosis[27,36]. If this is validated in further clinical trials, we would recommend preoperative medical treatment to achieve nutritional and immunological levels that optimize the PNI, and then perform surgery at the optimal time.

The new index that we propose here, Canton score, is a superior prognostic factor compared to PNI, NLR, or PLR alone, as it better represents the relative contribution of each of these indices. In addition to PNI and NLR, which include TLC and ALB levels, together with the neutrophil count, Canton score also includes the PLT level, which was recently found be associated with tumor development. Platelets can secrete angiogenic factors and hence promote tumor growth by stimulating angiogenesis and are also involved in tumor invasion by binding to tumor cells *via* the adhesion molecules found in their alpha-granules. Further studies are needed to fully evaluate the predictive value of Canton score in cancer.

Several limitations of our study need to be considered. This was a retrospective observational study in which we detected a significant association between PNI and OS in gastric cancer, but we could not prove this association. Additionally, patients with different disease stages were not equally distributed, with relatively few cases of stage Ⅰ disease, thus probably skewing the results.

In conclusion, PNI, but not NLR or PLR, is an independent prognostic factor for OS in gastric cancer, especially amongst patients with advanced disease, deep tumor invasion, or lymph node metastasis. However, more studies with larger sample sizes are needed to explore the prognostic value of PNI in gastric cancer and the benefit of intervention to improve immunological and nutritional status in order to achieve a favorable PNI. Further, we showed that Canton score could be a novel preoperative prognostic index in gastric cancer.

COMMENTS

Background

Gastric cancer is a major cause of morbidity and mortality worldwide, especially in developing countries. However, the indices used to evaluate the optimal timing of surgery or to predict survival preoperatively are still limited. Prognostic nutritional index (PNI), neutrophil-lymphocyte ratio (NLR), and platelet-lymphocyte ratio (PLR) were recently used to predict prognosis in a number of malignancies, including pancreatic, hepatocellular, and colorectal carcinomas. In this study, the authors aimed to assess the prognostic significance of these immunological and nutritional-based indices in gastric cancer.

Research frontiers

For gastric cancer, it remains unclear about the prognostic significance of immuno-nutritional indices in patients with different disease stages. It is important to compare different indices to identify a more effective and convenient scoring system. And it is unknown whether there is an advantage to combining these prognostic indices.

Innovations and breakthroughs

PNI, but not NLR or PLR, is an independent prognostic factor for overall survival (OS) in gastric cancer, especially amongst patients with advanced disease, deep tumor invasion, or lymph node metastasis. Canton score, a combination of PNI, NLR and PLT, can be a novel preoperative prognostic index in gastric cancer for its better prognostic value than PNI.

Applications

PNI and canton score can be used to predict survival of patients with gastric cancer preoperatively, which helps in making key clinical decisions such as the timing of surgery and the correct preoperative medical treatment. More studies with larger sample sizes are needed to explore the prognostic value of these two indices in gastric cancer and the benefit of intervention to improve immunological and nutritional status.

Terminology

Canton score is an innovative prognostic index which derives from the combination of PNI, NLR and PLT. It is defined as the number of the following prognostic indexes (PNI > 48, NLR < 1.83 and PLT < 3 × 10^{11}/L) and thus has a value of 0, 1, 2, or 3. Higher value usually indicates better immuno-nutritional status.

Peer-review

Overall this is an interesting study and the work is generally clearly presented and described. The authors compared the prognostic significance of different immuno-nutritional indices including PNI, NLR and PLR in a large cohort of gastric cancer patients, and proposed a new index-Canton score, which is superior to other indexes in predicting OS. The article has an adequate bibliography, the manuscript is correctly written and the conclusions are justified by the results found in the study.

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ORIGINAL ARTICLE

Retrospective Study

Clinicopathologic features of remnant gastric cancer over time following distal gastrectomy

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Author contributions: Zhang DW and Dai DQ conceived the study; Zhang DW, Li Z, and Dong B collected and analyzed the data; Zhang DW, Dong B, and Dai DQ designed the study and participated in writing the paper; Zhang DW submitted the final manuscript and all authors read and approved the final manuscript.

Ethics approval: This retrospective study was approved by the Ethics Committee of The Fourth Affiliated Hospital, China Medical University. All patient records and information were anonymized and deidentified prior to analysis.

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

Conflict-of-interest: All the authors declare that they have no conflict of interest.

Data sharing: Technical appendix, statistical methods, and some datasets are available from the corresponding author at syzhangdewei@sohu.com.

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Abstract

AIM: To investigate remnant gastric cancer (RGC) at various times after gastrectomy, and lay a foundation for the management of RGC.

METHODS: Sixty-five patients with RGC > 2 years and < 10 years after gastrectomy (RGC Ⅰ) and forty-nine with RGC > 10 years after gastrectomy (RGC Ⅱ) who underwent curative surgery were enrolled in the study. The clinicopathologic factors, surgical outcomes, and prognosis were compared between RGC Ⅰ and RGC Ⅱ.

RESULTS: There was no significant difference in surgical outcomes between RGC Ⅰ and RGC Ⅱ. For patients reconstructed with Billroth Ⅱ, significantly more patients were RGC Ⅱ compared with RGC (71.9% *vs* 21.2%, $P < 0.001$), and more RGC Ⅱ patients had anastomotic site locations compared to RGC Ⅰ (31.0% *vs* 56.3%, $P = 0.038$). The five-year survival rates for the patients with RGC Ⅰ and RGC Ⅱ were 37.6% and 47.9%, respectively, but no significant difference was observed. Borrmann type and tumor stage were confirmed to be independent prognostic factors in both groups.

CONCLUSION: RGC Ⅱ is located on the anastomotic site in higher frequency and more cases develop after Billroth Ⅱ reconstruction than RGC Ⅰ.

Key words: Clinical pathology; Recurrence; Remnant gastric cancer; Survival

Core tip: This article is an important paper about clinicopathologic features of remnant gastric cancer (RGC) and the comparison of RGC with time interval of > 2 and ⩽ 10 years (RGC Ⅰ) after prior gastrectomy for gastric cancers. RGC after 10 years was easier

to locate on the anastomotic site than RGC Ⅰ. The predominant reconstruction type of the first operation is Billroth Ⅰ for RGC Ⅰ and Billroth Ⅱ for RGC Ⅱ. There may be different pathogeneses in different subgroups of RGC.

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INTRODUCTION

Remnant gastric cancer (RGC) refers to a carcinoma detected in remnant stomach more than five years after primary surgery for a benign disease. Many studies have paid attention to this disease[1-4]. It has been reported to account for 2.4% to 5% of all gastric cancers[5,6]. In recent decades, due to improved outcomes of medical treatment for gastric or duodenum ulcer, the number of patients undergoing gastrectomy for benign disease has decreased by a wide margin. Hence, the incidence of RGC strictly according to the definition is on the downside.

On the contrary, the number of patients with RGC following gastrectomy for gastric cancer has progressively increased as a result of improved outcomes for patients with gastric cancer and the increasing proportion of patients diagnosed with early gastric cancer[7]. Recently, several reports have used RGC to define all cancers arising from the remnant stomach after partial gastrectomy, regardless of the initial disease[7-10]. Several studies evaluated the clinicopathologic characteristics and surgical outcomes of patients with RGC after gastric cancer and compared them with the RGC after benign disease[11-13], and observed no significant differences.

The preferred explanation for the pathogenesis of RGC is that Billroth Ⅱ reconstruction produces a typical model of carcinogenesis. Gastroduodenal reflux and *Helicobacter pylori* colonization in the remnant stomach promote the development of RGC[14,15]. Because the gastric stump is constantly under carcinogenic influence, the time interval is one of the most important factors for the development of RGC. For the patients with RGC after benign disease, the average latency time is reported to be 20-27 years, and may go up to 40 years. Most authors have reported a steep increase in the risk of developing gastric stump cancer from the 20th year after the first gastrectomy[16-18]. Nevertheless, we consider that there maybe some differences in clinical pathology and prognosis between the RGC patients with a recurrence interval shorter than 10 years and those longer than 10 years.

In this study, we divided RGC following distal gastrectomy for gastric cancer into two subgroups: RGC Ⅰ (2-10 years post-gastrectomy) and RGC Ⅱ (> 10 years). The clinicopathologic features, type of operation, and the long-term survival results of the two subgroups were investigated and compared.

MATERIALS AND METHODS

Patients

Sixty-five patients with RGC Ⅰ and forty-nine with RGC Ⅱ underwent treatment at the Department of Gastrointestinal surgery, cancer institute of China Medical University from January 1980 to December 2010. The patients whose cancers were detected in the distal stomach after proximal gastrectomy were excluded. To exclude the residual cancer in the initial surgery, the patients with time interval ⩽ 2 years between the two cancers were excluded, though the proximal and distal resection margins were evaluated intraoperatively to confirm freedom from carcinoma at the initial surgery. Of them, ninety-five patients underwent surgical treatment and nineteen patients underwent non-surgical treatment for the distant metastasis or poor physical conditions. Seventy-four patients (RGC Ⅰ: 42, RGC Ⅱ: 32) underwent curative operation.

Pathologic examination and classification

Clinicopathologic data were recorded based on the second English edition of the Japanese Classification System for Gastric Cancer, edited by the Japanese Gastric Cancer Association. The formalin-fixed specimens, containing the carcinoma lesions together with the surrounding gastric wall, were cut into multiple slices, principally parallel to the lesser curvature, at an interval of 5 mm. The hematoxylin and eosin-stained sections of tumor were initially examined independently by two pathologists and further confirmed by an additional expert pathologist for a final diagnosis. If there was disagreement in the diagnosis, the slides were rechecked by all three pathologists.

Clinicopathologic features, including age, sex, tumor location, tumor size, Borrmann type, histologic grade, Lauren grade, tumor stage, and type of operation, were investigated and compared between the two groups. The tumor locations of RGC were classified as anastomotic site, non-anastomotic site, and total stump. The total number of retrieved lymph nodes in all patients were > 15, so the tumors were staged according to the International Union Against Cancer (UICC, 7th edition) classification. Tumor histologic grade was classified into two groups: differentiated, which included papillary adenocarcinoma and moderately or well-differentiated adenocarcinoma, and undifferentiated, which included poorly or undifferentiated adenocarcinoma, mucinous carcinoma, and signet ring cell carcinoma. The type of the second resection included palliative resection, total

Table 1 Number of patients according to the different periods and treatment type

Year	RGC Ⅰ			RGC Ⅱ		
	Curative resection	PR or no resection	No operation	Curative operation	PR or no resection	No operation
1980-1990	6	6	5	3	3	4
1990-2000	12	3	3	11	2	2
2000-2010	22	4	2	18	3	3
Total	42	13	10	32	8	9

PR: Palliative resection; RGC Ⅰ: Remnant gastric cancer 2-10 years after gastrectomy; RGC Ⅱ: Remnant gastric cancer > 10 years after gastrectomy.

Table 2 Surgical outcomes of the patients *n* (%)

Parameters	RGC Ⅰ (*n* = 55)	RGC Ⅱ (*n* = 40)	*P* value
Type of surgery			0.803
No resection	5 (9.1)	4 (10.0)	
Bypass surgery	2	3	
Open biopsy only	3	1	
Palliative resection	8 (14.5)	4 (10.0)	
Curative resection	42 (76.4)	32 (80.0)	
Resection type			0.189
Resection of RG or gastrectomy	30	26	
Extended gastrectomy	12	6	
Splenectomy	3	2	
Whipple	4	0	
Segmental T-colon resection	2	2	
Distal pancreatectomy	0	2	
Left lateral sectionectomy of liver	2	0	
Diaphragm excision	1	0	

RG: Remnant gastric; RGC Ⅰ: Remnant gastric cancer 2-10 years after gastrectomy; RGC Ⅱ: Remnant gastric cancer > 10 years after gastrectomy.

gastrectomy, and extended gastrectomy. Extended gastrectomy included splenectomy, segmental T-colon resection, distal pancreatectomy, left lateral sectionectomy of liver, and diaphragm excision. For the patients with Billroth Ⅱ reconstruction, the resection of segmental jejunum connected to the gastric stump was not recorded as extended gastrectomy.

Follow-up

All patients with RGC who underwent curative resection operations were followed-up. Patients were evaluated by chest radiograph, ultrasonography, abdominopelvic CT scan, serum tumor markers, and endoscopy to detect recurrence every 3 mo in the first two years. After the first two years, a telephone interview was conducted every 2 mo. Follow-up was complete for the entire study population to December 2013. At the end of follow-up, seven patients were lost. The rate of follow-up was 94.6%.

Statistical analysis

All the statistical analyses were performed by SPSS 16.0 statistical package (SPSS Inc., Chicago, IL, United States). Overall survival rates were determined using the Kaplan-Meier estimator method. The log-rank test was used to identify differences between the survivals of the two subgroups. In univariate analysis, two-tailed χ^2 tests for categorical variables and 2 *t* tests for continuous variables were employed for statistical comparisons. In multivariate analysis, Cox's proportional hazard model was used to identify independent factors correlated with prognosis. A $P < 0.05$ was considered statistically significant.

The statistical methods of this study were reviewed by Bo Qu, (professor and biostatistician), China Medical University.

RESULTS

There were 79 men and 35 women enrolled in the study with a mean age of 61 ± 12 years. The number of the patients with RGC according to the different periods and treatment type is shown in Table 1.

Of the 114 patients, 95 underwent surgical treatment. The types of surgery according to the different subgroups are listed in Table 2. A total of 74 patients (70.7%) received a curative resection. Because of peritoneal seeding, liver metastasis and serious adjacent organ invasion, 12 patients (RGC Ⅰ: 8, RGC Ⅱ: 4) underwent a palliative resection and 9 (RGC Ⅰ: 5, RGC Ⅱ: 4) patients underwent a non-resective operation such as bypass surgery or diagnostic laparotomy. In the RGC Ⅰ group, 42 patients underwent curative resection.

Clinicopathologic features of the 74 patients with curative resection are shown in Table 3. The second cancer was more frequently located on the anastomotic site in the patients with RGC Ⅱ than that in the patients with RGC Ⅰ ($P = 0.038$). Twenty-three patients with RGC Ⅱ underwent Billroth Ⅱ reconstruction in the first operation, which was significantly more than that in the patients with RGC Ⅰ ($P < 0.001$). There were no significant differences in age, tumor size, Borrmann type, histologic grade, Lauren grade, or tumor stage between the two groups. The tumor stage of the initial cancer was only known for 33 patients with RGC Ⅰ and 22 with RGC Ⅱ because some clinical data were lost. There was a significant difference in the number of patients with stage Ⅲ or Ⅳ initial cancers, observed in 60.6% of RGC Ⅰ patients *vs* 22.7% of RGC Ⅱ patients cases ($P = 0.006$).

The overall five-year survival rate of patients with RGC was 43.6%. As shown in Figure 1, the five-year survival rates were 37.6% and 47.9% for the patients with RGC Ⅰ and RGC Ⅱ, respectively.

Table 3 Clinicopathologic features *n* (%)

Parameters	RGC Ⅰ	RGC Ⅱ	*P* value
Sex			0.805
Male	29 (69.0)	21 (65.6)	
Female	13 (31.0)	11 (34.4)	
Age (yr), mean ± SD	60.5 ± 11.6	62.3 ± 13.4	0.103
Tumor size, cm			0.301
< 4.0	16 (38.1)	15 (46.9)	
⩾ 4.0	26 (61.9)	17 (53.1)	
Tumor location			0.038
Anastomotic site	13 (31.0)	18 (56.3)	
Non-anastomotic site	19 (45.2)	6 (18.7)	
Total stump	10 (23.8)	8 (25.0)	
Reconstruction of 1st operation			
Billroth Ⅰ	31 (78.8)	9 (28.1)	< 0.001
Billroth Ⅱ	11 (21.2)	23 (71.9)	
Borrmann type			0.322
Ⅰ + Ⅱ	15 (35.7)	14 (43.7)	
Ⅲ + Ⅳ	27 (64.3)	18 (56.3)	
Histology grade			0.421
Differentiated	19 (45.2)	12 (37.5)	
Undifferentiated	23 (54.8)	20 (62.5)	
Lauren grade			0.308
Intestinal	20 (47.6)	18 (56.3)	
Diffuse	22 (52.4)	14 (43.7)	
Tumor stage			0.106
Ⅰ + Ⅱ	15 (35.7)	16 (50.0)	
Ⅲ + Ⅳ	27 (64.3)	16 (50.0)	
1st tumor stage			0.006
Ⅰ + Ⅱ	13 (39.4)	17 (77.3)	
Ⅲ + Ⅳ	20 (60.6)	5 (22.7)	

RGC Ⅰ: Remnant gastric cancer 2-10 years after gastrectomy; RGC Ⅱ: Remnant gastric cancer > 10 years after gastrectomy.

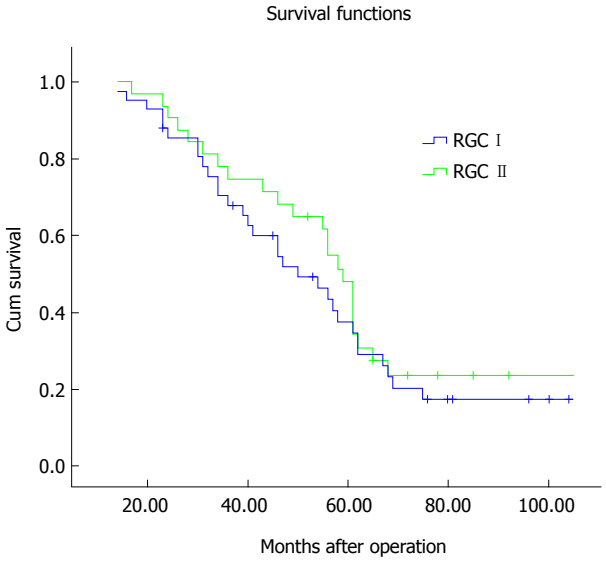

Figure 1 Survival curves. The five-year survival rate for patients with remnant gastric cancer 2-10 years after gastrectomy (RGC Ⅰ) was 37.6%, and was 47.9% for those with remnant gastric cancer > 10 years after gastrectomy (RGC Ⅱ) (*P* = 0.440).

Univariate analyses identified tumor size, Borrmann type, histologic grade, Lauren grade, and tumor stage as factors associated with prognosis (Table 4). In multivariate analysis, Borrmann type and tumor stage were confirmed as independent factors.

Table 4 Analysis of the prognostic factors

Parameters	RGC Ⅰ			RGC Ⅱ		
	5-YSR	*P* value		5-YSR	*P* value	
	(%)	UA	MA	(%)	UA	MA
Sex		0.574			0.179	
Male	31.9			51.0		
Female	51.9			43.6		
Age, yr		0.854			0.467	
< 60	40.8			44.4		
⩾ 60	34.2			49.2		
Tumor size (cm)		0.018	0.235		0.009	0.653
< 4.0	59.8			60.0		
⩾ 4.0	23.7			35.2		
Reconstruction of 1st operation		0.875			0.644	
Billroth Ⅰ	34.4			44.4		
Billroth Ⅱ	45.5			49.0		
Borrmann type		0.015	0.004		0.031	0.013
Ⅰ + Ⅱ	57.4			66.7		
Ⅲ + Ⅳ	25.9			36.9		
Histologic grade		0.035	0.532		0.044	0.254
Differentiated	53.3			66.7		
Undifferentiated	24.3			36.9		
Lauren grade		0.026	0.108		0.032	0.321
Intestinal	56.9			55.6		
Diffuse	22.9			36.7		
Tumor stage		0.015	< 0.001		0.009	< 0.001
Ⅰ + Ⅱ	67.0			67.3		
Ⅲ + Ⅳ	18.1			27.5		

MA: Multivariate analysis; RGC Ⅰ: Remnant gastric cancer 2-10 years after gastrectomy; RGC Ⅱ: Remnant gastric cancer > 10 years after gastrectomy; UA: Univariate analysis; 5-YSR: Five-year survival rate.

DISCUSSION

Although the mortality of gastric cancer has substantially decline because of early diagnosis, radical surgery, and the development of adjuvant therapies, the deaths that do occur almost invariably follow tumor recurrence[19,20]. RGC following distal gastrectomy for gastric cancer is a frequent type of tumor recurrence. Operation is the most effective treatment for RGC. Some reports proposed different operative method for the curative resection of RGC[13,21]. We performed mainly total gastrectomy plus D2 lymphadenectomy and adjacent organ resection regardless of RGC Ⅰ or RGC Ⅱ. The reports concerning the rate of resectability for RGC have some discordant opinions. It had often been described as having low resectability rates (< 70%)[22,23]. It was also reported that the rate of curative resection was 60% in patients with RGC after benign disease, 78% in patients with RGC after gastric cancer, and 70% in total patients with RGC[24]. Our study reports higher rates, as the rate of curative resection was 76.4% for RGC Ⅰ and 80.0% for RGC Ⅱ, with an overall rate of 77.9%. The reason for this might be that some patients with distant metastasis or poor physical condition were recommended to undergo

treatment in the department of medical oncology.

Some studies report a high rate (40%-70%) of combined organ resection, which was attributed it to the high incidence of adjacent organ invasion and the need for lymphadenectomy[25-27]. In the present study, although the combined resection of involved organs was more frequently selected in patients with RGC Ⅰ than those in the patients with RGC Ⅱ (25.8% *vs* 12.5%; *P* = 0.189), the rates are much lower than what has been reported. This might be induced by the different standard in the preoperational assessment. In this investigation, Whipple resection was conducted in four patients with RGC Ⅰ, but not in patients with RGC Ⅱ. This might account for the difference of the adjacent organs, which could be attributed to different reconstruction type.

According to a previous study, gastritis cystica polyposa, which was suspected to be of great relevance to cancer development in the remnant stomach, was detected more often on the anastomotic site, and more often in patients with Billroth Ⅱ reconstruction than in those with Billroth Ⅰ [24]. These suggest that long-term exposure of the gastric mucosa to duodenal contents is one of the major causes of RGC after distal gastrectomy and the anastomotic site, which is the most direct site for this disadvantage, and is understandably the most common location for the RGC. However, the time interval from the first resection to development of RGC varied to a great extent. The average latency time was reported to be 20-27 years, and up to 40 years. As the risk of developing gastric stump cancer increases after 20 years[16-18], the time interval appears to be one of the most important factors for the development of RGC. A reason for this is because the remnant stomach is constantly under carcinogenic influence. This is only applicable for RGC after benign disease, as the anastomotic site of Billroth Ⅱ reconstruction is frequently involved by RGC 15 years or more after the first gastrectomy for benign disease[4,28]. In the present study, more than half of RGC following gastric cancer surgery developed less than 10 years after the first operation. The time interval did not seem to be enough for the development of RGC. Meanwhile, RGC Ⅰ was more frequently located on the non-anastomotic site and more cases of them developed after Billroth Ⅰ reconstruction. Thus, we infer that there might be some different pathogeneses for RGC Ⅰ. It is well known that mucosal changes, such as atrophic gastritis and intestinal metaplasia, are often observed in tumor-adjacent tissues. Denervation during initial gastric cancer surgery might damage the defense mechanisms of the gastric mucosa, which facilitates the development of cancer from precancerous lesions[29]. We therefore also infer that preexisting mucosal changes in the remnant stomach, such as atrophic gastritis and intestinal metaplasia, rather than gastroduodenal reflux, are more relevant to the development of RGC Ⅰ. On the other hand, RGC Ⅰ was more common in patients with a more advantaged tumor stage of the first cancer. This may suggest that the development of RGC Ⅰ is be due to residual carcinomas ignored at the initial operation. Although resection margins are deemed histologically free of tumor involvement at initial operation, tumor cells still may remain in the remnant stomach.

It has been reported that the incidence of synchronous multiple gastric cancers are approximately 4%-7% of surgically resected cases[30]. One study conducted a comprehensive evaluation of serial sections from the entire stomach and found an incidence of 13.2%-14.6%, suggesting a higher incidence of latent lesions[30]. According to another previous study[31], patients with multiple gastric cancers are more likely to develop secondary gastric cancers in the remnant stomach. All the above suggest that RGC Ⅱ after Billroth Ⅱ reconstruction might develop from a typical model of carcinogenesis for gastroduodenal reflux. However, preexisting mucosal changes or residual carcinomas ignored at initial treatment might be more responsible for RGC Ⅰ. In patients whose re-oncogenesis locations were non-anastomotic sites and total stump, we did not observe other sites of re-oncogenesis *via* preoperative gastroscopy and intraoperative check, therefore, we surmise that the re-oncogenesis locations were associated with background mucosal change. Moreover, curative resection with a safe margin (all the margins have been proven postoperatively to be safe or called "negative margin") was achieved in all 42 of these patients. Thus, more studies should be conducted to find the most possible reason for their early re-oncogenesis after prior gastrectomy.

In conclusion, there is no significant difference in surgical outcomes and prognosis between RGC Ⅰ and RGC Ⅱ. However, in clinicopathologic features, RGC Ⅱ is more frequently located on the anastomotic site and more cases develop after Billroth Ⅱ reconstruction. Therefore, we propose that gastroduodenal reflux might induce the development of RGC Ⅱ. However, the development of RGC Ⅰ could also be attributed to preexisting mucosal changes or residual carcinomas ignored at initial treatment for synchronous multiple gastric cancers.

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COMMENTS

Background

The time interval between the primary cancer and the remnant gastric cancer (RGC) in patients is a key point in the definition of RGC. Several reports have used RGC to define all cancers arising from the remnant stomach after partial

gastrectomy, regardless of the initial disease.

Research frontiers

The preferred explanation for the pathogenesis of RGC is that Billroth Ⅱ reconstruction produces a typical model of carcinogenesis. Gastroduodenal reflux and *Helicobacter pylori* colonization in the remnant stomach promote the development of RGC. Because the gastric stump is constantly under carcinogenic influence, the time interval is one of the most important factors for the development of RGC.

Innovations and breakthroughs

Most authors have reported a steep increase in the risk of developing gastric stump cancer from the 20th year after the first gastrectomy. However, the authors consider that there may be some differences in clinical pathology and prognosis between the RGC patients with a recurrence interval shorter than 10 years and those longer than 10 years. The authors found that RGC that occurs after > 10 years is more frequently located on the anastomotic site and more cases develop after Billroth Ⅱ reconstruction. Therefore, the authors suggest that gastroduodenal reflux induces the development of these cases of RGC.

Applications

The development of RGC within 10 years after gastrectomy may result from preexisting mucosal changes or residual carcinomas ignored at initial treatment for synchronous multiple gastric cancers. This study may help to lay the foundation of the management of RGC.

Terminology

RGC refers to a carcinoma detected in remnant stomach more than five years after primary surgery for a benign disease.

Peer-review

The study is done carefully. This article is an important paper concerning clinicopathologic features of RGC. Original point of this paper is the comparison of RGC with time interval of > 2 and ⩽ 10 years after prior gastrectomy for gastric cancers.

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ORIGINAL ARTICLE

Clinical Trials Study

Phase Ⅰ trial of combination chemotherapy with gemcitabine, cisplatin, and S-1 in patients with advanced biliary tract cancer

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Ethics approval: The study was reviewed and approved by the Kitasato university hospital Institutional Review Board.
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Abstract

AIM: To evaluate the dose-limiting toxicities (DLTs) and determine the maximum-tolerated dose (MTD) and recommended dose (RD) of combination chemotherapy with gemcitabine, cisplatin and S-1 which is an oral fluoropyrimidine pro-drug in patients with advanced biliary tract cancer.

METHODS: Patients with histologically or cytologically confirmed unresectable or recurrent biliary tract cancer were enrolled. The planned dose levels of gemcitabine (mg/m^2), cisplatin (mg/m^2), and S-1 (mg/m^2 per day) were as follows: level -1, 800/20/60; level 0, 800/25/60; level 1, 1000/25/60; and level 2, 1000/25/80. In each cycle, gemcitabine and cisplatin were administered intravenously on days 1 and 15, and S-1 was administered orally twice daily on days 1 to 7 and days 15 to 21, every 4 wk.

RESULTS: Twelve patients were enrolled, and level 0 was chosen as the starting dose. None of the first three patients had DLTs at level 0, and the dose was escalated to level 1. One of six patients had DLTs (grade 4 febrile neutropenia, leucopenia, and neutropenia; grade 3 thrombocytopenia) at level 1. We then proceeded to level 2. None of three patients had DLTs during the first cycle. Although the MTD was not determined, level 2 was designated at the RD for a subsequent phase Ⅱ study.

CONCLUSION: The RD was defined as gemcitabine 1000 mg/m^2 (days 1, 15), cisplatin 25 mg/m^2 (days 1, 15), and S-1 80 mg/m^2 per day (days 1-7, 15-21), every 4 weeks. A phase Ⅱ study is planned to evaluate the effectiveness of combination chemotherapy with

gemcitabine, cisplatin, and S-1 in advanced biliary tract cancer.

Key words: Gemcitabine; Cisplatin, S-1; Advanced biliary tract cancer

Core tip: This Phase Ⅰ trial revealed that combination therapy with gemcitabine, cisplatin, and S-1 was well tolerated and feasible in patients with advanced biliary tract cancer. We are now proceeding to a phase Ⅱ study to investigate the efficacy of this combination regimen in advanced biliary tract cancer.

Watanabe A, Kida M, Miyazawa S, Iwai T, Okuwaki K, Kaneko T, Yamauchi H, Takezawa M, Imaizumi H, Koizumi W. Phase Ⅰ trial of combination chemotherapy with gemcitabine, cisplatin, and S-1 in patients with advanced biliary tract cancer. *World J Gastroenterol* 2015; 21(19): 5979-5984 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5979.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5979

INTRODUCTION

Biliary tract cancer is more common in East Asia and Latin America than in other continents[1]. Despite recent remarkable progress in diagnostic procedures, most cases are advanced at initial diagnosis and are thus treated by chemotherapy. Moreover, even if surgery, the only potentially curative treatment, can be performed, relapse often occurs, and 5-year survival rates are not high (ampullary cancer, 52.8%; gallbladder cancer, 41.6%; bile duct cancer, 33.1%)[2].

Gemcitabine, cisplatin, and fluorouracil (including their pro-drugs) are widely used to treat biliary tract cancer. Gemcitabine is used throughout the world as a key drug for the management of biliary tract cancer because clinical trials have confirmed its effectiveness, with a response rate (RR) of 17.5% and a mean survival time (MST) of 7.6 mo[3]. In addition, the ABC-02 study, a phase Ⅲ randomized controlled trial comparing gemcitabine alone with gemcitabine plus cisplatin (GC), reported that MST was significantly longer for the combination regimen (gemcitabine, 8.1 mo *vs* GC, 11.7 mo, $P < 0.001$)[4]. These results established GC combination therapy as a standard treatment for advanced biliary tract cancer.

S-1 is an oral fluoropyrimidine pro-drug that has been confirmed to be effective against various types of solid tumors, both alone and in combination with other cytotoxic drugs[5-12]. S-1 has also been confirmed to be effective against biliary tract cancer. Two phase 2 clinical trials reported RRs of 21.1% and 35.0% with MSTs of 252 d and 287 d, respectively[13,14]. However, these results remain unsatisfactory.

Available evidence suggests that a three-drug combination regimen of gemcitabine, cisplatin, and S-1 might further enhance response and improve outcomes. However, the effectiveness of combination therapy with gemcitabine, cisplatin, and S-1 has not been evaluated previously in advanced biliary tract cancer. We designed this phase Ⅰ study to evaluate the safety and determine the maximum-tolerated dose (MTD) and recommended dose (RD) of this triplet combination in patients with advanced biliary tract cancer.

MATERIALS AND METHODS

Patient eligibility

Patients with histologically or cytologically confirmed biliary tract cancer were eligible for enrollment if they met the following criteria: unresectable or recurrent disease; no prior therapy (radiation or chemotherapy) other than surgery; 20-79 years of age; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; and adequate bone marrow function (white blood cell count 3500-12000/mm^3, neutrophil count $\geqslant$ 2000/mm^3, platelet count $\geqslant$ 100000/mm^3, and hemoglobin $\geqslant$ 10 g/dL), adequate liver function (aspartate aminotransferase/alanine aminotransferase (AST/ALT) $\leqslant$ three times the upper limit of normal (ULN) (in patients with obstructive jaundice, $\leqslant$ five times the ULN after biliary drainage), and total bilirubin $\leqslant$ 2 mg/dL (in patients with obstructive jaundice, $\leqslant$ 3 mg/dL after biliary drainage), adequate renal function (creatinine clearance $\geqslant$ 60 mL/min; 24-h urine collection was recommended, or the Cockcroft-Gault formula could be used if 24-h collection was not possible), and adequate heart function (practically normal); and adequate oral intake. All patients provided written informed consent. The exclusion criteria were as follows: the presence of another cancer; severe complications (for example, congestive heart disease, coronary artery disease, active arrhythmias, a history of cerebral infarction or hemorrhage, active gastrointestinal bleeding or ulcer, uncontrollable diabetes mellitus, renal failure, active hepatitis, liver cirrhosis, or liver failure); the presence of a fever with suspected infection; paresis, peripheral neuropathy, or edema unrelated to biliary tract cancer; severe pleural or pericardial effusion; moderate or severe ascites; pregnancy or nursing infants, women of childbearing age; pulmonary fibrosis or interstitial pneumonia; severe mental disorders; a history of severe allergy or allergies to the drugs used in this study; treatment with another fluoropyrimidine cytotoxic agent; and treatment with flucytosine. All procedures were performed in accordance with the 1964 Declaration of Helsinki.

Study design

This dose-escalating, single-center phase Ⅰ study was performed at Kitasato University East Hospital in

Table 1 Doses and treatment schedules for each level

	Gemcitabine (mg/m^2, Days 1, 15)	Cisplatin (mg/m^2, Days 1, 15)	S-1 (mg/d, Days 1-7, 15-21) BSA < 1.25	1.25 < BSA < 1.5	BSA > 1.5
Level -1	800	20	60	80	100
Level 0	800	25	60	80	100
Level 1	1000	25	60	80	100
Level 2	1000	25	80	100	120

BSA: Body surface area.

Japan. The protocol was approved by the institutional review board of the hospital. Patient registration and data management were conducted at the Department of Gastroenterology, Kitasato University School of Medicine. All laboratory tests required to assess eligibility were completed within 14 d before starting the protocol treatment. The doses and treatment schedules of each level are summarized in Table 1; these recommendations were based on previous studies evaluating gemcitabine, cisplatin, and S-1 in advanced biliary tract cancer[3,4,13-15].

Dose-limiting toxicities (DLTs) were defined according to the Common Terminology Criteria for Adverse Events (CTCAE), version 4.0, as the following events: grade 4 leucopenia, neutropenia, or anemia; grade 3 neutropenia complicated by fever (> 38 ℃) persisting for more than 2 d; grade 3 thrombocytopenia; any other grade 3-4 non-hematologic toxicity, with the exception of alopecia, anorexia, fatigue, nausea, and vomiting; a delay of more than 2 wk in starting the second cycle of chemotherapy; and a delay of more than 2 wk in the administering the cytotoxic agents scheduled to be given on day 15. At least three patients were enrolled at each dose level. If DLT occurred in one patient during the first cycle, three additional patients were enrolled at the same dose level. If only one of the six patients had DLT, the dose was escalated to the next level. There was no dose escalation in individual patients. MTD was defined as the dose that caused DLT in two or more of the first six patients or in two initially treated patients. If the MTD was defined as level 0, which was used as the starting dose, the dose was de-escalated to level -1. RD was defined as one dose lower than the MTD, given the toxicity and tolerability of treatment in this study. If no patient had DLT at level 2, level 2 was defined as the RD.

Treatment

All patients received the first course of chemotherapy in an inpatient clinic to closely monitor toxicity. Chemotherapy was started on day 1 in eligible patients. Treatment was repeated on day 15 or subsequently, provided that all of the following criteria were met: white-cell count > 3000/mm^3; neutrophil count > 1500/mm^3; platelet count > 75000/mm^3; no fever (> 38 ℃) due to infection; hemoglobin > 9 mg/dL; AST/ALT < five times the ULN (patients without biliary drainage) or < three times the ULN (patients with biliary drainage); total bilirubin < 3 mg/dL (patients without biliary drainage) or < 2 mg/dL (patients with biliary drainage); creatinine clearance > 60 mL/min; no diarrhea/fatigue/mucositis or oral/peripheral neuropathy of grade 2 or higher; no non-hematologic toxicities of grade 3 or higher (except for abnormal blood test results not relevant to the study drugs). If the patient did not meet the above criteria, chemotherapy was postponed by several days to 3 wk until recovery. If chemotherapy was delayed by more than 3 wk, the protocol therapy was discontinued. S-1 was discontinued if the patient met any of the following criteria during the treatment course: white-cell count < 2000/mm^3; neutrophil count < 1000/mm^3; platelet count < 75000/mm^3; fever (> 38 ℃) due to infection; hemoglobin < 9 mg/dL; AST/ALT > five times the ULN (patients without biliary drainage) or >three times the ULN (patients with biliary drainage); total bilirubin > 3 mg/dL (patients without biliary drainage) or > 2 mg/dL (patients with biliary drainage); creatinine clearance < 60 mL/min; diarrhea/fatigue/oral mucositis of grade 2 or higher; or non-hematologic toxicities of grade 3 or higher (excluding abnormal blood test results not relevant to the study drugs). Because this was a dose-escalation study a reduction in dosage was not allowed. If dose reduction was required, the protocol therapy was discontinued.

Pretreatment and follow-up evaluations

Pretreatment evaluations included a complete medical history, physical examinations, blood tests, imaging studies by contrast-enhanced computed tomography, electrocardiography, and chest radiography. Creatinine clearance was evaluated using 24-h urine specimens (by the Cockcroft-Gault formula if impossible). During protocol treatment, physical examinations and blood tests were scheduled every week. Carcinoembryonic antigen and carbohydrate antigen 19-9 (CA19-9) were measured at the time of enrollment in the study and every month thereafter. Toxicity was evaluated according to the CTCAE, version 4.0. In patients with measurable target lesions, the objective RR was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1, and imaging tests were planned after the first cycle. Additional imaging tests were performed if clinically indicated or at the discretion of the treating physician.

RESULTS

Characteristics

Twelve patients were enrolled between June 2011 and January 2014 (Table 2). The median age was 69 years (range, 44-77 years), and no patient had recurrent disease. Seven patients had gallbladder cancer (58%), three (25%) had extrahepatic bile duct cancer, and two

Table 2 Patient characteristics

Characteristic	*n* (%)
Sex	
Male	10 (83)
Female	2 (17)
Median age	69 (range 44-77)
Primary lesion	
Intrahepatic	2 (17)
Extrahepatic	3 (25)
Gallbladder	7 (58)
Ampulla of vater	0 (0)
Disease status	
Unresectable	12 (100)
Recurrent	0 (0)
Performance status (0/1)	12/0
Biliary drainage	6 (50)
Median CEA (ng/mL)	3 (range 1.1-33.4)
Median CA19-9 (U/mL)	156.5 (range 1.0- > 10000)

CEA: Carcinoembryonic antigen; CA: Carbohydrate antigen.

Table 3 Dose-limiting toxicities at each level

Level	Age	Sex	Primary lesion	Biliary drainage	DLT	Response (RECIST)
0	71	M	Extrahepatic	Yes	None	PR
0	73	M	Extrahepatic	Yes	None	SD
0	63	F	Gallbladder	No	None	PD
1	77	M	Intrahepatic	Yes	Gr 4 febrile neutropenia and leucopenia, Gr 3 thrombocytopenia	PD
1	67	M	Gallbladder	Yes	None	NE
1	64	M	Gallbladder	No	None	SD
1	70	M	Extrahepatic	Yes	None	SD
1	72	M	Gallbladder	No	None	PR
1	74	M	Gallbladder	No	None	PR
2	58	M	Intrahepatic	No	None	PR
2	68	F	Gallbladder	No	None	SD
2	44	M	Gallbladder	Yes	None	PD

DLT: Dose-limiting toxicities; Gr: Grade; NE: Not evaluable; PD: Progressive disease; PR: Partial response; SD: Stable disease.

(17%) had intrahepatic bile duct cancer. Six patients (50%) required biliary drainage before starting treatment.

DLTs

DLTs are summarized according to dose level in Table 3. Level 0 was chosen as the starting dose. Three patients were assigned to level 0, and no patient had DLT. Therefore, the dose was escalated to level 1. At level 1, DLT occurred in one of the first three patients, and three additional patients were assigned to this level. In total, one of the six assessable patients had DLTs (grade 4 febrile neutropenia, leucopenia and neutropenia; grade 3 thrombocytopenia), and the dose was further escalated to level 2. At level 2, DLT did not occur in the first three assessable patients. Therefore, level 2 was designated as the RD.

Table 4 Hematologic adverse events during the first cycle

	Level 0 (*n* = 3)		Level 1 (*n* = 6)		Level 2 (*n* = 3)	
	Gr 1-2	Gr 3-4	Gr 1-2	Gr 3-4	Gr 1-2	Gr 3-4
Neutropenia	0	0	0	1	0	1
Leucopenia	1	0	2	1	1	0
Thrombocytopenia	2	0	1	1	0	0
Anemia	2	0	1	0	0	0
Febrile neutropenia	NA	0	NA	1	NA	0

NA: Not applicable.

Toxicity

Common hematologic and non-hematologic adverse events occurring during the first cycle of chemotherapy are summarized in Tables 4 and 5. Grade 3-4 neutropenia, leucopenia, thrombocytopenia, and anemia occurred in 2, 1, 1, and 0 patients (17%, 8%, 8%, and 0%), respectively. Febrile neutropenia occurred in one patient at level 1. Common non-hematologic adverse events were anorexia (5 cases, 42%), nausea (2 cases, 17%), vomiting (1 case, 8%), fatigue (2 cases, 17%), constipation (2 cases, 17%), and elevation of AST (5 cases, 42%) or ALT (4 cases, 33%). In addition, hyperbilirubinemia (4 cases, 33%) was common; however, this adverse event was attributed primarily to obstruction of the biliary tract caused by the primary disease. Among these adverse events, the incidences of grade 3-4 events were generally low (Table 5). On the basis of the incidences of DLTs and adverse events, we selected level 2 as the RD for a phase Ⅱ study designed to evaluate the effectiveness of a combination of gemcitabine, cisplatin, and S-1.

Response

Although tumor response was not the primary endpoint of this study, imaging studies to evaluate tumor response were planned after the first cycle. Eleven of the 12 patients were assessable for response according to RECIST; four patients had a partial response (one at dose level 0, two at dose level 1, and one at dose level 2), four patients had stable disease (one at dose level 0, two at dose level 1, and one at dose level 2), and three patients had disease progression (one at each dose level), resulting in an overall RR of 33.3%.

DISCUSSION

This phase 1 dose-escalation study was designed to define the MTD and RD of combination chemotherapy with gemcitabine, cisplatin, and S-1 in patients with advanced biliary tract cancer. Dose level 2 (gemcitabine 1000 mg/m^2, cisplatin 25 mg/m^2, S-1 80 mg/m^2 per day) was designated as RD; however, the MTD could not be estimated.

Table 5 Non-hematologic adverse events during the first cycle

	Level 0 (n = 3)		Level 1 (n = 6)		Level 2 (n = 3)	
	Gr 1-2	Gr 3-4	Gr 1-2	Gr 3-4	Gr 1-2	Gr 3-4
Anorexia	2	0	1	0	2	0
Nausea	1	0	1	0	0	0
Vomiting	1	0	0	0	0	0
Fatigue	0	0	0	0	2	0
Constipation	1	0	1	0	0	0
Fever	1	0	2	0	0	0
Biliary tract infection	NA	3	NA	0	NA	1
Infections (others)	0	0	0	2	0	0
AST	3	0	2	0	0	0
ALT	2	0	2	0	0	0
Hyperbilirubinemia	2	0	1	0	1	0
Creatinine	0	0	3	0	0	0

NA: Not applicable; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

We expected that our triple-drug regimen for chemotherapy would enhance effectiveness as compared with previously studied singlet or doublet regimens, because previous clinical trials obtained low RRs and short MSTs. In a phase 2 study of gemcitabine alone, Okusaka *et al*[3] obtained an RR of 17.5% and an MST of 7.6 mo. The ABC-02 study was reported that MST of patients who received GC (11.7 mo) was significantly longer than that of patients who received gemcitabine alone (8.1 mo, $P < 0.001$)[4]. Two phase 2 clinical trials showed that S-1 monotherapy has clinically significant antitumor activity with mild toxicity[13,14]. Kanai *et al*[15] conducted a phase 2 study of gemcitabine plus S-1 (GS) in patients with advanced biliary tract cancer and reported this regimen provided a promising survival benefit with acceptable toxicity.

The efficacy and tolerability of triplet chemotherapy regimens for other solid cancers were reported recently. Vermorken *et al*[16] conducted a clinical trial comparing a combination of docetaxel, cisplatin, and fluorouracil (DCF) with cisplatin plus fluorouracil in patients with head and neck cancer. DCF significantly improved median progression-free survival as compared with cisplatin plus fluorouracil (DCF, 11.0 mo *vs* cisplatin plus fluorouracil, 8.2 mo, $P = 0.007$) and had tolerable toxicities. Furthermore, Conroy *et al*[17] compared FOLFILINOX (a combination of fluorouracil, oxaliplatin, and irinotecan) with gemcitabine alone. Although triplet therapy was significantly more effective (MST: FOLFILINOX 11.1 mo *vs* gemcitabine 6.8 mo, $P < 0.001$), FOLFILINOX had increased toxicity[17]. Koizumi *et al*[18] conducted a phase 2 study of combination therapy with docetaxel, cisplatin and S-1 in advanced gastric cancer and reported that this regimen was highly active and well tolerated. These triplet regimens with high RRs have been suggested to be useful for neoadjuvant chemotherapy[19,20]. The findings of these previous studies support our concept of combination therapy with gemcitabine, cisplatin, and S-1.

However, multiple-drug regimens for chemotherapy probably increase the risk of severe adverse events. We based the treatment schedule of our regimen on the results of previous pivotal clinical trials. First, in the ABC-02 trial, the GC group received gemcitabine 1000 mg/m^2 and cisplatin 25 mg/m^2 on days 1 and 8 every 3 wk. Adverse events of grade 3 or higher were neutropenia (25.3%), thrombocytopenia (8.6%), and anemia (7.6%). Second, as for GS, we referred to the results of a study of GS performed by Ookawa *et al*[21] in patients with pancreatic cancer, because fewer studies of GS have been reported for biliary tract cancer than for pancreatic cancer. In that study, gemcitabine 1000 mg/m^2 was given on day 1, and S-1 80 or 100 mg/m^2 was given orally on days 1 to 7, every 2 wk. Adverse events or grade 3 or higher were only leucopenia (25%) and neutropenia (20%); moreover, there were no grade 4 events. On the basis of these findings, we decided to administer gemcitabine and cisplatin on days 1 and 15 and S-1 on days 1 to 8 and 15 to 21 every 4 wk because the triple-drug combination of gemcitabine, cisplatin, and S-1 was based on the GC and GS regimens and was expected to have a higher risk of adverse events.

In conclusion, our results showed that combination therapy with gemcitabine, cisplatin, and S-1 was well tolerated and feasible in patients with advanced biliary tract cancer. We are now proceeding to a phase Ⅱ study to investigate the efficacy of this combination regimen in advanced biliary tract cancer.

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COMMENTS

Background

Biliary tract cancer is more common in East Asia and Latin America than in other continents. Despite recent remarkable progress in diagnostic procedures, most cases are advanced at initial diagnosis and are thus treated by chemotherapy. Moreover, even if surgery, the only potentially curative treatment, can be performed, relapse often occurs, and 5-year survival rates are not high. Much chemotherapy has been reported, but their efficacies are not satisfactory.

Research frontiers

Gemcitabine, cisplatin, and fluorouracil (including their pro-drugs, for example S-1) are widely used to treat biliary tract cancer. Especially, gemcitabine is used throughout the world as a key drug for the management of biliary tract cancer because clinical trials have confirmed its effectiveness. In addition, cisplatin and S-1 have been reported their efficacy both in alone and some combination chemotherapies.

Innovations and breakthroughs

Available evidence suggests that a three-drug combination regimen of gemcitabine, cisplatin, and S-1 might further enhance response and improve outcomes. However, the effectiveness of combination therapy with gemcitabine,

cisplatin, and S-1 has not been evaluated previously in advanced biliary tract cancer.

Applications

This trial showed that combination therapy with gemcitabine, cisplatin, and S-1 was well tolerated and feasible in patients with advanced biliary tract cancer.

Peer-review

This paper reported the results of a phase I trial of combination chemotherapy with gemcitabine, cisplatin, and S-1 in patients with advanced biliary tract cancer. The clinical trial was well designed and got expected results. The results provide a possible improvement for advanced biliary tract cancer treatment although more data are needed to support the conclusion. The manuscript was well organized and the language is good.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis

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Author contributions: Yoshimatsu Y and Suzuki Y contributed equally to this work; Yoshimatsu Y and Suzuki Y designed research; Yoshimatsu Y, Yamada A, Furukawa R, Sono K, Osamura A, Nakamura K, Aoki H, Tsuda Y, Hosoe N and Takada N performed research; Yoshimatsu Y and Suzuki Y contributed new reagents/analytic tools; Yoshimatsu Y and Suzuki Y analyzed data; and Yoshimatsu Y and Suzuki Y wrote the paper.

Ethics approval: The study was reviewed and approved by the ethical committee of Toho University Sakura Medical Center.

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

Conflict-of-interest: Yasushi Yoshimatsu has no conflict of interest.

Data sharing: No additional data are available.

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Abstract

AIM: To evaluate the effectiveness of probiotic therapy for suppressing relapse in patients with inactive ulcerative colitis (UC).

METHODS: Bio-Three tablets, each containing 2 mg of lactomin (*Streptococcus faecalis* T-110), 10 mg of *Clostridium butyricum* TO-A, and 10 mg of *Bacillus mesentericus* TO-A, were used as probiotic therapy. Sixty outpatients with UC in remission were randomly assigned to receive 9 Bio-Three tablets/day (Bio-Three group) or 9 placebo tablets/day (placebo group) for 12 mo in addition to their ongoing medications. Clinical symptoms were evaluated monthly or on the exacerbation of symptoms or need for additional medication. Fecal samples were collected to analyze bacterial DNA at baseline and 3-mo intervals. Terminal restriction fragment length polymorphism and cluster analyses were done to examine bacterial components of the fecal microflora.

RESULTS: Forty-six patients, 23 in each group, completed the study, and 14 were excluded. The relapse rates in the Bio-Three and placebo groups were respectively 0.0% *vs* 17.4% at 3 mo (P = 0.036), 8.7% *vs* 26.1% at 6 mo (P = 0.119), and 21.7% *vs* 34.8% (P = 0.326) at 9 mo. At 12 mo, the remission rate was 69.5% in the Bio-Three group and 56.6% in the placebo group (P = 0.248). On cluster analysis of fecal flora, 7 patients belonged to cluster Ⅰ, 32 to cluster Ⅱ, and 7 to cluster Ⅲ.

CONCLUSION: Probiotics may be effective for

maintaining clinical remission in patients with quiescent UC, especially those who belong to cluster I on fecal bacterial analysis.

Key words: Ulcerative colitis; Probiotics; Inflammatory bowel disease; Cluster analysis

Core tip: We conducted a single-center, randomized, double-blind, placebo-controlled study to examine whether 12 mo of probiotic therapy was useful for preventing relapse of ulcerative colitis (UC) in patients who were already in remission. The relapse rates in the probiotic therapy group and placebo group were respectively 0.0% *vs* 17.4% at 3 mo (P = 0.036), 8.7% *vs* 26.1% at 6 mo (P = 0.119), and 21.7% *vs* 34.8% (P = 0.326) at 9 mo. At 12 mo, the remission rate was 69.5% in the probiotic therapy group and 56.6% in the placebo group (P = 0.248). Therefor probiotics may be effective for maintaining clinical remission in patients with quiescent UC.

Yoshimatsu Y, Yamada A, Furukawa R, Sono K, Osamura A, Nakamura K, Aoki H, Tsuda Y, Hosoe N, Takada N, Suzuki Y. Effectiveness of probiotic therapy for the prevention of relapse in patients with inactive ulcerative colitis. *World J Gastroenterol* 2015; 21(19): 5985-5994 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5985.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5985

INTRODUCTION

Ulcerative colitis (UC) is a chronic, idiopathic, refractory, inflammatory bowel disease (IBD) characterized by inflammatory mucosal injury of the colon, with repeated periods of remission and relapse. The cause and etiology of UC remain unclear. The mainstay of treatment for UC is sulfasalazine- or mesalazine-based therapy. In patients with moderate to severe UC, steroids are used concurrently to attempt to induce remission. However, a considerable number of cases are resistant to steroids. Patients with steroid-resistant disease are given immunosuppressants and newly developed biological preparations to promote remission induction. Although these new treatments have enhanced the remission induction rate as compared with conventional therapy, achievement of a high long-term rate of remission maintenance remains a largely unattained goal. Steroids are very effective for the induction of remission, but do not contribute to remission maintenance. In addition, long-term treatment with high doses of steroids is associated with high rates of various adverse effects, seriously impairing the quality of life of patients. Sulfasalazine, mesalazine, and immunomodulators promote remission maintenance, but are not adequately effective. Moreover, an appreciable number of patients cannot tolerate these drugs, and immunomodulators can cause serious adverse events, necessitating close follow-up. Therefore, the development of new remission maintenance treatments that are very effective and safe with good compliance when used on a long-term basis has been eagerly awaited.

Recently, probiotic therapy has been acknowledged to be potentially effective and safe in patients with UC. Probiotics are defined as a live microbial feed nutritional supplement that beneficially affects the host by improving the balance of the intestinal flora. Studies of animal models of colitis have suggested that the intestinal flora has an important role in the pathogenesis of colitis. In IBD-sensitive knockout or transgenic mice, colitis develops in the presence of a normal intestinal flora, but not in mice raised in a germ-free environment, strongly suggesting that the intestinal flora participates in the development of colitis[1,2]. Therefore, probiotic therapy designed to correct the intestinal flora is expected to be useful for preventing colitis.

Many studies have examined the effects of specific bacterial strains or species in active UC. However, very few studies have reported on the relation between the intestinal flora as a whole (including microorganisms that are difficult or impossible to culture) and the pathological characteristics of UC[3-7]. In a previous study, we therefore gave probiotic or synbiotic therapy for 4 wk to 20 patients with mild to moderate UC who did not respond to, or could not tolerate, standard therapy [oral mesalamine preparations, sulfasalazine, azathiopurine (AZA)/6-mercaptopurine (6-MP), and mesalamine enemas]. Our results confirmed that such therapy can improve clinical symptoms and endoscopic findings and provided evidence that remission induction is promoted by a certain improvement in the intestinal flora. We also reported that probiotic therapy might be effective for maintenance of remission[8]. On the basis of the results of our previous study, we conducted a single-center, randomized, double-blind, placebo-controlled study to examine whether 12 mo of probiotic therapy is useful for preventing relapse of UC in patients who were already in remission.

MATERIALS AND METHODS

Patients

The study group comprised patients with UC in remission who were receiving treatment on an outpatient basis at Sakura Medical Center, Toho University. UC was diagnosed in accordance with the diagnostic criteria proposed by the Survey Research Group of Intractable Inflammatory Intestinal Disorders/Specified Diseases, Japanese Ministry of Health, Labour and Welfare. Patients 13 years or older in whom the CAI was maintained at 5 or less while receiving drugs

such as mesalazine, salazosulfapyridine, or steroids, with no change in treatment regimens within 4 wk before study entry, were enrolled in this randomized, double-blind, placebo-controlled study.

Patients were excluded if they had serious cardiac disease, serious renal disease, hypotension (systolic blood pressure, ≤ 80 mmHg), a history of shock during extracorporeal circulation, serious infections such as sepsis or pneumonia, or a serum hemoglobin concentration of less than 10 g/dL. We also excluded patients who newly began treatments such as leukocytapheresis, granulocyte adsorptive apheresis, or immunosuppressant therapy with drugs such as 6-mercaptopurine, azathioprine, and cyclosporine to improve symptoms, as well as patients who had milk allergy or a CAI of 6 or higher. Pregnant women were also excluded. All procedures were in accordance with the Helsinki Declaration of 1975, as revised in 1983.

Study probiotic

Bio-Three tablets (Toa Pharmaceutical Co., Ltd., Toyama, Japan), a live microbial preparation, and matching placebo tablets (Toa Pharmaceutical Co., Ltd.) were used as the study preparations. Bio-Three tablets were granted manufacturing approval in 1963. Each tablet contains 2 mg of lactomin (*Streptococcus faecalis* T-110), 10 mg of *Clostridium* (*Clostridium butyricum* TO-A), and 10 mg of *Bacillus* (*Bacillus mesentericus* TO-A), combined with potato starch and lactose. This preparation is effective for resolving various symptoms caused by abnormal intestinal flora and mainly improves bowel movement disorders. Placebo tablets were prepared by substituting equivalent amounts of starch for the probiotic powder. Placebo tablets were identical to Bio-Three tablets and could not be distinguished from the active preparation on the basis of appearance.

Study design and treatment

At the start of the study, 30 outpatients were randomly assigned to the Bio-Three group and 30 to the placebo group by means of a computer-generated scheme. The study protocol was reviewed and approved by the Ethics Committee of Sakura Medical Center, Toho University.

In both the Bio-Three group and the placebo group, patients orally received 3 tablets 3 times daily. In principle, the duration of treatment was 12 mo. Fecal samples were collected immediately before and 3, 6, 9, and 12 mo after the start of treatment. Fecal samples for measurement of organic acids were preserved by freezing, without modification. Fecal samples used for DNA extraction were suspended in GTC solution (100 mmol/L Tris-HCl, pH 9.0; 40 mmol/L Tris-EDTA, pH 8.0; and 4 mol/L guanidine thiocyanate) and were preserved at 4 ℃. As for concomitant medication (therapy), the use of mesalazine and salazosulfapyridine was unrestricted, but steroids could not be used as remission maintenance therapy. The use of drugs with similar effects as the study drug, potentially affecting the evaluation of effectiveness (*i.e.*, other active live microbial preparations, laxatives, *etc.*) was prohibited from 1 wk before study entry to the completion of the study. In principle, the use of oral antibiotics was also prohibited, but the use of topical antibiotics other than oral preparations was not particularly restricted. If a patient received a new treatment in addition to their basic therapy with drugs such as mesalazine or salazosulfapyridine, relapse was diagnosed, and the study treatment and fecal sample collection were discontinued.

Analysis of intestinal microflora

DNA extraction: About 800 μL of the fecal sample suspension preserved at 4 ℃ was transferred to a tube containing zirconia beads (Nippon Gene Co., Ltd., Tokyo, Japan), and the cells were processed with the use of FastPrep FP120A cell disruptor (MP Biomedicals, Irvine, CA). After cooling on ice, the specimen was centrifuged at 5000 rpm for 1 min. DNA was automatically extracted from the processed supernatant with the use of a 12GC and GC series Magtration-MagaZorb DNA Common Kit 200N (Precision System Science, Chiba, Japan). The final concentration of the extracted DNA was adjusted to 10 ng/μL.

Terminal restriction fragment length polymorphism analysis: Terminal restriction fragment length polymorphism (T-RFLP) analysis was performed as described by Nagashima *et al*[9]. The 16S rRNA gene was amplified with the use of primer sets516F (5′-TGCCAGCAGCCGCGGTA-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The 5′-end of the forward primer 516F was labeled with 6′-carboxyfluorescein. Amplified polymerase chain reaction (PCR) products were refined with the use of MultiScreen® PCRμ96 filter plates (Millipore, Tokyo, Japan).

The refined PCR products (about 3 μL) were digested for 3 h at 55 ℃ with 10 U of *BslⅠ* restriction enzyme (New England Biolabs, Inc., Ipswich, MA, United States). The length of the separated fluorescent PCR fragment was determined with an ABI PRISM 3130xl genetic analyzer (Applied Biosystems, Tokyo, Japan), and the data were analyzed with GeneMapper® software. MapMarker® X-Rhodamine Labeled 50-1000bp (BioVentures, Inc., Murfreesboro, TN, United States) was used as a size standard marker.

Cluster analysis: To objectively interpret differences in T-RFLP patterns, NTSYSpc software (Exeter Software, Setauket, NY, United States) was used to perform cluster analysis. Each terminal restriction fragment (T-RF) was expressed as a percentage of the peak area of all T-RFs. Disparity in similarity

Table 1 Baseline characteristics of the study group

	Bio-three (n = 23)	Placebo (n = 23)
Male/female	16/7	12/11
Age (yr, mean ± SD)	44.8 ± 13.8	42.9 ± 15.9
Age of onset (yr)	37.1 ± 14.4	36.0 ± 14.2
Disease duration (yr, mean ± SD)	8.0 ± 6.3	6.7 ± 5.9
Left colon	6	9
Proctosigmoiditis	6	5
Total/subtotal	11	9
Concomitant drug		
Pentasa	11	13
Salazopyrin	10	9
Pentasa + salazopyrin	1	0
Nothing	1	1

among fecal samples in individual patients was calculated using a correlation matrix and was presented graphically on tree diagrams with the use of a weighted pair-group method with arithmetic mean (WPGMA) clustering[10].

High-performance liquid chromatography analysis of fecal organic acids: Organic acid concentrations in fecal samples were measured as follows. About 0.1 g of fecal sample was measured and combined with trans-crotonic acid as an internal standard substance, and extraction was performed twice with 0.6 mL of 0.25% ammonia solution. After adding a 0.3-fold dilution of 10% (w/v) perchloric acid, deproteinization was performed by centrifugation. The solution was filtered and analyzed with a post-column high-performance liquid chromatography (HPLC) system (Waters, Milford MA, United States). Organic acids in the sample were separated with an ion exchange column (organic acid column, 7.8 mm i.d. × 300 mm long; Waters). The reaction temperature in the column and post-column was 60 ℃. The mobile phase was 0.08% perchloric acid delivered at a flow rate of 0.8 mL/min. The solution eluted from the column was allowed to react with BTB solution (0.2 mmol/L bromothymol blue, 5.2 mmol/L sodium hydroxide, and 15 mmol/L disodium hydrogen phosphate), delivered at a flow rate of 0.8 mL/min. The absorbance was quantitatively measured at 445 nm using an ultraviolet visible spectrophotometer (2487 Dual λ UV/Vis Detector; Waters).

Statistical analysis

Clinical data were statistically analyzed with SAS software (version 8.2, SAS Institute Inc., Cary, NC, United States). For patients with UC in remission, the Kaplan-Meier method was used to compare the cumulative non-relapse rate over the course of 12 mo between the Bio-Three group and placebo group. The statistical significance of differences between groups was evaluated with the log-rank test and the generalized Wilcoxon test, and 95% confidence intervals were calculated.

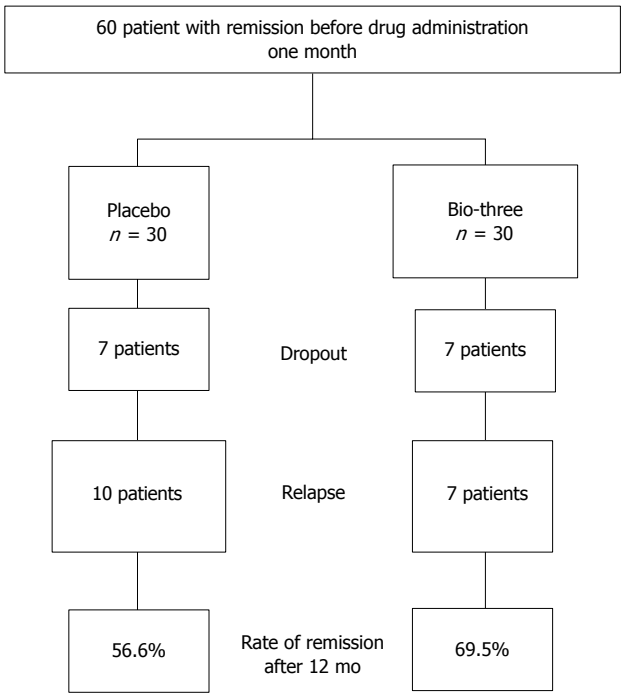


Figure 1 Clinical outcomes of patients according to treatment received.

RESULTS

Patient characteristics

At the start of the study, 30 patients each were randomly assigned to the Bio-Three group and the placebo group. After randomization, the baseline characteristics of sex, age, age at disease onset, disease duration, disease extent, and concomitant treatment did not differ between the groups (Table 1). Among the enrolled subjects, 7 patients in each group were excluded because they met the exclusion criteria specified in the protocol, such as the use of prohibited drugs or refusal to participate in the study. Treatment was actually begun in 23 patients in the Bio-Three group and 23 in the placebo group (Figure 1).

Clinical results

After the study began, the number of patients who had relapse was 2 at 6 mo, 5 at 9 mo, and 7 at 12 mo among the 23 patients in the Bio-Three group and 4 at 3 mo, 6 at 6 mo, 8 at 9 mo, and 10 at 12 mo among the 23 patients in the placebo group. Kaplan-Meier curves were plotted, and relapse rates at each time point were compared between the groups with the use of the χ^2 test (two-tailed, α = 0.05). The P value was 0.0363 at 3 mo, 0.1197 at 6 mo, and 0.3259 at 9 mo. The cumulative remission maintenance rate at 12 mo was 56.6% (n = 12) in the placebo group and 69.5% (n = 16) in the Bio-Three group (Figure 2). When remission maintenance rates were compared between the treatment groups with the use of the log-rank test, the P value was 0.302, with a hazard ratio of 0.607 (95%CI: 0.23-1.59). On the generalized Wilcoxon test, the P value was 0.248. During our study, no adverse

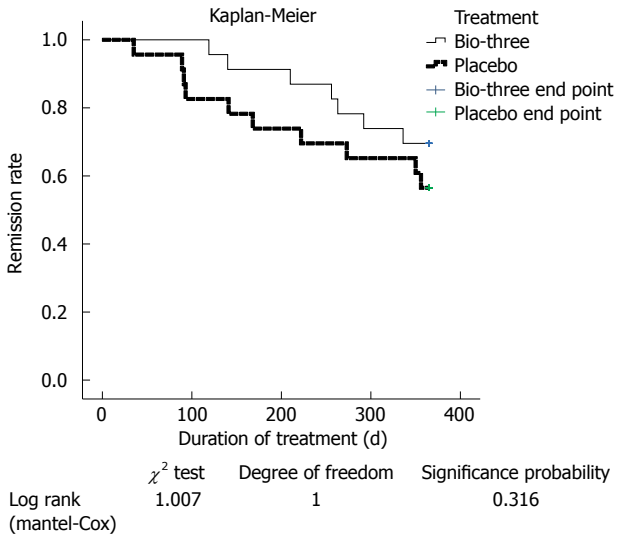


Figure 2 Cumulative remission maintenance rate in the Bio-Three group and placebo group.

changes were observed in the Bio-Three group, as compared with the placebo group, indicating that there is no problem with the safety of Bio-Three.

Bacteriological analysis

In our previous study evaluating the effectiveness of Bio-Three for inducing remission in patients with active UC[8], a cluster analysis was performed to evaluate fluctuations in fecal flora, using the T-RF data derived by digestion of the PCR products with *BslⅠ* restriction enzyme, and several findings were obtained. In the present study of the effectiveness of Bio-Three for remission maintenance, the PCR products were similarly digested with *BslⅠ* restriction enzyme, and cluster analysis of the T-RF data showed that the fecal flora can be divided into 3 clusters, consistent with the results of our previous study. To confirm whether the 3 clusters in the present study correspond to the 3 clusters in our previous study, the fecal sample data used in our previous study of remission induction were linked to the fecal sample data in the present study, and another cluster analysis was performed. The results showed that each of the fecal samples from our previous study belonged to the same clusters as those in our previous analysis. Therefore, for convenience the same names of the clusters in our previous study were used in the present study, *i.e.*, the cluster in the upper part of the figure was named cluster Ⅱ, the cluster in the middle part was named cluster Ⅲ, and the other cluster was named cluster Ⅰ (Figure 3, Table 2).

A total of 138 fecal samples belonged to cluster Ⅱ. The clinical outcomes of the 32 subjects who belonged to cluster Ⅱ at the start of treatment were remission maintained in 11 of the 16 patients in the Bio-Three group and 10 of the 16 patients in the placebo group. This difference was not significant. As a characteristic of T-RF in cluster Ⅱ, OTU124 accounted for a significantly higher proportion of total T-RF peak area in cluster Ⅱ than in the other clusters.

A total of 28 fecal samples belonged to cluster Ⅲ. The clinical outcomes of the 7 subjects who belonged to cluster Ⅲ at the start of treatment were remission maintained in 2 of the 4 patients in the Bio-Three group and 2 of the 3 patients in the placebo group. The difference between the groups was not significant.

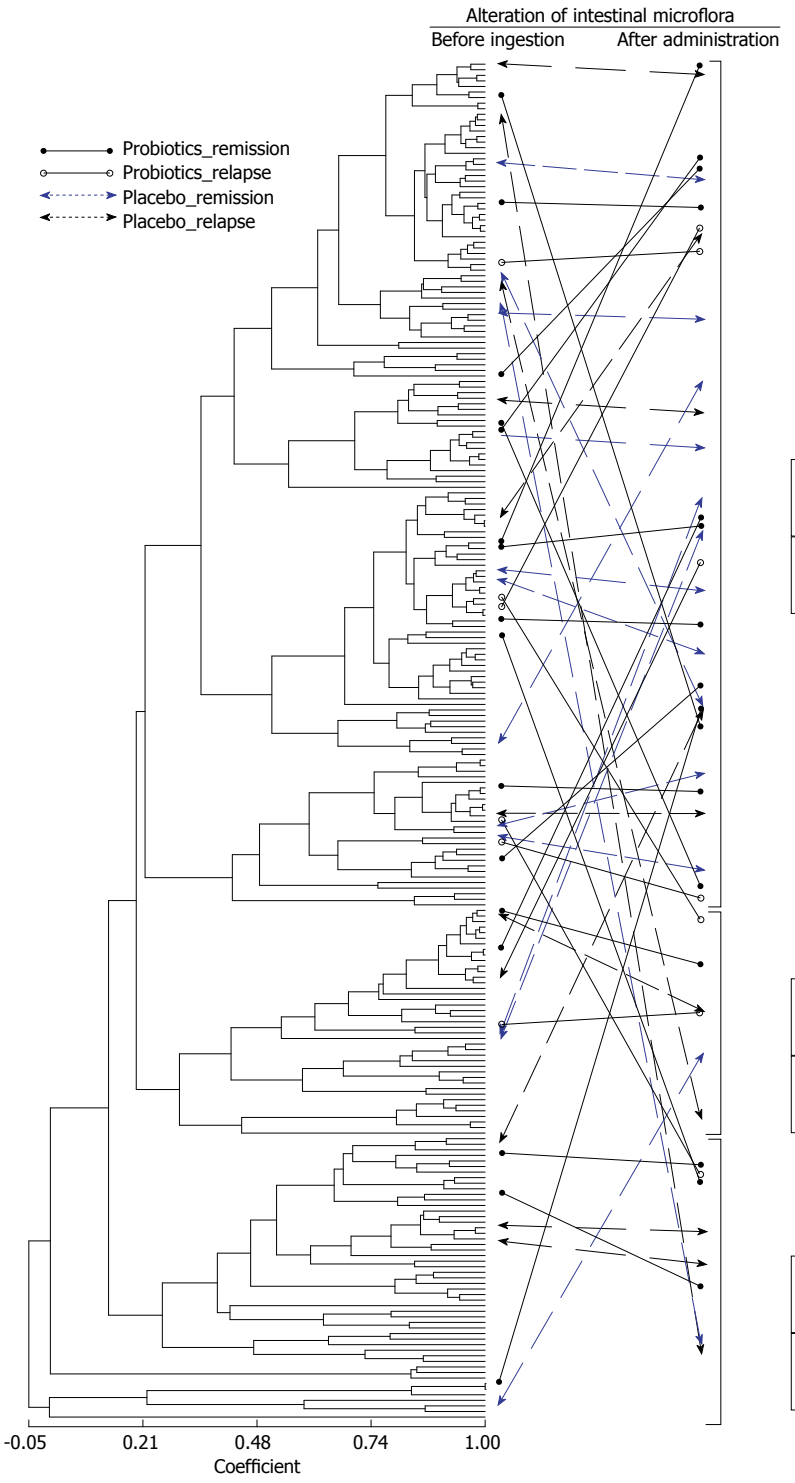
A total of 39 fecal samples belonged to cluster Ⅰ. The clinical outcomes in the 7 subjects who belonged to cluster Ⅰ at the start of treatment were remission maintained in all 3 patients in the Bio-Three group, as compared with only 1 of the 4 patients in the placebo group. Among the 39 fecal samples that belonged to cluster Ⅰ, 19 fecal samples were derived from patients in the Bio-Three group, and 17 of these samples were from patients who had a final evaluation of remission maintained. The other 20 fecal samples were from patients in the placebo group, and 8 of these samples were from patients who had a final evaluation of remission maintained. When these data were compared with the use of Pearson's χ^2 test, the *P* value was 0.0013, indicating that the rate of remission maintenance was significantly higher in the Bio-Three group than in the placebo group, and the relapse rate was significantly lower in the Bio-Three group than in the placebo group.

HPLC analysis of fecal organic acids

The fecal concentrations of short-chain fatty acids did not differ significantly between the Bio-Three group and the placebo group at any time during treatment. On comparison of the clusters derived by T-RFLP analysis, the ratio of the concentration (mmol/L) of butyrate to that of acetate (Bu/Ac ratio) was significantly higher in cluster Ⅰ than in clusters Ⅱ and Ⅲ. When fecal organic acids were compared according to clinical outcomes (*i.e.*, between fecal samples obtained at each of the specified times from patients in whom remission was maintained for 1 year and fecal samples from patients who had relapse within 6 mo after fecal collection), the concentrations of butyric acid and other short-chain fatty acids did not differ significantly between the groups. However, the Bu/Ac ratio at each of the sampling times was significantly higher in fecal samples obtained from patients who had relapse within 6 mo after fecal collection than in those obtained from patients who remained in remission (Table 3).

DISCUSSION

T-RFLP is a molecular technique that allows the diversity and colony structure of microbial complexes to be promptly compared and the diversity of ecosystems to be evaluated[11,12]. Recently, several studies have performed T-RFLP in patients with UC.



Cluster Ⅱ	No. of patients (at the start)	Clinical effectiveness (after treatment)
Probiotics	16	Remission: 11 Relapse: 5
Placebo	16	Remission: 10 Relapse: 6

Cluster Ⅲ	No. of patients (at the start)	Clinical effectiveness (after treatment)
Probiotics	4	Remission: 2 Relapse: 2
Placebo	3	Remission: 2 Relapse: 1

Cluster Ⅰ	No. of patients (at the start)	Clinical effectiveness (after treatment)
Probiotics	3	Remission: 3 Relapse: 0
Placebo	4	Remission: 1 Relapse: 3

Figure 3 Alteration of intestinal microflora after treatment in the Bio-Three group and placebo group. The dendrogram indicates the similarity of individual intestinal microflora T-RFLP patterns of fecal samples obtained after 0, 3, 6, 9, and 12 mo of treatment. Solid line and circles, Bio-Three group and remission; double line and open circles, Bio-Three group and relapse; solid dotted line and arrows, placebo group and remission; double dotted line and arrows, placebo group and relapse. The first and last samples from the same individuals are connected with lines. The tables indicate clinical effectiveness according to the cluster that the subjects belonged to at the start of this study.

However, few studies have examined time-series samples obtained from the same subjects. Most previous investigations have evaluated fecal samples obtained at a specific time[13-17].

We previously studied the effectiveness of probiotic therapy for inducing remission in patients with flare-ups of UC. On T-RFLP cluster analysis, fecal flora could be divided into 3 clusters, designated as clusters Ⅰ, Ⅱ, and Ⅲ. The flora of patients whose UC disease activity index (UCDAI) was improved by probiotic therapy was centered around cluster Ⅱ. Cluster Ⅱ flora was characterized by a high ratio of OTU124[8].

Table 2 Comparison of characteristics of fecal samples (according to cluster)

Group (sample number)	OTU124/all T-RFs (%)	Butyrate/acetate ratio (mol)
Cluster Ⅰ	2.8 ± 3.6	0.254 ± 0.172
Cluster Ⅱ (n = 138)	27.6 ± 12.0	0.184 ± 0.147
Cluster Ⅲ (n = 28)	18.0 ± 10.8	0.075 ± 0.126

Data are expressed as mean ± SD. Because equal variances were rejected for each variable, multiple comparisons were performed with Dunnett's T3 test. Different letters indicate the presence of a significant difference (P < 0.05). T-RF: Terminal restriction fragment.

Table 3 Comparison of characteristics of fecal samples (according to clinical outcomes)

Group (sample number)	OTU124/all T-RFs (%)	Butyrate/acetate ratio (mol)
Remission (before treatment) (n = 29)	22.7 ± 13.8	0.165 ± 0.143
Remission (months 3 and 6) (n = 58)	22.0 ± 14.6	0.145 ± 0.136
Remission (months 9 and 12) (n = 56)	21.7 ± 14.1	0.160 ± 0.146
Relapse (within 6 mo) (n = 28)	20.7 ± 14.7	0.260 ± 0.185

Data are expressed as mean ± SD. Because each variable had equal variances, multiple comparisons were performed with Tukey's honestly significant difference test. Different letters indicate the presence of a significant difference (P < 0.05). "Relapse (within 6 mo)" means that fecal samples were obtained within 6 mo before relapse (*i.e.*, relapse occurred within 6 mo after collection of fecal samples).

Nagashima *et al*[9], who proposed the T-RFLP technique used in our study, reported that OTU124 is derived from *Bifidobacteria*. Many studies have examined the relation between *Bifidobacteria* and UC, and most have reported that *Bifidobacteria* mitigates inflammation associated with UC[18-21]. On the basis of these findings, we previously concluded that cluster Ⅱ flora is "healthy intestinal flora (appropriate intestinal flora)" in patients with UC. Probiotic therapy may therefore be less effective for improving flora in patients who initially have cluster Ⅱ flora, leading only to marginal improvement clinically[8].

The results of the present study similarly suggest that cluster Ⅱ represents "healthy intestinal flora". Consequently, probiotic therapy is expected to be of the greatest potential benefit for intestinal flora that is most dissimilar to cluster Ⅱ. This speculation is supported by the following two points. First, in our previous study of patients with "flare-ups" of UC, only 6 (30%) of the 20 subjects had intestinal flora belonging to cluster Ⅱ before treatment[8]. In the present study of patients with UC "after induction of remission", 32 (70%) of the 46 subjects had intestinal flora belonging to cluster Ⅱ. Given that the proportion of patients with "appropriate intestinal flora" is expected to be higher among patients with remission induction and remission maintenance than among those with relapse, the fact that the majority of patients in the present study, who were already in remission, belonged to cluster Ⅱ supports our speculation that probiotic therapy is potentially most beneficial for patients with intestinal flora most dissimilar to cluster Ⅱ. Second, among the 32 subjects who belonged to cluster Ⅱ at the start of treatment in the present study, remission was maintained in 11 of the 16 patients in the Bio-Three group and 10 of the 16 patients in the placebo group. There was no difference in the remission maintenance rate between these groups. In contrast, among the 7 subjects who belonged to cluster Ⅰ (*i.e.*, the cluster farthest from cluster Ⅱ) before treatment, remission was maintained in all 3 patients in the Bio-Three group, whereas relapse occurred in 3 of the 4 patients in the placebo group. These findings indicate that probiotic therapy was less effective in patients who initially belonged to cluster Ⅱ ("appropriate intestinal flora"), with no difference from placebo. In contrast, probiotic therapy was potentially most beneficial for patients with intestinal flora belonging to cluster Ⅰ flora before treatment, the cluster that is farthest from cluster Ⅱ. Consequently, probiotic therapy was more effective than placebo for maintaining remission in this subgroup of patients. These results were very interesting and are consistent with the findings of our previous study[8].

The fecal Bu/Ac ratio differed between patients with relapse and those in whom remission was maintained for 12 mo and was significantly higher within 6 mo before relapse than at other times (Table 3). Interestingly, the Bu/Ac ratio tended to be higher in feces belonging to cluster Ⅰ than in the other clusters (Table 2). These findings may be attributed to the following mechanism. Butyrate serves as an energy source for intestinal epithelial cells and is known to induce apoptosis of colorectal cancer cells and the differentiation of intestinal epithelial cells. In addition, butyrate has been shown to inhibit the activation of nuclear factor kappa B (NF-κB) and to have anti-inflammatory properties[11,22,23]. On the other hand, the utilization efficiency of butyrate has been reported to be low in the colonic mucosa of patients with refractory UC. The anti-inflammatory activity of butyrate has prompted several studies of its effectiveness and mechanism of action in patients with UC[24-28].

In our previous study evaluating the effectiveness of Bio-Three for inducing remission in patients with UC, the decrease in the UCDAI after treatment (*i.e.*, the improvement in symptoms of UC) correlated with the decrease in the fecal butyrate concentration[8]. On the basis of this finding, we performed breath tests after administration of [1-^{13}C]-butyrate enemas in 10 patients with active UC and 12 with quiescent UC and confirmed that the utilization efficiency of butyrate was decreased in patients with high inflammatory activity[29-33]. These findings suggest that an increased Bu/Ac ratio resulting from decreased absorption of butyrate, an indicator of anti-inflammatory activity,

and an increase in fecal butyrate concentrations is associated with a higher risk of relapse in patients with UC. The mean rate of remission maintenance at 12 mo in patients who receive mesalazine preparations alone is estimated to be about 61% (range: 45%-71%) on the basis of the results of previous randomized controlled trials[34-41]. This rate is similar to the relapse rate among patients who received mesalazine preparations alone (56.6%) in the placebo group of our study.

We used the Kaplan-Meier method to compare relapse rates between the treatment groups. The relapse rate after 12 mo did not differ significantly between the Bio-Three group and the placebo group on either the log-rank test or generalized Wilcoxon test. However, detailed analysis showed that clinical effectiveness differed between the Bio-Three group and placebo group among patients who belonged to cluster Ⅰ. As mentioned above, probiotic therapy is most likely to be effective in patients with intestinal flora belonging to cluster Ⅰ, which is characterized by both a low ratio of OTU124 (which tended to be high in patients belonging to cluster Ⅱ, classified as "healthy intestinal flora") and a high fecal Bu/AC ratio (which was significantly higher in patients within 6 mo before relapse than in patients without relapse).

Combining probiotics or synbiotics with conventional drugs has been recommended as a safe and effective treatment for patients with active UC. For more than 10 years considerable attention has focused on the effectiveness of probiotic therapy for UC[42]. Nearly all studies have reported that probiotics such as VSL#3[43-45], BIFICO[46], and *E. coli* Nissle 1917[47] and prebiotics such as GBF[48-50] and BGS[51] are useful for maintaining remission maintenance and preventing relapse in patients with UC. In comprehensive Cochrane reviews of clinical studies evaluating the effectiveness of probiotics for UC, Mallon *et al*[52] and Naidoo *et al*[53] concluded that although probiotics are ineffective for the induction of remission, probiotics combined with conventional therapy are expected to be effective for maintenance of remission. A meta-analysis conducted by Sang *et al*[54]. reported that probiotics are slightly but not significantly effective for remission induction, but significantly contribute to remission maintenance.

The results of our study suggest that cluster analysis of patients' intestinal flora before treatment can contribute to the effective use of probiotic therapy. To our knowledge, our study represents an unprecedented attempt to define factors related to the effectiveness of Bio-Three for the prevention of relapse in patients with inactive UC. Not only the fecal flora, but also the fecal concentration of short-chain fatty acids differed between patients who had relapse within 1 year and those in whom remission was maintained for 1 year. This finding suggests that patient profiling on the basis of factors such as the results of cluster analysis of fecal flora and the fecal short-chain fatty acid concentration might facilitate prediction of the response to treatment and future clinical status in patients with UC.

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COMMENTS

Background

Ulcerative colitis (UC) is a chronic, idiopathic, refractory, inflammatory bowel disease (IBD) characterized by inflammatory mucosal injury of the colon, with repeated periods of remission and relapse. Recently, probiotic therapy has been acknowledged to be potentially effective and safe in patients with UC. Probiotics are defined as a live microbial feed nutritional supplement that beneficially affects the host by improving the balance of the intestinal flora.

Research frontiers

Studies of animal models of colitis have suggested that the intestinal flora has an important role in the pathogenesis of colitis. In IBD-sensitive knockout or transgenic mice, colitis develops in the presence of a normal intestinal flora, but not in mice raised in a germ-free environment, strongly suggesting that the intestinal flora participates in the development of colitis. Therefore, probiotic therapy designed to correct the intestinal flora is expected to be useful for preventing colitis.

Innovations and breakthroughs

The results confirmed that probiotic therapy can improve clinical symptoms and endoscopic findings and provided evidence that remission induction is promoted by a certain improvement in the intestinal flora. The authors also reported that probiotic therapy might be effective for maintenance of remission.

Applications

The authors conducted a single-center, randomized, double-blind, placebo-controlled study to examine whether 12 mo of probiotic therapy is useful for preventing relapse of UC in patients who were already in remission.

Peer-review

In this study, the authors designed the randomized double-blind controlled study to evaluate the effectiveness of probiotic therapy for suppressing relapse in patients with UC. They concluded that probiotics may be effective for maintaining clinical remission in patients with quiescent UC, especially those who belong to cluster Ⅰ on fecal bacterial analysis. This paper is well written and the results of the study are clinically interesting.

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ORIGINAL ARTICLE

Randomized Clinical Trial

Efficacy and safety of a patient-positioning device (EZ-FIX) for endoscopic retrograde cholangiopancreatography

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Abstract

AIM: To assess the efficacy and safety of a patient-positioning device (EZ-FIX) for endoscopic retrograde cholangiopancreatography (ERCP).

METHODS: A total of 105 patients were randomized to the EZ-FIX (n = 53) or non-EZ-FIX (n = 52) group in this prospective study. Midazolam and propofol, titrated to provide an adequate level of sedation during therapeutic ERCP, were administered by trained registered nurses under endoscopist supervision. Primary outcome measures were the total dose of propofol and sedative-related complications, including hypoxia and hypotension. Secondary outcome measures were recovery time and sedation satisfaction of the endoscopist, nurses, and patients.

RESULTS: There was no significant difference in the rate of hypoxia, but there was a statistical trend (EX-FIX group; n = 4, 7.55%, control group; n = 6, 11.53%, P = 0.06). The mean total dose of propofol was lower in the EZ-FIX group than in the non-EZ-FIX group (89.43 ± 49.8 mg *vs* 112.4 ± 53.8 mg, P = 0.025). In addition, the EZ-FIX group had a shorter mean recovery time (11.23 ± 4.61 mg *vs* 14.96 ± 5.12 mg, P < 0.001). Sedation satisfaction of the endoscopist and nurses was higher in the EX-FIX group than in the non-EZ-FIX group. Technical success rates of the procedure were 96.23% and 96.15%, respectively (P = 0.856). Procedure-related complications did not differ by group (11.32% *vs* 13.46%, respectively, P = 0.735).

CONCLUSION: Using EZ-FIX reduced the total dose of propofol and the recovery time, and increased the satisfaction of the endoscopist and nurses.

Key words: EZ-FIX; Patient-positioning device; Propofol; Sedation; Endoscopic retrograde cholangiopancrea-

tography

Core tip: Although the incidence of sedation-related complications is low, it is closely associated with endoscopy-related morbidity and mortality. Many studies on the efficacy and safety of various sedative drugs have been conducted, but none used a patient-positioning device. We planned this study to improve the safety and efficacy of sedation during endoscopy by using a patient-positioning device (EZ-FIX).

Lee S, Han JH, Lee HS, Kim KB, Lee I, Cha EJ, Shin YD, Park N, Park SM. Efficacy and safety of a patient-positioning device (EZ-FIX) for endoscopic retrograde cholangiopancreatography. *World J Gastroenterol* 2015; 21(19): 5995-6000 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/5995.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.5995

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is an advanced upper endoscopic procedure, and is useful for the diagnosis and treatment of pancreatobiliary disorders[1]. However, ERCP is an invasive procedure of considerable duration and causes substantial discomfort to patients. Thus, a deeper level of sedation may be necessary to ensure the success and safety of the procedure. Deep sedation during ERCP with careful monitoring minimizes patient movement, allowing the endoscope to be manipulated precisely with little interruption. Although the incidence of sedation-related complications is low, it is closely associated with endoscopy related morbidity and mortality. EZ-FIX is a patient-positioning device that uses polyethylene particles and compressed air that is expected to contribute to sedation safety by fixing the position of the patient. Such fixation avoids the transient failure of maintenance of sedation that can occur during ERCP procedures. Studies on the efficacy and safety of various sedative drugs have been published, but none of these studies used a patient-positioning device. This study was designed to assess the efficacy and safety of the EZ-FIX during ERCP.

MATERIALS AND METHODS

Patients and study design

This prospective, randomized trial included 105 patients who underwent therapeutic ERCP between April 2013 and March 2014, at the Chungbuk National University Hospital. The sample-size calculation was based on a preliminary study. Consecutive patients who provided informed consent and were to undergo ERCP were assigned randomly to the EZ-FIX group (n = 53) or the non-EZ-FIX group (n = 52). Patients were excluded if they were younger than 18 years of age, had an ASA classification of "V", had a history of complications during sedative endoscopy, adverse reactions to propofol or midazolam injection, severe obstructive sleep apnea, pregnancy, or an allergy to eggs or soybeans.

Therapeutic ERCP was performed by one experienced endoscopist who had performed over 1000 therapeutic ERCP procedures during the previous 5 years (Han JH). Trained registered nurses, with experience of over 100 therapeutic ERCP procedures, administered all medications, monitored patients, and prepared medical records under the supervision of the physician performing the ERCP. They were assisted during ERCP by one gastroenterology fellow and two other nurses.

A patient-positioning device (EZ-FIX, Arlico Medical, South Korea) was used in this study. EZ-FIX is filled with tiny polystyrene particles and compressed air and is covered with polyurethane. First, the EZ-FIX is inflated after setting the device on the procedure table in a loose shape. The patient is then placed on the EZ-FIX in the desired posture. Air is removed from the EZ-FIX by vacuum to "copy" and fix the shape of the patient's posture (Figure 1). Sedatives were then administered after placement of monitoring devices.

The level of sedation was defined according to the ASA classifications: minimal, moderate, deep sedation, and general anesthesia[2]. Minimal sedation is a state in which the patient can respond normally to verbal stimulation. With moderate sedation, the patient can respond purposefully to verbal commands, either alone or accompanied by light tactile stimulation. Under deep sedation, the patient cannot be aroused easily, but responds purposefully following repeated or painful stimulation. In this study, the target level of sedation was deep sedation, which is used for advanced endoscopic procedures, including ERCP and endoscopic submucosal dissection.

The initial intravenous bolus dose of midazolam was 0.05 mg/kg; a half dose was used in those over 70 years old and ASA Ⅲ-Ⅳ patients. A 20-mg propofol bolus was also injected when a patient failed to achieve sedation 3 min after the first injection, and 20-mg doses of propofol were injected repeatedly at 1-min intervals until sedation was achieved. Additional midazolam 0.025 mg/kg was administered when the procedure time was predicted to be excessive or the demand for propofol was too frequent. All the subjects gave informed consent, and the study was approved by the Institutional Review Board at the Chungbuk National University (CBNUH201304018002) and is registered at the Clinical Research Information Service (CRIS, KCT000137).

Outcome measures

Primary outcome measures were the total dose of propofol and sedative-related complications, including

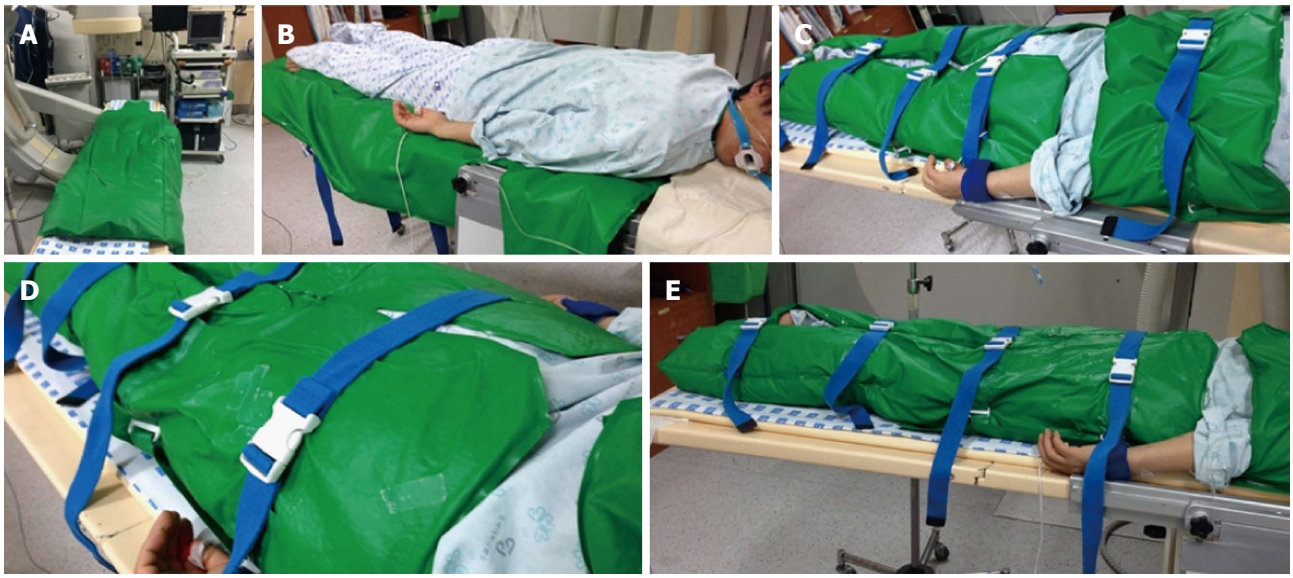

Figure 1 Preparation process for endoscopic retrograde cholangiopancreatography using EZ-FIX. EZ-FIX is filled with tiny polystyrene particles and compressed air. First, EZ-FIX was placed on the procedure table in a loose shape (A); Then, the patient was placed on the EZ-FIX, and the monitoring device was set up (B); All belts on the device were locked, including those for both hands (C); Inflation state of EZ-FIX after sedation (D); Deflation state of EZ-FIX. It fixes the shape of the patient's posture (E).

Table 1 Baseline characteristics of the EZ-FIX and non-EZ-FIX groups *n* (%)

	EZ-FIX (*n* = 53)	Non-EZ-FIX (*n* = 52)	*P* value
Age (yr)	68.0 ± 15.4	67.0 ± 14.0	0.837
Gender (male: female)	55:45	56:44	0.914
Weight (kg)	59.42 ± 10.36	60.79 ± 10.75	0.507
Procedure time (min)	24.66 ± 13.58	27.15 ± 11.73	0.317
ASA classification			0.458
1-2	42 (79.2)	38 (73.1)	
3-4	11 (20.8)	14 (26.9)	

hypoxia and hypotension. Secondary outcome measures were recovery time and sedation satisfaction of the endoscopist, nurses, and patients. Procedure time was counted from the dosing of sedatives until withdrawal of the endoscope. Recovery time was defined from the completion of the procedure until the Aldrete score reached 10 points. The total doses of midazolam and propofol administered during the procedure were measured. The endoscopists and sedation nurses completed a questionnaire, using a 10-cm visual analog scale (VAS), which assessed patient cooperation and overall satisfaction with sedation and the procedure (ranging from 0 = poor, to 10 = excellent). Safety was monitored by measuring systolic and diastolic pressure, heart rate, and oxygen saturation before and after the procedure and at 10-min intervals during the procedure. Procedure-related complications recorded included bleeding, perforation, and pancreatitis.

Statistical analysis

Baseline data from the patients in the two groups were compared using a χ^2 test or Fisher's exact test for categorical variables. The Student's t-test was used for normally distributed continuous variables and a Mann-Whitney *U*-test for non-normally distributed continuous variables. A *P* value of < 0.05 was considered statistically significant. The SPSS software (ver. 13.0; SPSS Inc., Chicago, IL, United States) was used for statistical analysis.

RESULTS

In total, 105 patients were enrolled in this prospective study and were randomized to the EZ-FIX group (*n* = 53) or the non-EZ-FIX group (*n* = 52). The characteristics of both groups are shown in Table 1. There was no significant difference between the two groups in terms of age, gender, body weight, duration of procedure, or ASA score.

Table 2 shows that the total dose of midazolam and the induction time were not significantly different between the groups (2.42 ± 1.88 mg *vs* 2.41 ± 1.91 mg, *P* = 0.976, and 3.37 ± 0.94 min *vs* 3.72 ± 1.02 min, *P* = 0.067, respectively). The mean total dose of propofol was lower in the EZ-FIX group than in the non-EZ-FIX group (89.43 ± 49.8 mg *vs* 112.4 ± 53.8 mg, *P* = 0.025). In addition, the EZ-FIX group had a shorter mean recovery time (11.23 ± 4.61 mg *vs* 14.96 ± 5.12 mg, *P* < 0.001; Figure 2). Based on the VAS, patient satisfaction scores did not differ significantly between the groups. However, the satisfaction scores of both the endoscopist and nurses were significantly higher in the EZ-FIX group than in the non-EZ-FIX group (8.11 ± 0.99 *vs* 6.54 ± 1.40, *P* < 0.001, and 7.81 ± 1.03 *vs* 6.02 ± 1.74, *P* < 0.001, respectively).

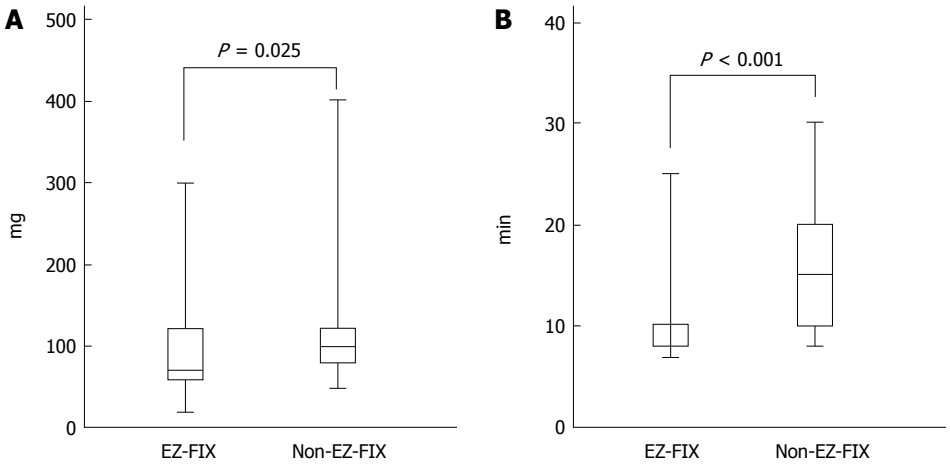

Figure 2 Comparisons of the efficacy profiles between the EZ-FIX and the non-EZ-FIX groups. A: Total dose of propofol administered; B: Recovery time of the patients.

Table 2 Efficacy in the EZ-FIX and non-EZ-FIX groups

	EZ-FIX (*n* = 53)	Non-EZ-FIX (*n* = 52)	*P* value
Total dose of midazolam	2.42 ± 1.88	2.41 ± 1.91	0.976
Total dose of propofol	89.43 ± 49.8	112.4 ± 53.8	0.025
Induction time, min	3.37 ± 0.94	3.72 ± 1.02	0.067
Recovery time, min	11.23 ± 4.61	14.96 ± 5.12	< 0.001
Pt. satisfaction score	7.26 ± 2.25	7.29 ± 2.47	0.722
Dr. satisfaction score	8.11 ± 0.99	6.54 ± 1.40	< 0.001
Nr. satisfaction score	7.81 ± 1.03	6.02 ± 1.74	< 0.001

Table 3 Safety index and complications during and after endoscopic retrograde cholangiopancreatography *n* (%)

	EZ-FIX (*n* = 53)	Non-EZ-FIX (*n* = 52)	*P* value
Below 90% in SaO_2 for ⩾ 30 s	4 (3.8)	6 (5.7)	0.060
↓ 20 mmHg in BP	8 (7.6)	9 (8.6)	0.800
↓ 20% in PR	3 (2.9)	3 (2.9)	1
Death	-	-	-
Procedure-related complications			0.735
Pancreatitis	5	4	
Bleeding	1	2	
Perforation	-	1	

BP: Blood pressure; PR: Pulse rate.

Adverse events are shown in Table 3. There were four cases of hypoxemia in the EZ-FIX group and six cases in the non-EZ-FIX group (*P* = 0.06). Severe hypotension was not observed in either group and no patient required artificial ventilation such as mask bagging and intubation. Procedure-related complications were similar between the two groups (6 *vs* 7, *P* = 0.775).

DISCUSSION

Since its introduction in 1968, endoscopic retrograde cholangiography (ERCP) has become a commonly performed endoscopic procedure[1]. The diagnostic and therapeutic utility of ERCP for a variety of disorders, including the management of biliary and pancreatic neoplasms, and the postoperative management of biliary perioperative complications, has been demonstrated[3-5]. However, ERCP is an uncomfortable and high-risk therapeutic procedure that cannot be performed without adequate sedation or general anesthesia. Patients must be administered agents to induce moderate-to-deep sedation to tolerate the ERCP procedure. The use of propofol for endoscopic sedation has increased markedly in recent years, mainly due to its favorable pharmacokinetic profile compared with "traditional" endoscopy sedation drugs, such as benzodiazepines and opioids[6-10]. When administered intravenously, propofol rapidly crosses the blood-brain barrier and activates aminobutyric acid to induce sedation, amnesia, and sleep. Sedative effects occur within, on average, 30-60 s after Ⅳ administration, and patients recover rapidly due to the short 1.3-1.4-min half-life[11]. However, propofol has no reversal agent; moreover, negative cardiac inotropy, including decreases in cardiac output, systemic vascular resistance, arterial pressure, and respiratory depression can occur[12,13]. Thus, it is recommended that propofol should be administered only by anesthesia specialists[14]. Although a large volume of data shows the safety of endoscopist-directed propofol[15-23], it is important to be aware of sedation-related complications due to the increase in the number of elderly patients with multiple underlying comorbidities and its nature as an advanced endoscopic technique that demands longer procedure times and deeper sedation. In clinical practice, it is common to combine propofol with additional drugs (benzodiazepine/opioid/ketamine), so-called "balanced propofol sedation" (BPS), which allows the propofol dose to be reduced and seems to be associated with less-frequent need for assisted ventilation, although this has not been demonstrated in head-to-head comparisons[23-27]. In an effort to reduce the incidence

of sedation-related complications, we performed this study to evaluate the efficacy and safety of a patient-positioning device.

Respiratory depression is more frequent in ERCP because the procedure is performed in the prone position. Several devices can be used, such as cushions to maintain the airway by lifting the right shoulder, but these are not commercial products. Recently, a transformable patient-positioning device (EZ-FIX, Arlico Medical, South Korea) was designed to make the procedure more efficient. EZ-FIX contains polystyrene balls in a soft and non-slip polyurethane cover, which provides flexibility in the changing postures of patients. Inflation with air and deflation with a vacuum facilitate rapid changes in and fixation of the posture of the patient. Patients are more comfortable in the desired position and maintain a stable posture during the procedure. In addition, EZ-FIX exerts a body-temperature-protecting effect and assists in turning the patient over.

Our study demonstrated that using EZ-FIX reduced both the total dose of propofol and the recovery time. The advantages of propofol sedation are the rapid onset and short recovery time. However, it is difficult to predict the timing when patients who have long procedure times are released from sedation, and this sometimes leads to a dangerous situation. Therefore, propofol is frequently administered before a return to minimal sedation, which can lead to over-sedation. However, these intermittent propofol injections in advance are not necessary with EZ-FIX, due to the immobilization of patients. Thus, the propofol dose can be reduced and the recovery time shortened. The higher satisfaction of the endoscopist and nurses in the EZ-FIX group was thought to be due to inhibition of unpredictable movements of the patient. A significant reduction in the amount of propofol required for deep sedation could theoretically decrease the risk of sedation-related complications. However, in this study, the rate of neither hypotension nor hypoxemia differed significantly between the groups. EZ-FIX may lead to a lower incidence of sedative-related complications in a larger-scale study. The rate of procedure-related complications did not differ between the groups, possibly due more to the type of procedure performed and underlying diseases than sedation methods.

This study had several limitations in that the sample size was small, and patients with ASA V, severe sleep apnea, and pulmonary disease were excluded. A double-blind design was not possible in that the endoscopist and nurses could see the device, which might have caused bias in the satisfaction scores. Further studies in patients with various characteristics are needed to further evaluate the efficacy and safety of EZ-FIX.

In conclusion, the results of this study showed that use of the EZ-FIX provided increased satisfaction for the endoscopist and nurses, reduced the total dose of propofol, and decreased recovery time. Thus, we suggest that the EZ-FIX may be valuable in advanced endoscopic procedures, including ERCP.

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COMMENTS

Background

Advanced endoscopic procedures such as endoscopic retrograde cholangiopancreatography (ERCP) are long, and patients require deep consistent sedation. The use of propofol for endoscopic sedation has increased due to its useful pharmacokinetic profile. However, no reversal agent is available and cardiovascular and respiratory complications can result.

Research frontiers

Propofol has been combined with other drugs to decrease sedation-related complications; this also allows the propofol dose to be reduced. However, studies on relevant patient-positioning devices have not yet appeared.

Innovations and breakthroughs

This is the first study to evaluate the efficacy and safety of a patient-positioning device (EZ-FIX) for ERCP. EZ-FIX increased the satisfaction levels of both the endoscopist and nurses, reduced the total dose of propofol required, and decreased recovery time. It is important to consider sedative drugs and external devices in combination to improve the efficacy and safety of sedation.

Applications

This study suggests that the EZ-FIX patient-positioning device could be valuable in advanced endoscopic procedures, such as ERCP, endoscopic submucosal dissection, and peroral endoscopic myotomy.

Terminology

EZ-FIX is a patient-positioning device (Arlico Medical, South Korea) that is filled with tiny polystyrene particles and compressed air and is covered with polyurethane.

Peer-review

This study affords useful information and suggests that a patient-positioning device could be valuable during ERCP. The authors show that this device (EZ-FIX) could reduced the total dose of propofol required, and decreased recovery time. EZ-FIX might be a valuable aid during advanced endoscopic procedures.

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ORIGINAL ARTICLE

Prospective Study

Assessment of disease specific knowledge and health-related quality of life among United States military veterans with inflammatory bowel disease

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

AIM: To evaluate the association between patient disease knowledge of inflammatory bowel disease (IBD) and health related quality of life (HRQoL) and identify patient and disease related predictors of patient knowledge of IBD.

METHODS: We performed a cross-sectional study of IBD patients with an established diagnosis of IBD longer than 3 mo prior to enrollment. The Crohn's and colitis knowledge score (CCKNOW) and short inflammatory bowel disease questionnaire (SIBDQ) were self-administered to assess patient knowledge of IBD and HRQoL, respectively. Demographic and disease characteristics were abstracted from the electronic medical record. The correlation between CCKNOW and SIBDQ scores was assessed by a linear regression model. Associations of patient knowledge and the variables of interest were calculated using ANOVA.

RESULTS: A total of 101 patients were recruited. Caucasian race, younger age at diagnosis, and having

a college or post-graduate degree were significantly associated with higher CCKNOW scores. Patients with CD had higher CCKNOW scores compared to patients with ulcerative colitis and inflammatory bowel disease type unclassified, $P < 0.01$. There was no significant correlation between overall CCKNOW and SIBDQ scores ($r^2 = 0.34$, $P = 0.13$). The knowledge sub-domain of diet in CCKNOW was negatively correlated with HRQoL ($r^2 = 0.69$, $P < 0.01$).

CONCLUSION: IBD diagnosis at a younger age in addition to Caucasian race and higher education were significantly associated with higher knowledge about IBD. However, patient knowledge of IBD was not correlated with HRQoL. Further studies are required to study the effect of patient knowledge of IBD on other clinical outcomes.

Key words: Crohn's disease; Ulcerative colitis; Crohn's and Colitis Knowledge Score; Short inflammatory bowel disease questionnaire; Health related quality of life

Core tip: No prior study on inflammatory bowel disease (IBD) has attempted to determine if there is a correlation between a patient's knowledge about his/her disease and their health related quality of life. Furthermore, no such study attempting to quantify a patient's knowledge of their IBD has been performed in the United States. While we found no statistically significant association, we did find several predictors of a patient's knowledge about their disease as well as disparities in knowledge. Through this study, we hope to bring to light these predictors and disparities in hopes of providing targeted opportunities for patient directed education tools.

Hou JK, Turkeltaub JA, McCarty III TR, El-Serag HB. Assessment of disease specific knowledge and health-related quality of life among United States military veterans with inflammatory bowel disease. *World J Gastroenterol* 2015; 21(19): 6001-6007 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6001.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6001

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the gastrointestinal tract resulting in a marked decrease in health related quality of life (HRQoL)[1-4]. Despite advances in pharmacologic and surgical treatment strategies, patient understanding and knowledge of their disease varies widely[5]. It would appear that these advances have yet to translate into a meaningful improvement in patients' understanding of their disease[6]. Poor knowledge and understanding about IBD may impair a patient's ability to be an active participant in his/her own management. In one study, only 14% of ulcerative colitis (UC) patients in a tertiary care hospital were aware UC was associated with an increased risk of colorectal cancer (CRC)[7]. A working knowledge of their disease and its management is essential for patients with chronic disorders such as IBD[8]. Among patients with type 1 diabetes mellitus, higher levels of patient knowledge were associated with higher medication compliance and lower glycosylated hemoglobin levels[9,10].

Although a validated patient knowledge score for IBD has been developed (the Crohn's and colitis knowledge score (CCKNOW), IBD patient knowledge has not been assessed in the United States patient population, nor has it been studied in relationship to HRQoL. Prior studies in the United Kingdom have demonstrated considerable gaps in patient knowledge specifically in medication options and IBD related complications[8]. Demographic and disease-related factors may potentially be used to identify patients at risk for non-adherence, though published studies have reported conflicting data[11,12]. Additionally, disease related knowledge of IBD among patients may affect adherence to medications or coping skills, and hence affect HRQoL.

We hypothesized that IBD-specific knowledge is associated HRQoL. The primary aim of this study was to identify associated patient and disease related factors associated with IBD knowledge. Secondary aims of this study were to quantify disease related knowledge among United States military veterans with IBD receiving care from the veterans affairs (VA) health system and to assess the association between knowledge and HRQoL. Identification of deficits by knowledge domain may provide opportunities for focused interventional patient education, and provide a baseline measurement against which future programs can be measured.

MATERIALS AND METHODS

Study population

Patients were recruited from the IBD clinic at the Michael E. DeBakey VA Medical Center in Houston, TX. Inclusion criteria: (1) diagnosis of IBD ascertained by a gastroenterologist based on clinical, endoscopic, and radiographic data[13]; (2) a diagnosis of IBD greater than 3 mo from enrollment; and (3) at least one prior outpatient clinic visit with a gastroenterologist. Patients who declined consent were excluded. Patients were recruited in a consecutive fashion and were asked to complete two self-assessed questionnaires at the time of their clinic visit.

Data collection

The CCKNOW and short inflammatory bowel disease questionnaire (SIBDQ) were prospectively completed at the time of clinic encounter. Another

Table 1 Demographic features and their association with patient knowledge (Crohn's and Colitis knowledge score)

		CCKNOW score, mean ± SD	*P* value
Total	101%	11.5 ± 5.2	
Gender			0.06
Female	9%	14.8 ± 3.2	
Male	91%	11.2 ± 5.2	
Race			0.02
Caucasian	33.70%	12.5 ± 5.2	
Non-caucasian	66.30%	9.6 ± 4.7	
Age at diagnosis (yr)			< 0.01
< 17	1%	14 ± 0.0	
17-40	56.4%	13.1 ± 4.8	
> 40	42.6%	9.2 ± 7.8	
Level of education			0.07
No college	65%	10.7 ± 4.1	
College/ post-graduate	35%	13.5 ± 5.4	

CCKNOW: Crohn's and Colitis knowledge score.

published IBD questionnaire, the Jones Knowledge Questionnaire, has limited evidence to support its use whereas the CCNOW has been embraced internationally[14]. Therefore the Jones Knowledge Questionnaire was not administered. The CCKNOW is a 30-item questionnaire that quantifies the disease-related knowledge of patients with IBD based on four domains: general knowledge, medication, diet, and complications of IBD. CCKNOW has been shown to be readable and reliable[8]. Permission to use the SIBDQ was obtained from McMaster University. The SIBDQ uses 10 questions derived from the original 32 item full Inflammatory Bowel Disease Questionnaire to subjectively assess the HRQoL in patients with IBD[15,16]. The SIBDQ examines four domains: bowel symptoms, systemic symptoms, emotional function, and social function. Each question is scored from 0 to 7 with a total score ranging from 10 (worst health) to 70 (best health). The total score is then divided by 10.

Medical chart review was performed by two of the investigators (JT and TM) using a standardized data collection form. Data were collected for care documented in the VA clinical encounter when the patient was enrolled. IBD diagnosis was confirmed based on endoscopic, histologic or radiologic findings consistent with standard clinical criteria[13]. Demographic data (race and ethnicity, gender, education), disease characteristics (age at diagnosis, IBD type, disease location, extra-intestinal manifestations), surgical history, and IBD related hospitalizations were abstracted. Education was classified as no high school, high school graduate, college degree, and post-graduate degree. Ethnicity was based on self-reported classification as Caucasian, Hispanic, African-American, Asian, other, and unknown. IBD age of diagnosis, location, and behavior were described according to the Montreal classification[17]. Bowel resection was defined as any small bowel or colonic resection, excluding perianal surgery. Perianal surgery included fistulotomy, abscess drainage, and seton placement. Extraintestinal manifestations (EIM) were defined as involvement of skin (pyoderma gangrenous or erythema nodosum), eye (uveitis, iritis, or episcleritis), or joint (inflammatory arthritis) and primary sclerosing cholangitis (PSC). Physician global assessment (PGA) was specifically noted in progress notes as quiescent, mild, moderate or severe.

Statistical analysis

The correlation between CCKNOW and SIBDQ scores was assessed by a linear regression model, and expressed as the Pearson correlation coefficient. Categorical variables were defined as age of diagnosis (< 17 years old, 17-40 years old, > 40 years old); current age (< 65 years old, ⩾ 65 years old); education (college degree *vs* no college degree); race (Caucasian *vs* non-Caucasian); previous number of surgeries (0 or ⩾ 1); disease duration (< 5, ⩾ 5 years); and disease activity (remission *vs* active disease by PGA). The association of patient knowledge and the variables of interest were calculated ANOVA. Statistical analysis was performed using Stata version 11 software.

RESULTS

A total of 101 IBD patients were recruited consisting of 49 patients with UC (48.5%), 43 patients with Crohn's disease (CD) (42.6%), and 9 patients with inflammatory bowel disease type unclassified (IBDU) (8.9%) (Table 1). The patients were 91% male, 66% Caucasian, and 34% non-Caucasian (27% African-American, 5% Hispanic, and 2% Asian). The mean age at time of IBD diagnosis was 39.6 years [standard deviation (SD) 14.6], and the mean age at time of enrollment was 51.5 years (SD 13.3) (Table 2). The average duration of disease of these patients was 11.8 years (SD 11.5). Of the 52 patients who provided their level of education, 35% had a college or post-graduate degree. The average CCKNOW score was 11.5 (SD 5.2), representing correct answers to 38% of questions.

Association of patient knowledge and HRQoL

There was no significant correlation between overall CCKNOW and SIBDQ scores (r^2 = 0.34, P = 0.13) (Figure 1). On analysis of CCKNOW sub-domains, higher diet knowledge scores were moderately associated with lower HRQoL (r^2 = 0.69, P < 0.01). There were no associations between the general knowledge, treatment, and complication sub-domains with SIBDQ scores (r^2 = 0.49, P = 0.25; r^2 = 0.41, P = 0.67; r^2 = 0.44, P = 0.59, respectively).

Patient factors associated with patient knowledge

Caucasian patients had higher CCKNOW overall scores as well as general knowledge sub-domain CCKNOW

Table 2 Inflammatory bowel disease characteristics and their association with patient knowledge (Crohn's and Colitis knowledge score)

Characteristics		CCKNOW Score, mean ± SD	*P* value
Disease duration (yr)			0.02
< 5	37.6%	9.9 ± 5.2	
⩾ 5	62.4%	12.5 ± 4.9	
Type of IBD			< 0.01
UC	48.5%	10.2 ± 4.7	
CD	42.6%	13.6 ± 5.0	
IBDU	8.9%	8.8 ± 5.9	
UC location			0.53
Proctitis	18.4%	9.5 ± 2.9	
Left colon	22.4%	9.8 ± 5.5	
Pancolitis	53.1%	11.1 ± 4.9	
NA	6.1%	7 ± 2.7	
CD location			0.92
Proximal	0	-	
Ileal	21%	13.1 ± 2.6	
Ileocolonic	58%	13.9 ± 5.2	
Colonic	21%	13.4 ± 6.6	
Family history of IBD			0.69
Yes	14.9%	12.6 ± 5.9	
No	80.1%	11.3 ± 5.1	
Unknown/adopted	5%	10.7 ± 5.8	
Tobacco			0.14
Never	39.6%	12.6 ± 4.9	
Current	18.8%	12.1 ± 5.8	
Quit	41.6%	10.2 ± 4.9	
IBD-related Hospitalizations			0.23
0	62.4%	11 ± 5.2	
⩾ 1	37.6%	12.4 ± 5.1	
Previous IBD-related surgeries			0.02
0	65.3%	10.5 ± 5.0	
⩾ 1	34.7%	13.3 ± 5.1	

CCKNOW: Crohn's and Colitis knowledge score; SD: Standard deviation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; IBDU: Inflammatory bowel disease unclassified.

scores (7.75 ± 3.20) compared to non-Caucasians (6.00 ± 2.98), P = 0.03 (Table 1). While Caucasian patients had numerically higher CCKNOW scores in diet, treatment, and complication domains compared to non-Caucasians, these were not statistically significant (Table 3).

Higher levels of education were numerically but not statistically significantly associated with overall higher CCKNOW scores, P = 0.22. Patients with a college or post-graduate degree had a higher diet subdomain CCKNOW scores (1.16 ± 0.38) compared to patients with no college degree (0.82 ± 0.39) P < 0.01. While patients with a college or post-graduate degree had higher numerical CCKNOW scores in general knowledge, treatment, and complications domains, these were not statistically significant.

Younger age of diagnosis was associated with higher CCKNOW scores. This association was seen across all knowledge sub-domains [general knowledge (P < 0.01), treatment (P = 0.02) and complications (P = 0.03)], except for diet (P = 0.12). Longer disease duration was also associated with higher CCKNOW scores (9.91 *vs* 12.54, for < 5 and ⩾ 5 years, respectively, P = 0.02). Neither family history nor tobacco use was associated with CCKNOW scores.

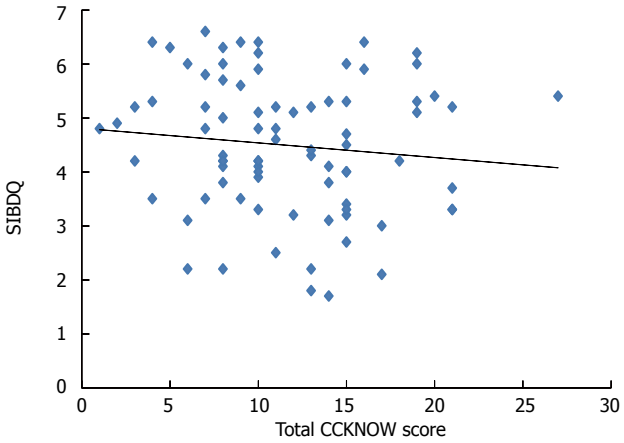

Figure 1 Correlation of quantified patient knowledge (Crohn's and Colitis knowledge score) and health related quality of life (short inflammatory bowel disease questionnaire).

IBD factors associated with patient knowledge

CD patients had higher CCKNOW scores (13.58; SD 4.95) compared to patients with UC (10.24 ± 4.65) and IBDU (8.75 ± 5.90), P < 0.01. Patients with a history of IBD related surgeries also had a higher CCKNOW (13.33 ± 5.09) compared to those without surgery (10.51 ± 4.98), P = 0.02. However, prior hospitalization, disease location, and disease activity were not associated with CCKNOW score.

Patients with joint EIMs had significantly higher CCKNOW scores (13.50 ± 5.54) compared to patients without joint manifestations (10.81 ± 4.89), P = 0.03. Other EIMs were not associated with CCKNOW score.

DISCUSSION

Caucasian race, younger age at time of diagnosis, and having a college or post-graduate degree were statistically significantly associated with higher CCKNOW scores. The patient populations of greatest IBD-specific knowledge deficiencies were in patients who were non-Caucasian, did not have a college or post-graduate degree, and at an older age at IBD diagnosis. However, we observed no significant correlation between overall patient knowledge of IBD and HRQoL, although higher diet related knowledge was associated with lower HRQoL.

We observed similar IBD-specific patient knowledge scores to prior studies in non- United States populations, ranging from a median CCKNOW score of 4 in Iran, to 7-10 in the United Kingdom, to 13 in Canada[18-20]. Caucasian race was associated with higher disease related knowledge in our study. In our study, all patients were native English speakers

Table 3 Inflammatory bowel disease characteristics and Crohn's and Colitis knowledge score sub-domains

Characteristics	General knowledge	SD	*P* value	Diet	SD	*P* value	Treatment	SD	*P* value	Complications	SD	*P* value
Gender			0.09			0.06			0.56			0.04
Male	43%	20.39		45.15%	23.51		25.47%	20.43		29.56%	19.25	
Female	55.47%	10.47		61.11%	33.33		29.63%	20.03		43.75%	12.40	
Race			0.03			0.26			0.28			0.08
Caucasian	47.34%	20.00		48.51%	24.57		27.44%	21.13		33.33%	20.81	
Non-caucasian	37.5%	18.64		42.65%	25.02		22.73%	18.55		26.04%	14.63	
Education			0.10			< 0.01			0.48			0.15
No college	39.54%	15.52		41.18%	19.35		26.26%	20.85		31.25%	15.70	
College/post-graduate	48.75%	21.16		58.33%	19.17		30.56%	20.00		40%	25.04	
IBD type			< 0.01			0.52			0.15			0.17
UC	39.49%	17.67		45.92%	22.45		20.92%	19.64		28.03%	18.89	
CD	51.92%	19.88		45.35%	28.48		30.49%	20.38		35%	19.17	
IBDU	31.25%	20.59		55.56%	16.67		20.37%	21.70		25%	23.57	

CCKNOW: Crohn's and Colitis Knowledge score; SD: Standard deviation; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; IBDU: Inflammatory bowel disease type unclassified.

and therefore we do not believe the racial disparity observed can be attributed to a language barrier. Our observation of racial disparity in IBD knowledge is in contrast to a prior study from Australia using CCKNOW among 258 IBD patients recruited from ambulatory clinics and outpatient offices which showed no difference in knowledge based on race comparing Caucasians and non-Caucasians[21]. However, our observation is in line with those observed in other disease states. A study by Abubakari *et al*[22] evaluating disease knowledge among patients with type 2 diabetes found that white-British patients had a higher disease-specific knowledge compared to black-African and black-Caribbean counterparts. Additionally, the study found that the latter two populations had high levels of misconceptions regarding their disease compared to the white-British patients. Racial disparities in disease specific knowledge have been attributed to level of education as well as health literacy[18,19]. Racial disparities in IBD-specific knowledge, as identified in our study, highlight potential areas of educational intervention, specifically targeting non-Caucasian populations.

We observed that a younger age at time of IBD diagnosis was associated with greater disease-specific knowledge. This was independent from duration of disease, which was not significantly associated with IBD-specific knowledge. Younger patients may be more likely to seek information on their own or have easier access to information outside of the medical setting. Presumably, younger patients may possess an increased access to online research enabling them to ascertain more validated or accurate disease information. However, patient access to potentially inaccurate internet information may be damaging[23-25]. Our observations suggest educational programs and materials may need to be tailored to reach older patients with IBD.

Although we did not observe a correlation between patient knowledge and HRQoL, areas of knowledge deficiency may still have a significant clinical impact and role in patient activation and self-care. One possible reason for our finding is that patients lack self-efficacy - even if aware of their disease state, they may lack the means to change their health and be active members in medical decisions making. It is also possible that increased knowledge about one's disease may result in depression, anxiety, or fear about the disease. Larsson and colleagues demonstrated that patients with high levels of anxiety did not exhibit improved angst after participating in educational programs about their disease[26]. Despite this, non-adherence has not been shown to be associated with anxiety and depression or disease-related patient knowledge[27].

Patients have been shown to rely on clinicians for their information needs[28]. A study assessing the effective measure of patient education to improve IBD-specific reproductive knowledge, found a single group-delivered session increased patient understanding and knowledge regarding the implications of disease for fertility and pregnancy[29]. This study used another precise questionnaire developed by Selinger entitled the Crohn's and Colitis Pregnancy Knowledge Score (CCPKnow)[30]. Of the CCKNOW sub-domains, higher diet knowledge scores were significantly correlated with lower HRQoL. This finding may in part reflect the diet specific questions on the CCKNOW that refer to enteral nutrition and lactose intolerance, which may bias towards patients with a history of more severe disease.

Our study has several limitations. Level of education was missing in 49% of patients who declined to answer that question on the survey. However, we were still able to detect differences in level of education associated with disease-specific knowledge. The surveys were self-administered and health literacy may impact the assessment of disease-specific knowledge and decrease the scores of CCKNOW. It is also important to note that half of patients felt CCNOW questions were too difficult as documented

in a previous study by Elkjaer *et al*[31]. Lastly, the patient population in this study was predominantly male, which is typically expected in the VA patient population. Therefore, conclusions regarding gender may not be generalizable. However, CCKNOW scores observed in this study parallel those of studies in other patient populations. Despite these limitations, our study has several strengths. This is the first study to use both the CCKNOW and SIBDQ questionnaires to evaluate patient knowledge and HRQoL in patients with IBD in the United States and also within the Veteran population. The Veteran patient population also represents a relatively diverse IBD population, both in race and age, relative to other frequently studied IBD populations. SIBDQ and CCKNOW were obtained prospectively from a non-tertiary practice and may therefore more closely represent IBD patients in the general population compared to complicated patients referred to tertiary IBD referral centers.

In conclusion, a cohort of United States military veterans with IBD, disease-specific knowledge of IBD was not correlated with HRQoL. Variations in disease-specific knowledge of IBD were observed based on age at IBD diagnosis, level of education, and race. These disparities may provide targeted opportunities for patient directed education tools. Further studies are required to study the effect of patient knowledge of IBD on other clinical outcomes.

COMMENTS

Background

Amongst a United States military veteran population with inflammatory bowel disease (IBD), disease-specific knowledge of IBD was not correlated with health related quality of life (HRQoL). While no correlation was shown, specific variables including Caucasian race, younger age at time of diagnosis, and having a college or post-graduate degree were significantly associated with higher Crohn's and colitis knowledge score (CCKNOW) scores.

Research frontiers

Although disease-related IBD knowledge may not be directly correlated with overall HRQoL as observed in this study, the wide variation in disease-specific knowledge provides important insight into opportunities to address knowledge gaps and improve other clinical outcomes.

Innovations and breakthroughs

Despite the existence of a validated patient knowledge score in the CCKNOW in Europe, IBD patient knowledge has not been assessed in the United States to date. This is the first study to explore the correlation between CCKNOW and SIBDQ questionnaires to evaluate patient knowledge and HRQoL in patients with IBD in the United States and also within the Veteran population.

Applications

Given the identified variations in disease-specific IBD knowledge based upon race, age of diagnosis, and education level, future studies and projects may shed light on expanding programs to narrow this gap in an effort to improve a range of clinical outcomes.

Peer-review

This study is interesting and useful for the medical practice.

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ORIGINAL ARTICLE

Prospective Study

Risk of venous congestion in live donors of extended right liver graft

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Data sharing: Consent was not obtained but the presented data are anonymized and risk of identification does not exist.

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Abstract

AIM: To investigate middle hepatic vein (MHV) management in adult living donor liver transplantation and safer remnant volumes (RV).

METHODS: There were 59 grafts with and 12 grafts without MHV (including 4 with MHV-5/8 reconstructions). All donors underwent our five-step protocol evaluation containing a preoperative protocol liver biopsy Congestive *vs* non-congestive RV, remnant-volume-body-weight ratios (RVBWR) and postoperative outcomes were evaluated in 71 right graft living donors. Dominant *vs* non-dominant MHV anatomy in total liver volume (d-MHV/TLV *vs* nd-MHV/TLV) was constellated with large/small congestion volumes (CV-index). Small for size (SFS) and non-SFS remnant considerations were based on standard cut-off- RVBWR and RV/TLV. Non-congestive RVBWR was based on non-congestive RV.

RESULTS: MHV and non-MHV remnants showed no significant differences in RV, RV/TLV, RVBWR, total bilirubin, or INR. SFS-remnants with RV/TLV < 30% and non-SFS-remnants with RV/TLV ⩾ 30% showed

no significant differences either. RV and RVBWR for non-MHV (n = 59) and MHV-containing (n = 12) remnants were 550 ± 95 mL and 0.79 ± 0.1 mL *vs* 568 ± 97 mL and 0.79 ± 0.13, respectively (P = 0.423 and P = 0.919. Mean left RV/TLV was 35.8% ± 3.9%. Non-MHV (n = 59) and MHV-containing (n = 12) remnants (34.1% ± 3% *vs* 36% ± 4% respectively, P = 0.148. Eight SFS-remnants with RVBWR < 0.65 had a significantly smaller RV/TLV than 63 non-SFS-remnants with RVBWR ⩾ 0.65 [SFS: RV/TLV 32.4% (range: 28%-35.7%) *vs* non-SFS: RV/TLV 36.2% (range: 26.1%-45.5%), P < 0.009. Six SFS-remnants with RV/TLV < 30% had significantly smaller RVBWR than 65 non-SFS-remnants with RV/TLV ⩾ 30% (0.65 (range: 0.6-0.7) *vs* 0.8 (range: 0.6-1.27), P < 0.01. Two (2.8%) donors developed reversible liver failure. RVBWR and RV/TLV were concordant in 25%-33% of SFS and in 92%-94% of non-SFS remnants. MHV management options including complete MHV *vs* MHV-4A selective retention were necessary in n = 12 *vs* n = 2 remnants based on particularly risky congestive and non-congestive volume constellations.

CONCLUSION: MHV procurement should consider individual remnant congestive- and non-congestive volume components and anatomy characteristics, RVBWR-RV/TLV constellation enables the identification of marginally small remnants.

Key words: Living donor liver transplantation; Liver volume; Remnant volume; Small-for-size; Small-for-size syndrome

Core tip: Prevention of liver failure in middle hepatic vein (MHV) inclusive right graft donors involves consideration of both congestive and non-congestive remnant volumes. MHV management should be individually based on MHV anatomy characteristics. Non-congestive volumes represent an important safety parameter in MHV management, especially in the setting of small for size remnants. The remnant-volume-body-weight ratios - remnant volumes/total liver volume constellation seems to have a synergistic (complementary) capacity for the identification of marginally small remnants with the highest risk potential of postoperative liver failure.

Radtke A, Sgourakis G, Molmenti EP, Beckebaum S, Cicinnati VR, Schmidt H, Peitgen HO, Broelsch CE, Malagó M, Schroeder T. Risk of venous congestion in live donors of extended right liver graft. *World J Gastroenterol* 2015; 21(19): 6008-6017 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6008.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6008

INTRODUCTION

The precise determination of graft and remnant volumes constitutes the most important parameter to prevent postoperative donor and recipient liver failure in adult live donor liver transplantation (ALDLT)[1-3]. Middle hepatic vein (MHV)-containing grafts are associated with small remnants whose function may be further impaired by early postoperative venous congestion of their medial sector (segment 4A/B)[1,4,5]. The occurrence of small-for-size syndrome (SFSS) in donors as a result of inadequate functional remnant volume is a constant reminder of the controversy surrounding venous congestion and MHV management. The commonly accepted definitions for small-for-size-(SFS)-remnants do not even consider remnant volume values[4,6-11]. To date, there are no published reports correlating the extent of functional impairment and parenchymal congestion in non-MHV containing remnants, and remnant volume limits for safe MHV inclusion with the right graft are still undefined.

In the present series, we evaluated our experience with liver failure in right graft donors. Our goal was to analyse the impact of MHV-containing right grafts on remnant volume (RV) and function. We considered the ratios remnant-volume-body-weight-ratio (RVBWR) and remnant volume percentage of total liver volume (RV/TLV) as a way to discriminate between SFS- and non-SFS remnants based on commonly accepted cut off values[4,8]. The following queries were addressed: (1) How concordant are these ratios in assessing SFS-remnants and determining their volume limits? (2) Is MHV procurement with right grafts associated with substantial loss of remnant volume? (3) Does inclusion of the MHV in right grafts impact remnant liver function and donor morbidity as a result of venous congestion? and (4) Does MHV anatomy affect venous outflow (= congestive volume) and thereby influence MHV management?

We finally considered "reasonable" criteria for procurement of right grafts with/without complete MHV *vs* selective MHV-4A preservation in remnants based both on our own experience with donors without evidence of steatosis as well as on that of others[4,6-8,11-16].

MATERIALS AND METHODS

Study population

From January 2003 to October 2007, 71 consecutive live donors (36 females and 35 males, mean age 37 ± 10.1 years) underwent right graft hepatectomy at the University Hospital Essen, Germany. There were 59 grafts with and 12 grafts without MHV (including 4 with MHV-5/8 reconstructions). All donors underwent our

Table 1 Etiology of liver disease among right graft recipients (*n* = 71)

Parameter	Number
Total	71
Male	43
Female	28
Autoimmune hepatitis	5
Hepatitis B	4
Hepatitis B associated with hepatocellular carcinoma (HCC)	7
Hepatitis C	8
Hepatitis C associated with HCC	10
Alcoholic	7
Alcoholic + associated with HCC	6
Morbus Wilson	2
Primary biliary sclerosis (PBC)	2
Primary sclerosing cholangitis (PSC)	7
HCC	4
Cryptogenic	6
Others[1]	3

[1]Neuroendocrine lever metastases (*n* = 2), liver metastases from insulinoma.

five-step protocol evaluation containing a preoperative protocol liver biopsy as previously described[14,17]. Biopsy results in all resected donors showed less than 10% steatosis and no evidence of hepatopathologic changes.

Recipient indications for liver transplantation

Sixty eight out of 71 right graft recipients (28 females and 43 males, mean age 50 ± 11.0 years) suffered from liver cirrhosis classified for Child-A score; *n* = 22, Child-B score; *n* = 33, Child-C score; *n* = 13, while in the remaining *n* = 3 cases with no cirrhosis the indication for liver transplantation were neuroendocrine liver metastases (*n* = 2) as well as liver metastases from insulinoma (*n* = 1, Table 1). The overall "Model of End-Stage Liver Diesease"-score (MELD) was of mean of 14 ± 8 (range: 11-40).

All-in-one protocol of multiphasic computed tomography scan

Computed tomography (CT) imaging was performed using a 16-row-Multidetector-CT-Scanner (Sensation16®, Siemens, Erlangen, Germany) as originally published by our group[18].

3D-CT-imaging analysis and -volumetry

CT images were analyzed with the software assistant HepaVision® (MeVis institute, Bremen, Germany)[19,20].

Liver volume definitions

RV: Congestive and non-congestive volumes.

Congestive volume: Venous congestion volume resulting from the detachment of left sided MHV-(4A/B) tributaries draining the left medial sector.

Non-congestive volume: Volume safely drained by the left hepatic vein (LHV) tributaries.

Congestive volume-index: Percentage of volume with venous congestion.

Donor RV/TLV: Remnant volume percentage of total liver volume considering the remnant volume with intact bi-sectorial venous outflow *via* the middle (MHV)- and LHV tributaries.

Donor RVBWR: (Safely drained by MHV and LHV) *vs* non-congestive *RVBWR* (safely drained by LHV) were calculated according to the Heinemann formula[9].

3-D virtual liver partition

The "carving" transection plane followed the course of the MHV, exposing it on the resection surface of either graft (MHV-procurement) or remnant (MHV-retention) livers[21]. The MHV trunk served as a reproducible surgical landmark for the exact extrapolation (by means of color doppler scanning, IOUS) of the 3-D liver model onto the operative field.

SFS vs non-SFS remnants

We evaluated the correlation between RVBWR and RV/TLV as a way to distinguish between SFS- and non-SFS remnant status based on the following cut off values: SFS-remnant: RVBWR < 0.65 *vs* non-SFS-remnant RVBWR ⩾ 0.65[8]. SFS-remnant: RV/TLV< 30% *vs* non-SFS-remnant RV/TLV ⩾ 30%[4].

SFSS definition

SFSS was defined as either poor initial remnant function or prolonged remnant dysfunction as a result of inadequate functional liver mass in the absence of other causative factors. This definition was based on criteria for both LDLT donors and recipients likewise tumor hepatectomy patients[22,23]. SFSS was characterised by the presence of at least two of the following symptoms within the first four post-operative weeks: encephalopathy (stage ⩾ 2), progressive intrahepatic cholestasis [Bilirubin > 5.0 (reference value: 0.2-1.2)], prolonged severe coagulopathy (INR > 2.2), excessive intractable ascites (> 3 L/d).

Hepatic vein dominance in total liver

Hepatic vein with the largest percentage of total liver volume (TLV) as originally classified by our group[20].

Statistical analysis

The non-parametric Sign test was used for two variables lacking normal distribution. The non-parametric Wilcoxon matched pairs test was applied to test the hypothesis that two variables (lacking normal distribution) were drawn from the same distribution.

Table 2 Concordant *vs* non-concordant interrelation between remnant volume (donor) body weight ratio and remnant volume percentage of total liver volume in discriminating small-for-size from non-small-for-size remnants according to two different cut-off values

Remnant status defined by Rvbwr		Remnant status defined by Rv/tlv	
SFS	Non SFS	SFS	Non SFS
RVBWR < 0.65	RVBWR ⩾ 0.65	RV/TLV < 30%	RV/TLV ⩾ 30%
n = 8	*n* = 63	*n* = 6	*n* = 65
RV/TLV < 30%	RV/TLV < 30%	RVBWR < 0.65	RVBWR < 0.65
Concordant	Non-concordant	Concordant	Non-concordant
2/8	4/63	2/6[1]	5/65[2]
25%	6%	33%	8%
RV/TLV ⩾ 30%	RV/TLV ⩾ 30%	RVBWR ⩾ 0.65	RVBWR ⩾ 0.65
Non-concordant	Concordant	Non-concordant	Concordant
6/8	59/63	4/6	60/65
75%	94%	67%	92%

[1]incl. 2 remnants with RVBWR = 0.60; [2]incl. 1 remnant with RVBWR = 0.60. SFS: Small-for-size; RV/TLV: Remnant volume percentage of total liver volume; RVBWR: Remnant volume (donor) body weight ratio.

The Mann-Whitney *U* Test was used to test the significance of the difference between two independent samples of an ordinal variable as well as differences in the shape of the distributions (not just the location of the ranks) of the two groups. Significance was considered at a level < 0.05. Statistical release 7 (Statsoft) was used for statistical analysis.

RESULTS

RV and RVBWR

Mean overall RV and RVBWR were 565 ± 97 mL and 0.79 ± 0.12, respectively. RV and RVBWR for non-MHV (*n* = 59) and MHV-containing (*n* = 12) remnants were 550 ± 95 mL and 0.79 ± 0.1 mL *vs* 568 ± 97 mL and 0.79 ± 0.13, respectively (*P* = 0.423 and *P* = 0.919, Mann Whitney *U* test).

RV/TLV

Mean left RV/TLV was 35.8% ± 3.9%. Non-MHV (*n* = 59) and MHV-containing (*n* = 12) remnants (34.1% ± 3% *vs* 36% ± 4% respectively, *P* = 0.148 Mann Whitney *U* test) showed no significant differences.

Correlation between donor RVBWR vs RV/TLV in defining SFS-remnants

We assessed the concordance between RVBWR < 0.65 and RV/TLV < 30% in all 71 right graft donors (Table 2).

Twenty-five percent (*n* = 2/8) of SFS-remnants had RVBWR < 0.65 with RV/TLV < 30%. 94% (*n* = 59/63) of non-SFS-remnants had RVBWR ⩾ 0.65 and RV/TLV ⩾ 30%. Eight SFS-remnants with RVBWR < 0.65 had a significantly smaller RV/TLV than 63 non-SFS-remnants with RVBWR ⩾ 0.65 [SFS: RV/TLV 32.4% (range: 28%-35.7%) *vs* non-SFS: RV/TLV 36.2% (range: 26.1%-45.5%), *P* < 0.009, Mann Whitney *U* test] Figure 1A.

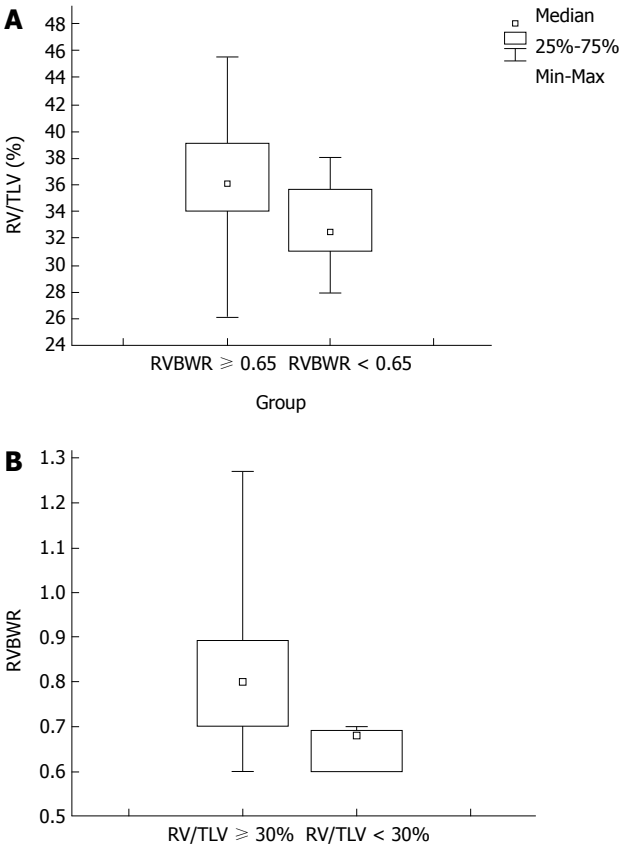

Figure 1 Correlation between remnant-volume-(donor) body-weight ratio (< 0.65 *vs* ⩾ 0.65) and remnant volume percentage of total liver volume in left remnants of 71 right graft donors (A) and correlation between remnant volume percentage of total liver volume (< 30% *vs* ⩾ 30%) and remnant-volume-(donor) body-weight ratio in the left remnants of 71 right graft donors (B). RV/TLV: Remnant volume percentage of total liver volume; RVBWR: Remnant-volume-(donor) body-weight ratio.

Thirty-three percent (*n* = 2/6) of SFS-remnants had RV/TLV < 30% with RVBWR < 0.65. 92% (*n* = 60/65) of non-SFS-remnants had RV/TLV ⩾ 30% and RVBWR ⩾ 0.65. Six SFS-remnants with RV/TLV < 30% had significantly smaller RVBWR than 65 non-SFS-remnants with RV/TLV ⩾ 30% [0.65 (range: 0.6-0.7) *vs* 0.8 (range: 0.6-1.27), *P* < 0.01, Mann Whitney *U* test] Figure 1B.

Congestive volume-index and non-congestive RVBWR

Mean overall congestive volume (CV) was 209.2 ± 77.6 mL (range: 40-459 mL) with a CV-index of 36.9 ± 11.6 %RV (range: 6.1-70.2 %RV). Mean non-congestive [safely drained by the left hepatic vein (LHV)] donor RVBWR (0.48 ± 0.12, range: 0.2-0.79) was significantly smaller than the corresponding donor RVBWR (safely drained by both MHV and LHV) (0.79 ± 0.12, range: 0.6-1.27, *P* < 0.0001, Wilcoxon's signed ranks test).

Liver function laboratory markers

Non-MHV containing remnants had a higher (although *P* values were very close) peak total bilirubin and INR

Table 3 Comparison of early postoperative biochemical liver function markers among right graft donors (n = 71)

Peak (mean ± SD)	Remnants MHV n = 12	Remnants Non-MHV n = 59	Remnants RV/TLV ⩾ 30% n = 65	Remnants RV/TLV < 30% n = 6
Bilirubin (0.2-1.2)	4.26 ± 2.86	5.54 ± 7.89	4.39 ± 3.92	5.1 ± 2.9
	P = 0.544		P = 0.27	
INR (1.0)	1.99 ± 0.52	2.15 ± 0.83	1.87 ± 0.44	2.02 ± 0.57
	P = 0.9		P = 0.587	

RV/TLV: Remnant volume percentage of total liver volume; MHV: Middle hepatic vein; INR: International normalized ratio; Bili: Total bilirubin (reference value 0.2-1.2).

than MHV-containing remnants (potentially suggesting a "negative effect" of venous congestion in the early postoperative liver function) (Table 3).

Postoperative donor morbidity

There were no donor deaths. Overall postoperative donor morbidity was 15.5% (n = 11), including 6 (8.4%) grade Ⅲ-Ⅳ Dindo-Clavien complications[23]. There was no significant difference among remnants with (n = 3, 25%) or without (n = 8, 13.6%) MHV under their diverse volume conditions (P = 0.4077, chi-square). Five medical complications included: 2 pleural effusions (1 in an MHV- and 1 in a non-MHV remnant) requiring drainage (D-Ⅱ), 1 pneumonia in a non-MHV remnant (D-Ⅱ), and 2 reversible liver failures (D-ⅣA). Six surgical morbidities included 2 bile leaks (1 in a non-MHV- and 1 in an MHV remnant) associated with bilomas and treated with percutaneous drainage (D-ⅢA), 1 ⅣC thrombosis treated surgically in a non-MHV remnant (D-ⅢB), 1 subphrenic abscess drained operatively in a non MHV remnant (D-ⅢB), and 2 superficial wound infections (D-ⅠA).

Association of MHV management and remnant liver failure in donors

Two (2.8%) donors developed reversible liver failure (see SFSS definition). Neither of them had a history of liver disease, experienced any adverse intraoperative events, or developed surgical/medical complications. Postoperative color doppler ultrasonography confirmed intact porto-arterial inflow and hepatic venous outflow.

Case-1: 40 year old female, BMI 26, liver biopsy < 10% steatosis, normal preoperative LFTs. MHV-containing remnant with safely (MHV + LHV)-drained-RVBWR of 0.63 (RV = 434 mL, RV/TLV = 35%). Postoperatively developed grade 2° encephalopathy, with peak Bilirubin of 26.5mg/dl and INR of 3.7. Recovered completely after two courses of plasmapheresis.

Case 2: 44-year-old male, BMI 27, liver biopsy < 10% steatosis, normal preoperative LFTs. Non-MHV-containing remnant with RVBWR of 0.65 (RV = 584 mL = RV/TLV 31%). RV safely drained by LHV of 344 mL (CV-index = 40.2%), with safely (LHV)-drained-RVBWR of 0.39. Postoperatively developed grade 2° encephalopathy, with peak bilirubin of 19.8 mg/dL and INR of 2.5. Recovered spontaneously after a hospital stay of 26 d.

MHV management in remnants with liver failure vs without liver failure

Our stepwise 3D-CT volumetry combined estimated left remnant congestive- and non-congestive volumes following virtual liver partition (Figure 2). Based on the experience of the Kyoto and Nagoya groups[12,13], the extremely low (25%-33%) concordance between donor RVBWR and RV/TLV, and the two reversible remnant liver failures in our series, we differentiated between right grafts inclusive of complete MHV and left remnants with selective MHV-4A retention by considering individual MHV anatomy patterns[16].

In 12 donors, the MHV was completely retained with the left remnants, providing an intact two-sectorial venous (MHV + LHV) drainage. In 10 cases, a risky dominant (d)-MHV type was preserved because of its particularly large congestive volume when compared to the non-dominant (nd)-MHV (d-MHV mean CV-index 41.2 ± 6.6 %RV *vs* nd-MHV mean CV-index 36.1 ± 12.2 %RV, P = 0.07, Mann-Whitney U test). In 2 donors with nd-MHV, the decision to retain the MHV with the left remnant was based on their small donor RVBWR-RV/TLV constellation (0.6/28.2% and 0.63/35%, Table 4).

The left sided MHV-4A drainage territory was preserved in 4 of 59 donors who underwent procurement of MHV-containing grafts as originally described by our group[15]. This decision was based on an extremely small non-congestive-RVGWR (0.2-0.27) (safely drained by LHV) in 2 cases (Table 4) and on the anatomical characteristics of the MHV-4A/MHV-8 confluence into the MHV trunk in the other 2 instances.

Two (20%) of ten donors with estimated very small RVBWR ⩽ 0.65 (inclusive of two with RV/TLV < 30%) developed reversible liver failure. The MHV was retained in two remnants (one with liver failure). Eight remnants (one with liver failure) had no MHV. In three non-liver failure remnants with extremely low non-congestive-RVBWR < 0.3 (safely drained by LHV), the MHV was completely retained or the MHV-4A drainage was preserved in the remnant liver (Table 4).

DISCUSSION

Although a RV/TLV of at least 30%-35% is usually required to avoid small-for-size syndrome (SFSS)[1,4,8], successful outcomes with RV/TLV < 30% have been reported in the setting of optimal liver quality[6,7,11,24]. Inclusion of the MHV with right grafts, which has been reported to optimize graft function[1,4,7,25] but to potentially impair remnant recovery[1,26-28], has both

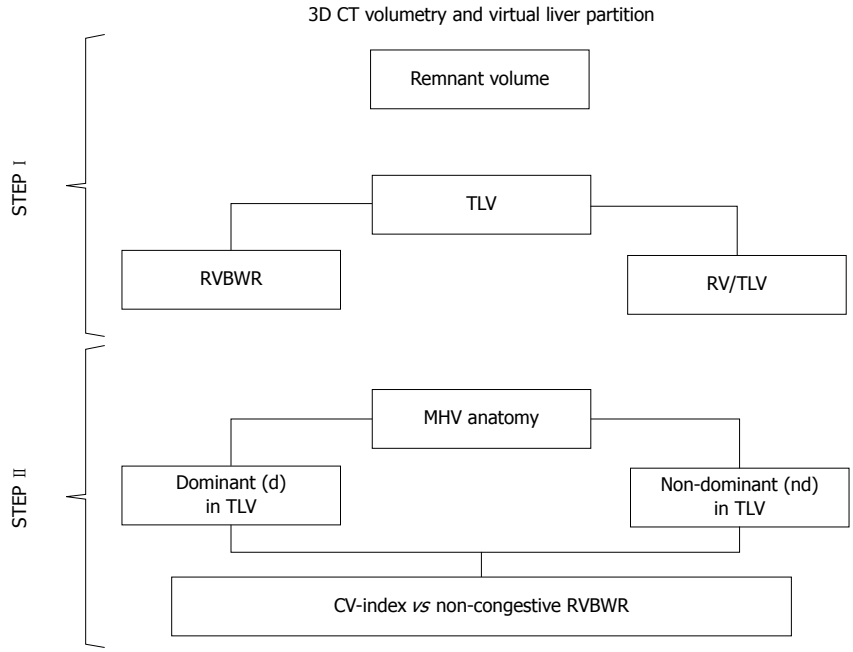

Figure 2 Stepwise 3D-computed tomography volumetry and virtual liver partition assessing congestive- and non-congestive (remnant) volumes. CV-index: Congestive volume percentage of remnant liver volume; 3-D: Three-dimensional; d: Dominant; MHV: Middle hepatic vein; nd: Non-dominant; RV/TLV: Remnant volume percentage of total liver volume; RVBWR: Remnant-volume-(donor) body-weight ratio; non-congestive-RVBWR: Safely LHV drained remnant-volume-body-weight ratio; TLV: Total liver volume.

Table 4 Middle hepatic vein management in small-for-size -remnants with remnant-volume (donor) body-weight-ratio ⩽ 0.65

Donor *n* = 71	Remnant Total MHV preserved	Remnant MHV-4A preserved	Remnant volume	Remnant RV/TLV	Remnant RVBWR	Remnant CV-index	Remnant nc-RVBWR (LHV)	SFSS
1	Yes	Yes	434 mL	35%	0.63	37.9%	0.38	Yes
2	No	No	584 mL	31%	0.65	40.2%	0.39	Yes
3	No	No	512 mL	32.9%	0.62	40.0%	0.40	No
4	No	No	500 mL	31.7%	0.64	37.2%	0.38	No
5	No	Yes	429 mL	35.1%	0.60	67.9%	0.20	No
6	No	No	506 mL	38%	0.65	14.8%	0.57	No
7	No	Yes	536 mL	35.6%	0.62	43.4%	0.27	No
8	No	No	505 mL	32%	0.63	39.4%	0.49	No
9	No	No	389 mL	28%	0.60	24.4%	0.41	No
10	Yes	Yes	464 mL	28.2%	0.60	55.4%	0.20	No

SFS: Small-for-size; SFSS: Small-for-size syndrome; LDLT: Live donor liver transplantation; MHV: Middle hepatic vein; MHV-4A: Branch of MHV draining left medial sector; LHV: Left hepatic vein; RVBWR: Remnant-volume (donor) body-weight-ratio; Non-congestive-(nc)-RVBWR: Volume safely drained by LHV; RV/TLV: Remnant volume percentage of total (donor) liver volume; CV-index: Potential congestion volume percentage of remnant liver volume.

supporters and detractors[2,7,8,27,29-34]. Currently, many centres encourage a selective MHV management policy based on individual graft/remnant characteristics[4,7,12].

Our series allowed us to conclude that procurement of right grafts including complete MHV itself did not cause a significant volume loss in remnants. Indeed, there were no significant RV, RV/TLV and RVBWR differences between remnants with and without MHV. We attributed this result to our "carving" liver partitioning technique, in which the transection plane exposed the MHV trunk on the resection surface of either graft (MHV-harvest) or remnant (MHV-retention) livers. There was no difference in donor morbidity attributable to SFS-remnant-status or MHV inclusion (even with RVBWR and RV/TLV below the respective marginal limits of < 0.6 and < 30%).

Our overall donor morbidity of 15.5% including 8.4% of Dindo-Clavien Ⅲ-Ⅳ type complications were comparable with the data reported in the literature[5,7,12].

In the Kyoto series overall 10% of donors suffered morbidity with similar incidence of complications who required treatment between (-) MHV (13%) *vs* (+) MHV (9%) remnants[12]. Comparable, in our donors the incidence of postoperative interventions did not considerably differ between the non-MHV (5.1%) and the MHV-contained (8.3%) remnants. In line with the cited reports, all our donors returned to

their pre-donation lifestyles. The subgroup analysis of the Istambul series[7] revealed a much higher overall complication rate in non-MHV (22.4%) *vs* MHV-contained remnants (7.8%) mirroring our own experience with the tendency for (-) MHV remnants to have more complications than (+) MHV ones (25% *vs*14%).

Furthermore, our experience as well as that of others did not reveal any late complications attributable to remnant size or MHV-status[7,35]. Our virtual data however, showed that the "sacrifice" of the left sided MHV-4A/B drainage in remnants due to MHV inclusion with the graft was associated with large congestive volumes (CV-index of 36.9 ± 11.6 %RV) that resulted in a significant reduction of non-congestive volumes (non-congestive-RVBWR) safely drained by LHV. We also observed a potentially (not statistically significant) detrimental effect of venous congestion in non-MHV remnants as illustrated by their elevated liver function markers (INR, total bilirubin) in the early postoperative period. These observations strongly correlate with previous published reports.

In the study of the Clischy group[5] segment Ⅳ congestion was never seen on the postoperative CT in donors who underwent a standard MHV-exclusive right graft harvest, while in the setting of the extended right graft inclusive of MHV procurements 84% remnants revealed venous congestion with the morbidity rate of 37%. Yaprak *et al*[11] had observed that RV/TLV ⩽ 30% impacted donor outcome (especially postoperative hyperbilirubinemia and major complications) irrespective of donor RVBWR (< 0.6 or > 0.6). In their experience, RVBWR < 0.6 significantly affected liver function but not donor morbidity. In a prospective study by Dayangac *et al*[7], procurement of right grafts inclusive of MHV was not associated with any additional donor risk except in SFS-remnants with RV/TLV < 30% (57% complication rate and prolonged postoperative hyperbilirubinemia). Others showed an association between small remnant volume and donor morbidity[1,11,36,37]. In our series the slightly higher bilirubin and INR levels in SFS-remnants probably resulted from a small RV.

The impact of remnant volume and remnant MHV-status on remnant regeneration has been extensively investigated. Belghiti *et al*[1] observed that a small RV accelerated early tissue regeneration, decreasing the proportion of functional liver tissue and increasing the risk of liver failure. Dayangac *et al*[7] showed that small non-MHV remnants had a significantly higher volume increase after the first postoperative week when compared to MHV remnants (76% and 50%, respectively). Similarly to our data, studies from several other groups showed that the volume regeneration rate of the total remnant liver (TLV) did not significantly differ among extended and regular right graft hepatectomies[7,38,39]. However, the observed compensatory lateral hyper-growth effect attributable to transient venous congestion in the MHV drainage area seems to reflect a competition between sectors, with the lateral one dominating regardless of remnant MHV status[40]. The development of a procoagulant state induced by the intense remnant regeneration as described by the Paris group might help explain the Ⅳ C thrombosis in one of our donors[1].

Our study revealed a very poor concordance between donor RVBWR and RV/TLV cut offs in SFS remnants (25%-33% in our series). Preoperative volume assessment based solely on RV/TLV can be misleading, particularly when compared to RVBWR of remnant volume and donor BMI[41]. RVBWR was also found to be more specific than RV/TLV as a predictor of postoperative outcomes in hepatic resections with SFS remnants[6]. Yigitler *et al*[42] observed a poor correlation between RV/TLV ⩽ 30% and RVBWR < 0.6 for SFS remnants after major hepatic resections. In a retrospective analysis by Yaprak *et al*[11], remnants with marginal RVBWR < 0.6 and RV/TLV ⩽ 30% constellation had the highest (52.2%) donor morbidity. However, their observation was not reproduced in our marginally small remnants.

Reversible liver failure occurred in MHV-inclusive as well as in non-MHV remnants with remnant volumes much above the commonly accepted limits (RVBWR 0.63-0.65 and RV/TLV 31%-35%). A retrospective analysis of virtual and clinical data confirming a non-steatosis in all donors on preoperative liver biopsy suggested that extensive venous congestion (CV-index of 40.2% RV) likely accounted for liver failure in case-2 [a non-MHV remnant with a tightly calculated functional reserve (non-congestive-RVBWR) of 0.39]. On the contrary, in case-1 (liver failure in an MHV-inclusive-remnant with intact bi-sectorial venous drainage *via* MHV + LHV, no plausible explanation could be found. A small-for-size syndrome (SFSS) is a multifactorial process primarily associated with insufficient functional liver mass that constitutes a life-threatening condition for both donors and recipients[22]. Although, a "safe" donor RVBWR-RV/TLV constellation seems to be the most effective parameter in donor selection and remnant MHV management, "liver quality" and "remnant volumes" are by no means dogmatic parameters[11,43]. The "venous congestion" and *vice versa* "non-congestive volume" association is potentially a strong additional factor[44].

The main goal of our study was to evaluate MHV management safety parameters to prevent life-threatening liver failure in MHV inclusive-right graft donors. As venous congestion in the drainage territories of MHV-4A/B branches can occur after procurement of right grafts containing MHV, congestive and non-congestive volume characteristics for each remnant should be carefully considered when making a decision on safe MHV management in donors.

Our study also showed that 10 of 12 retained MHV remnants had risky dominant d-MHV anatomy, with

considerably large CV-index when compared to nd-MHV, that required complete preservation of the MHV in the left remnants. Based on our learning curve experience (including two lethal SFSS grafts[14] and two reversible SFSS remnants) and the experiences of other groups[12,13], we followed an "exclusion" scheme aimed at identifying high risk donors unsuitable for MHV-inclusive grafts. The main finding distinguishing our series from previous ones is that MHV inclusion with right grafts is not (by itself) associated with prohibitively small remnant volumes. We individualized MHV management by determining MHV-4A/B drained congestive and safely LHV drained non-congestive volume components.

All donors with (extremely small) non-congestive-RVBWR < 0.3 underwent successfully either complete MHV- or MHV-4A remnant-preserving right graft procurements. The two donors with reversible liver failure in our series portray an enormous risk potential. Further validation of our findings with a systematic prospective clinical study will be required.

Our final conclusions include: (1) prevention of liver failure in MHV inclusive right graft donors involves consideration of both congestive and non-congestive remnant volumes; (2) MHV management should be individually based on MHV anatomy characteristics; (3) non-congestive volumes represent an important safety parameter in MHV management, especially in the setting of SFS remnants; and (4) the RVBWR-RV/TLV constellation seems to have a synergistic (complementary) capacity for the identification of marginally small remnants with the highest risk potential of postoperative liver failure.

COMMENTS

Background

The accurate magnitude of graft and remnant volumes comprises the most critical parameter to preclude postoperative donor and recipient liver failure in adult live donor liver transplantation (ALDLT). Middle hepatic vein (MHV)-containing grafts are correlated with small remnants whose function may be further compromised by immediate postoperative venous congestion of their medial sector (segment 4A/B). The incident of small-for-size syndrome (SFSS) in donors as a result of ineffective functional remnant volume is a steady notice of the dispute encompassing venous congestion and MHV management.

Research frontiers

Current virtual data, disclosed that the "sacrifice" of the left sided MHV-4A/B drainage in remnants due to MHV inclusion with the graft was related with large congestive volumes (CV-index of 36.9 ± 11.6 %RV) that gave rise to a significant reduction of non-congestive volumes (non-congestive-RVBWR) securely drained by LHV. The authors likewise noted a potentially harmful outcome of venous congestion in non-MHV remnants as demonstrated by their elevated liver function tests (INR, total bilirubin) in the early postoperative period.

Innovations and breakthroughs

As yet, there are no published studies connecting the magnitude of functional impairment and parenchymal congestion in non-MHV containing remnants, and remnant volume limits for secure MHV enclosure with the right graft are still indeterminate.

Applications

MHV management in adult live donor liver transplantation should be individually based on MHV anatomy characteristics.

Peer-review

This is a good paper, which investigates the outcome of RL-LDLT donors with remnant liver with or without the MHV trunk. It also analyzes the consistency between RVBWR and remnant volumes/total liver volume.

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ORIGINAL ARTICLE

Prospective Study

Vascular endothelial growth factor and tryptase changes after chemoembolization in hepatocarcinoma patients

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Author contributions: Ranieri G ideated and finalized the research and the manuscript; Ammendola M, Marech I and Sacco R performed literature research, data analysis, and made the figures and tables of the manuscript; Laterza A, Abbate I and Vacca A performed the enzyme-linked immuno-absorbent assay (ELISA) and fluoro-enzyme immunoassay (FEIA) for the assessment of VEGF and tryptase concentrations, respectively; Gadaleta CD performed trans-arterial chemoembolization; all authors wrote the manuscript and Oakley C edited the manuscript.
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Abstract

AIM: To evaluate vascular endothelial growth factor (VEGF) and tryptase in hepatocellular cancer (HCC) before and after trans-arterial chemoembolization (TACE).

METHODS: VEGF and tryptase serum concentrations were assessed from 71 unresectable HCC patients before and after hepatic TACE performed by binding DC-Beads® to doxorubicin. VEGF levels were examined for each serum sample using the Quantikine Human VEGF-enzyme-linked immuno-absorbent assay (ELISA), whereas tryptase serum concentrations were assessed for each serum sample by means of fluoro-enzyme immunoassay (FEIA) using the Uni-CAP100 tool. Differences between serum VEGF and tryptase values before and after TACE were evaluated using Student *t* test. Person's correlation was used to assess the degree of association between the two variables.

RESULTS: VEGF levels and serum tryptase in HCC

patients before TACE had a mean value and standard deviation (SD) of 114.31 ± 79.58 pg/mL and 8.13 ± 3.61 μg/L, respectively. The mean levels and SD of VEGF levels and serum tryptase in HCC patients after TACE were 238.14 ± 109.41 pg/mL and 4.02 ± 3.03 μg/L. The changes between the mean values of concentration of VEGF and tryptase before treatment and after treatment was statistically significant (P < 0.000231 and P < 0.00124, by Wilcoxon-Mann-Whitney respectively). A significant correlation between VEGF levels before and after TACE and between tryptase levels before and after TACE was demonstrated (r = 0.68, P = 0.003; r = 0.84, P = 0.000 respectively).

CONCLUSION: Our pilot results suggest that the higher serum VEGF levels and the lower tryptase levels following TACE may be potential biomarkers changing in response to therapy.

Key words: Serum levels; Tryptase; Vascular endothelial growth factor; Hepatocellular cancer; Chemoembolization

Core tip: Experimental data suggest that vascular endothelial growth factor (VEGF) and tryptase play a role in tumour angiogenesis. This study aims to assess VEGF and tryptase serum concentrations from 71 hepatocellular cancer patients before and after hepatic trans-arterial chemoembolization (TACE) by mean of enzyme-linked immuno-absorbent assay and fluoro-enzyme immunoassay methods respectively. Here, we demonstrated higher serum VEGF levels and lower tryptase levels following TACE as compared to pre-TACE levels. We suggest that changes of VEGF and tryptase levels may be biomarkers of response to therapy. In this context tryptase and VEGF receptor axis inhibitors may be evaluated as adjuvant therapies.

Ranieri G, Ammendola M, Marech I, Laterza A, Abbate I, Oakley C, Vacca A, Sacco R, Gadaleta CD. Vascular endothelial growth factor and tryptase changes after chemoembolization in hepatocarcinoma patients. *World J Gastroenterol* 2015; 21(19): 6018-6025 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6018.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6018

INTRODUCTION

Hepatocellular carcinoma (HCC) is a well-established hypervascular tumour with a high rate of angiogenesis[1]. Interestingly, published data suggest that the expression levels of classical surrogate angiogenic factors such as vascular endothelial growth factor (VEGF) or non-classical pro-angiogenic factors, such as tryptase, in primary tumour tissue or in circulating blood fractions could express the biological aggressiveness of malignancies and provide prognostic information[2-9].

For what concern VEGF it is a powerful angiogenic cytokine that induces proliferation of both endothelial cells and tumoural cells[10]. The soluble isoform of VEGF is a dimeric glycoprotein of 36-46 kDa, mainly induced by hypoxia in tumoural and stromal cells that in turn release VEGF. VEGF binds to two specific tyrosine-kinase receptors so called: VEGF-1 (flt-1) and VEGF-2 (KDR/flk1) respectively[11]. From a therapeutic point of view, the axis VEGF/VEGF-receptors is an important anti-angiogenic target for the treatment of HCC patients[12]. Several published studies suggest that the assessment of VEGF in the serum of HCC patients may predict the biological aggressiveness of tumours and indicate response to therapy. However, no conclusive data are available[13-17].

With special regard to tryptase, it is a neutral serine protease stored in the cytoplasmic granules of mast cells (MCs). To date, four different forms of tryptase have been identified: -γ, -β, -α and -δ[18]. Of these, α- and β-tryptase are the two best circulating isoforms described and they are released from MCs[19]. Interesting experimental studies show tryptase is as a potent angiogenic factor able to stimulate the neovascularization in both *in vitro* and *in vivo* laboratory experiments[20-28]. In particular, it induces vascular tube formation by either directly acting through mitogen action on endothelial cells[29-38]. Tryptase acts as an agonist of a receptor activated by an endogenous protease, PAR-2, which is expressed on endothelial cells and involved in their proliferation[39,40]. Interestingly, in several pet and human malignancies a correlation has also been demonstrated between angiogenesis and MCs positive to tryptase[41-46]. With particular reference to HCC cells, recently a possible role of tryptase positive MCs in the development of the disease has been suggested[47].

This prospective study aimed to assess levels of both VEGF and tryptase in HCC serum before and after hepatic trans-arterial chemoembolization (TACE) treatment to see if the above pro-angiogenic factors levels change in response to TACE. In addition the correlation between VEGF and tryptase each to other and important clinico-pathological features has been also analysed.

MATERIALS AND METHODS

Study population and treatment procedure

Between April 2008 and March 2012, 71 patients 22 females, 49 males, median age 74 years (range: 47-86 years) with intermediate grade [stage B according to the Barcelona Clinic Liver Cancer (BCLC) staging classification] unresectable HCC underwent TACE of the liver at the Interventional Radiology Unit with Integrated Section of Translational Medical Oncology of National Cancer Research Centre "Giovanni Paolo Ⅱ", Bari, Italy. All patients were enrolled in this prospective

Table 1 Eligibility criteria for treatment with trans-arterial chemoembolization

HCC
Cytohistologically confirmed
Unresectable (technical reasons, comorbidities, refusal of treatment)
Adequate liver function level
Child-Pugh class (A) or (B)
Bilirubin ⩽ 2.4 mg/dL
Absence of ascites
BCLC intermediate stage (B)
N1 tumor nodule → diameter > 3.0 cm
Max N3 tumor nodules → diameter ⩽ 3.0 cm
ECOG performance status of 0 to 2

TACE: Trans-arterial chemoembolization; HCC: Hepatocellular carcinoma; BCLC: Barcelona Clinic Liver Cancer; ECOG: European Cooperative Oncology Group.

Table 2 Base clinical characteristics of 71 patients with hepatocellular carcinoma

Age (yr)[1]	74 (47-87)
Sex (M/F)	42/29
Etiology (HCV/HBV/alcoholic/NASH)	52/8/7/4
Child-Pugh grade (A/B)	45/26
Serum AFP (ng/mL)[1]	79 (2-5967)
Serum bilirubin (mg/dL)[1]	1.7 (0.2-2.5)
Serum AST (IU/L)[1]	53 (18-201)
Serum ALT (IU/L)[1]	36.4 (8-187)

[1]Data are expressed as median values (range). HCV: Hepatitis C virus; HBV: Hepatitis B virus; NASH: Nonalcoholic steatohepatitis; AFP: Alpha-fetoprotein; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; IU: International unit.

study and underwent measurement of serum VEGF and tryptase before and after TACE. All participants signed a written informed consent. The pre-treatment evaluation included: biochemical liver function, complete blood count, coagulation profile, dose serum alpha-fetoprotein (AFP), chest X-ray, liver ultrasound with contrast medium (CEUS), and computed tomography (CT) scan of the abdomen. The diagnosis of HCC was histologically confirmed by echo-guided needle aspiration or, alternatively, on classic imaging findings for HCC associated with pathological increase of AFP levels higher than the cut-off 200 ng/mL.

The selection criteria for TACE at our institute includes: (1) absence of extrahepatic metastases; (2) patency of the portal vein; and (3) adequate functional reserve of the liver (stage A or B according to Child-Pugh classification, serum bilirubin ⩽ 2.4 mg/dL, absence of ascites and hepatic encephalopathy) as shown in Table 1.

The baseline clinical data of 71 patients studied are listed in Table 2. Fifty-two (73%) patients were positive for the hepatitis C antibody, eight (11%) patients were positive for the hepatitis B surface antigen (HBsAg), seven (10%) patients were affected by alcoholic liver disease and four (6%) were affected by nonalcoholic steatohepatitis (NASH). The serum AFP median level of patients was 79 ng/mL. Forty-eight (67%) patients had normal levels (< 20 ng/mL) of AFP, while the other twenty-three (33%) had higher levels.

TACE was performed under general anesthesia by binding DC-Beads® (Biocompatibles, Farnham, GB) to a total dose of doxorubicin of 100 mg/50 mL and injecting through the percutaneous insertion of a microcatheter into the femoral artery of the patient under fluoroscopic guidance (X-ray), corresponding to the artery of the liver. When possible, the artery that feeds the tumor was cannulated in a superselective approach.

Preparation of samples and biomarker assessment

All subjects avoided aspirin or non-steroidal anti-inflammatory drug ingestion for 4 wk before blood collection. The peripheral blood samples were taken between 7:00 am and 9:00 am after overnight fasting the day before and one day after TACE treatment. They were immediately dispensed in test tubes with serum separator tubes without additives (Becton Dickinson Vacutainer Systems Hemogard, Plymouth, United Kingdom) and left for at least 30 min at room temperature to allow for a complete clotting process. The samples were then centrifuged at 1500 × *g* for 15 min at room temperature and the supernatant recovered. Patient sera thus obtained were collected, aliquoted and frozen at -80 ℃ until the analysis phase.

VEGF levels were examined for each serum sample using the Quantikine Human VEGF-enzyme-linked immuno-absorbent assay (ELISA) (R&D Systems Inc., Minneapolis, MN, United States), which recognises VEGF-165[3,4] According to the manufacturer, the minimum detectable dose of VEGF is typically less than 9.0 pg/mL. Values below 9.0 pg/mL were considered as zero. Tryptase concentrations were assessed for each serum sample by means of fluoro-enzyme immunoassay (FEIA) using the Uni-CAP100 tool (Pharmacia Diagnostics AB, Uppsala, Sweden).

Statistical analysis

Statistical analysis was performed using SPSS software (version 17.0). Descriptive statistics of serum VEGF and tryptase levels were used to calculate means and ranges of distribution (range and standard deviation). Differences between serum VEGF and tryptase values before and after TACE were evaluated using Wilcoxon-Mann-Whitney test. Person's correlation was used to assess the degree of association between the two variables. A P value of < 0.05 was considered significant.

RESULTS

The levels of VEGF and serum tryptase in patients studied at the time of pre-treatment (24 h before TACE) had a mean value and standard deviation (SD) of 114.31 ± 79.58 pg/mL and 8.13 ± 3.61 μg/L

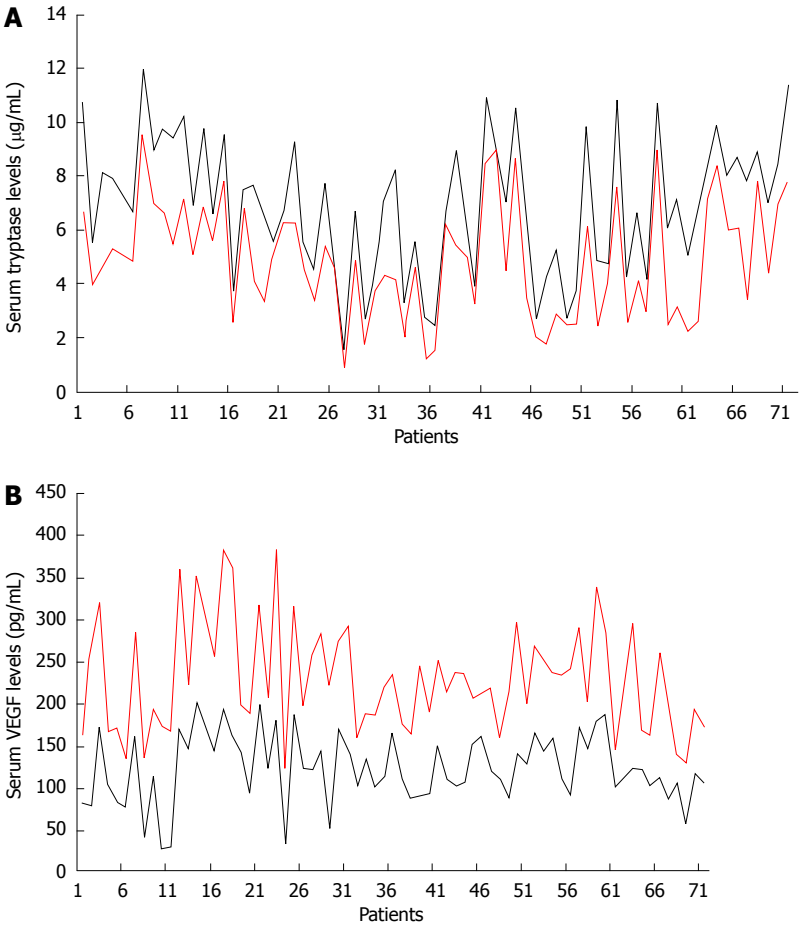

Figure 1 The levels of serum tryptase (A) and vascular endothelial growth factor (B) in patients. A: Shows changes between serum tryptase levels before trans-arterial chemoembolization (TACE) (blue line) and serum tryptase levels after TACE (red line). A clear decreased concentration is showed for each patient after TACE; B: Shows changes between serum vascular endothelial growth factor (VEGF) levels before TACE (blue line) and serum VEGF levels after TACE (red line). A clear increased concentration is showed for each patient after vascular endothelial growth factor.

Table 3 Serum vascular endothelial growth factor and tryptase levels in 71 patients with hepatocellular cancer measured 1 d prior and subsequent to treatment with trans-arterial chemoembolization

Sample collection time	*n*	Mean concentrations of serum VEGF ± SD (pg/mL)	Mean concentrations of serum tryptase ± SD (μg/L)
24 h before TACE	71	114.31 ± 79.58	8.13 ± 3.61
24 h after TACE	71	238.14 ± 109.41	4.02 ± 3.03
P value		< 0.000231	< 0.00124

VEGF: Vascular endothelial growth factor; HCC: Hepatocellular carcinoma; TACE: Trans-arterial chemoembolization; SD: Standard deviation.

respectively. No significant correlation between serum levels of VEGF and tryptase each to other was shown. Again no significant correlation between serum levels of VEGF and tryptase and the main clinico-pathological features was shown. The mean levels and SD of VEGF and tryptase in serum determined at the time of the post-treatment (+ 24 h after TACE) were 238.14 ± 109.41 pg/mL and 4.02 ± 3.03 μg/L. The changes between the mean values of concentration of VEGF and tryptase before treatment and after locoregional treatment was statistically significant significant ($P < 0.000231$ and $P < 0.00124$, by Wilcoxon-Mann-Whitney test respectively (Figure 1A and B; Table 3). A significant correlation between VEGF levels before and after TACE and between tryptase levels before and after TACE was demonstrated ($r = 0.68$, $P = 0.003$; $r = 0.84$, $P = 0.000$ respectively) (Figure 2A and B).

DISCUSSION

HCC is the fifth leading cause of cancer mortality in the world. The identification of serum biomarkers of easy execution and surrogate of the presence or persistence of disease may improve clinical outcome. To this end we analyzed VEGF and tryptase serum levels in HCC before and after TACE. Our data indicated the lack of correlation between serum tryptase and VEGF levels before TACE and between serum tryptase and VEGF levels after TACE suggesting an independent role of tryptase and VEGF in the angiogenic process. Interestingly, this data may be important from a therapeutic point of view suggesting that angiogenesis involved in HCC may be inhibited at two different molecular levels. Here, we also

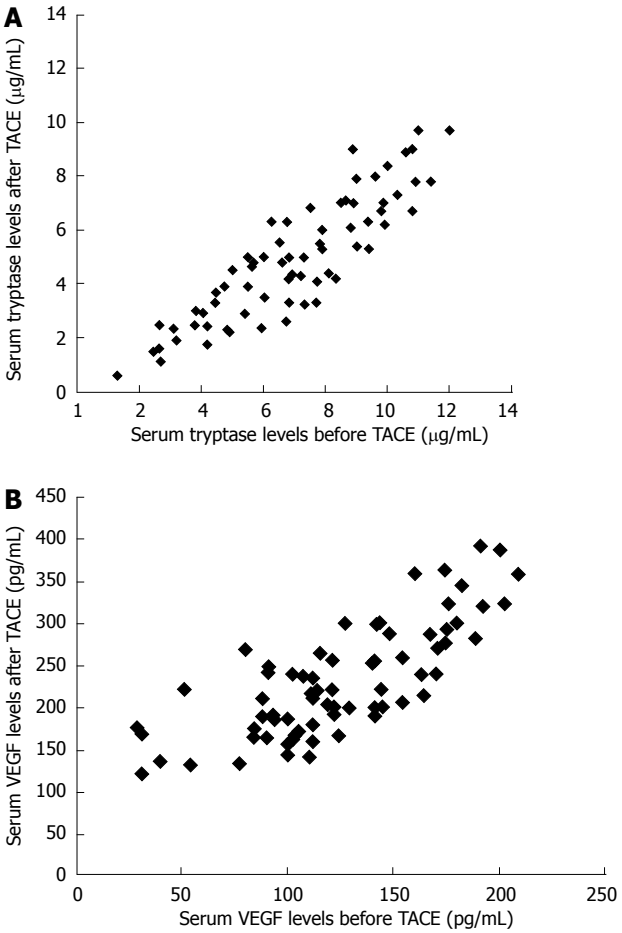

Figure 2 Serum tryptase (A) and trans-arterial chemoembolization (B) levels before TACE. A: Shows a strong correlation between serum tryptase levels before trans-arterial chemoembolization (TACE) and serum tryptase levels after TACE, in that a decreased tryptase levels is always present for each patient; B: Shows a good correlation between serum VEGF levels before TACE and serum VEGF levels after TACE, in that an increased VEGF levels is always present for each patient.

demonstrated that VEGF significantly increased after TACE (Figure 1A). Our data agree with results from Shim *et al*[15] and Sergio *et al*[17]. The first Author' s group found that VEGF levels were significantly higher 1-2 d after TACE performed with Doxorubicin plus lipiodol than at baseline. In this study, over-expression of VEGF was associated with extrahepatic metastases. The second Author's group also found that when TACE is not totally effective, it may induce a significant neoangiogenic reaction, as suggested by an increase in VEGF following treatment; this affected patient survival[17]. Differently to the above studies we detected VEGF after TACE performed with doxorubicin loaded with microspheres called DC-Bead. Although TACE represents the a main treatment for stage B HCC patients (BCLC classification), TACE also induces hypoxia and stimulates angiogenesis *via* VEGF expression that in turns may help residual cancer cells to survival[12,14,48,49]. Due to this VEGF serum rebound patients with higher serum VEGF levels post treatment may be select to receive an adjuvant therapy with sorafenib that inhibits the VEGF/VEGF-receptor axis.

With particular reference to tryptase, experimental data indicate that it plays an important role in tumour angiogenesis[37-43,50-54]. Our results show high basal levels of serum tryptase as compared to serum tryptase levels after TACE. It is therefore likely that the levels of serum tryptase may be a surrogate indicator of the magnitude of the angiogenic process and of the presence of HCC tumor tissue. Substantiating this assumption, our results showed that following TACE and subsequent tumor tissue necrosis serum tryptase levels decrease (Figure 1B), as if due to the destruction of MC content in the tumor nodule the source production of tryptase itself ceases. Hence, these serin-proteases may play a role as a predictive factor of response to locoregional treatment in HCC patients. In the present study, we measured tryptase level 24 h before treatment as a circulating biomarker surrogate for the presence of neoplastic disease, and again 24 h after treatment to evaluate the reduction of the concentration of the same. The rationale for assessing levels of tryptase after 24 h lies in the short life of tryptase, which is about 4 hours. Therefore, if the primary source of tryptase production no longer exists, after 24 h you would expect a significant reduction in serum concentration. This assumption was reflected in the data we obtained, as the difference between men pre-and post-treatment concentrations, expressed in g/L that was statistically significant. Should tryptase levels not fall during post-treatment in some patients, this could be indicative of residual disease and therefore patients should undergo further diagnostic studies and therapies. For these patients tryptase inhibitors, such as Nafamostat mesilate or Gabexate, may be evaluated in future awaited clinical trials. Although preliminary, and therefore worthy of further investigation, the results of this pilot study suggest a role of both VEGF and tryptase as possible biomarkers in HCC patients underwent to TACE able to select patients in which an adjuvant anti-angiogenic therapy may be recommended.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is a hypervascular tumour in which angiogenesis has a crucial role in progression, as already demonstrated in other tumours. Pro-angiogenic factors, such as vascular endothelial growth factor (VEGF) and tryptase, inducing proliferation of both endothelial and tumoural cells, may predict the biological aggressiveness of tumours and represent important anti-angiogenic targets in HCC. However, there are no conclusive data available about the assessment of VEGF serum levels of HCC patients.

Research frontiers

To establish the role of tryptase and VEGF in HCC progression angiogenesis-

mediated, by mean the assessment of their serum levels in HCC patients. To identificate serum biomarkers of easy execution and surrogate of the presence or persistence of disease that may improve clinical outcome. To determine if these pro-angiogenic factors may be considerable as possible biomarkers able to select patients in which an adjuvant anti-angiogenic therapy may be recommended after TACE in HCC patients.

Innovations and breakthroughs

Several published studies suggest that the assessment of VEGF in the serum of HCC patients may predict the biological aggressiveness of tumour, however, no conclusive data are available. This is the unique study, in which both serum levels of VEGF and tryptase were assessment after hepatic chemoembolization with DC-Beads® (DEB-TACE) in HCC patients and that they could be indicative of residual disease.

Applications

In this patients setting tryptase inhibitors (Nafamostat mesilate or Gabexate) or anti-VEGF/VEGFR therapy could slow HCC progression, even if these therapeutic approaches may be evaluated in future awaited clinical trials in HCC patients underwent to DEB-TACE.

Terminology

VEGF is an angiogenic cytokine (induced by hypoxia in tumoral microenvironment) that binding VEGF receptors-1/2 promotes proliferation of both endothelial and tumoural cells; Tryptase is an angiogenic serine protease (stored in mast cells) that binding a receptor activated by an endogenous protease stimulates proliferation of endothelial cells; TACE means trans-arterial chemoembolization (hepatic locoregional treatment used in stage B of HCC patients according to Barcelona Clinic Liver Cancer staging classification in which the blood supply to the tumor is blocked from chemotherapeutic agent (doxorubicin, mitomycin) plus lipiodol; DEB-TACE is TACE with drug-eluting beads (DC-Beads®) that combines the drug with the embolization device by using microsphere.

Peer-review

This pilot study could be interesting for the reader because provides good explanation of the potential benefits of pro-angiogenic factors with possible clinical impact in HCC patients undergone to DEB-TACE.

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META-ANALYSIS

Association of type 2 diabetes mellitus and the risk of colorectal cancer: A meta-analysis and systematic review

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Data sharing: Technical appendix and output files of the statistical analysis are available from the corresponding author at salmanguraya@gmail.com; Informed consent for data sharing was not obtained from the participants as the presented data are anonymized and the risk of identification is very low.

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Abstract

AIM: To provide a quantitative assessment of the association between type 2 diabetes mellitus (T2DM) and the risk of colorectal cancer (CRC).

METHODS: Systematic review was conducted thorough MEDLINE, EMBASE, Cochrane Library, and ISI Web of knowledge databases till 31st January 2014. This meta-analysis included the cohort studies that illustrated relative risk (RR) or odds ratio estimates with 95%CI for the predictive risk of CRC by T2DM. Summary relative risks with 95%CI were analyzed by using an effects summary ratio model. Heterogeneity among studies was assessed by the Cochran's Q and I^2 statistics.

RESULTS: The meta analysis of 8 finally selected studies showed a positive correlation of T2DM with the risk of CRC as depicted by effects summary RR of 1.21 (95%CI: 1.02-1.42). Diabetic women showed greater risk of developing CRC as their effect summary RR of 1.22 (95%CI: 1.01-49) with significant overall Z test at 5% level of significance was higher than the effect summary RR of 1.17 (95%CI: 1.00-1.37) of men showing insignificant Z test. The effect summary RR of 1.19 with 95%CI of 1.07-1.33 indicate a positive relationship between DM and increased risk of CRC with significant heterogeneity (I^2 = 92% and P-value < 0.05).

CONCLUSION: Results from this systematic review and meta-analysis report that diabetic people have an increased risk of CRC as compared to non-diabetics.

Key words: Colorectal cancer; Type 2 diabetes mellitus; Risk ratio; Gastrointestinal cancers; Cancer statistics

Core tip: The prevalence of diabetes for all age groups worldwide is estimated to be 2.8% in 2000 and 4.4% in 2030. Type 2 diabetes mellitus (T2DM) is associated with increased insulin resistance and insulin has been reported to exhibit procarcinogenic effects in a number of human systems including colon and rectum. This meta-analysis of 8 relevant cohort studies showed an increased risk for colorectal carcinoma by T2DM, and

this association was more evident in diabetic women than men.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) has been reported to increase the risks of a wide spectrum of cancers including kidney[1], non-small-cell lung[2], pancreas[3], early gastric[4], ovarian[5], prostate[6], and colorectal cancer (CRC)[7]. The hallmark of T2DM is its associated insulin resistance, and in the majority, with compensatory hyperinsulinemia. As of 2010, more than 250 million people are suffering from T2DM worldwide; and this number is expected to reach 380 million in 20 years[8] (Chowdhury, 2010 #887). CRC is the second leading cause of cancer-related deaths world-wide[9] and is the third most commonly diagnosed cancer in the United States for both men and women[10]. Because of the magnitude of the prevalence of T2DM and CRC and reports of published data suggesting a causal role of T2DM in the development of CRC[11,12], the frequency of relationship of these two illnesses needs to be investigated. Thus, exploring the association between T2DM and the risk of CRC is of clear significance.

Published data has shown inconsistent findings about the association of T2DM with the risk of CRC. There are also inconclusive results about the gender predominance and subsite in the colorectum harboring cancerous growths. This meta-analysis quantitatively assesses the results from published cohort studies to provide a more precise estimate of the association between T2DM as a possible predictor of the risk of CRC.

MATERIALS AND METHODS

Systematic review was conducted to explore the association of T2DM with the risk of CRC thorough MEDLINE, EMBASE, Cochrane Library, and ISI Web of knowledge databases till 31st January 2014. Only English language original studies conducted on human subjects were considered with the following eligibility criteria: (1) Cohort studies which explored the risk of CRC by DM; and (2) Empirical studies with appropriate data for investigating RR with relation to CRC and DM.

Data was retrieved by connecting MeSH terms ("colorectal cancer" and "diabetes" and "risk" or "colon cancer" or "rectal cancer") in Endnote X5 which retrieved 575 citations as shown in Figure 1. After analysis of abstracts and titles 520 studies were excluded as irrelevant because these studies did not meet the inclusion criteria. During full text analysis of the remaining 55 relevant studies, 32 case-control and 15 theoretical and review articles were furtherer excluded. Only 8 relevant cohort studies were selected for further analysis. In this study, meta-analysis was done by using Forest plot which graphically presents the consistency and reliability of the results of selected studies. Forest plot was developed through Review Manager 5.3 software by Cochrane Library[13]. In this plot, effect size of each study is computed as an outcome and pooled effect size is also calculated to observe the heterogeneity among studies. *Q* test was used as a tool for verifying the heterogeneity in selected studies and its null hypothesis was that "all studies are identical". The I^2 statistic is an excellent method to ensure the quantity of heterogeneity in percentage terms and consistency of results of the selected studies[14]. After carefully analyzing the heterogeneity, next step is to apply appropriate effect summary model fixed effects or random effects model. If heterogeneity is low then it's better to apply fixed effects model while random effects is most commonly used model when heterogeneity is higher. The level of significance in this study is 5% ($P < 0.05$).

RESULTS

Association of T2DM and the risk of CRC; research outcome

The Forest plot in Figure 2 portrays a series of estimates and their confidence intervals (CI) at 95% level. Each individual study's effect size (outcome) is shown by a square and their CIs are represented through horizontal lines. The Forest plot shows that the selected studies have wider confidence interval and inconsistent response rates which indicates the heterogeneity. In order to check heterogeneity statistically, the *Q* test and I^2 statistics were applied. The results of χ^2 test in Figure 2 showed a 5% level of significance, thus rejecting the null hypothesis "all studies are identical". The value of I^2 is 96%, again verifying the presence of considerable heterogeneity amongst the studies. On the basis of considerable heterogeneity, random effects model was most appropriate for this study.

The effect summary which represents through diamond has RR of 1.21 (95%CI: 1.02-1.42) indicating positive relationship between DM and increased risk of CRC. There is great heterogeneity among all studies as only two studies have RR ratio below; Bella *et al*[15] has RR 0.97 and Jarvandi *et al*[16] has RR 0.93, while the remaining studies lie on the right side of one difference line showing positive association between DM and increased risk of CRC. The *Z* test is also significant at 5% level of significance and depicts significantly higher risk of CRC in diabetics by random-effects model. In

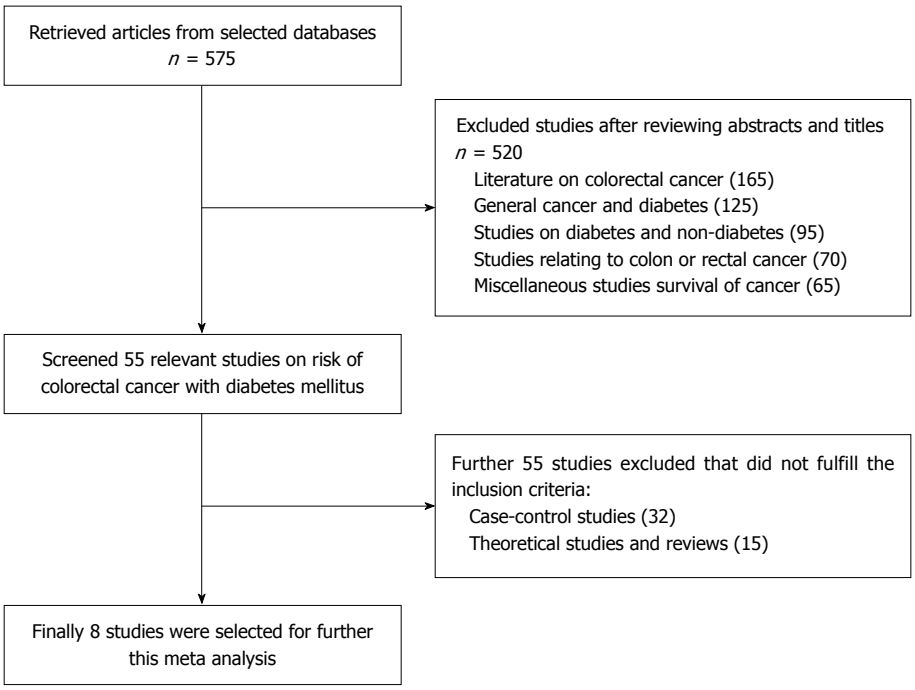

Figure 1 Flow diagram of the literature search mechanism used in the meta analysis.

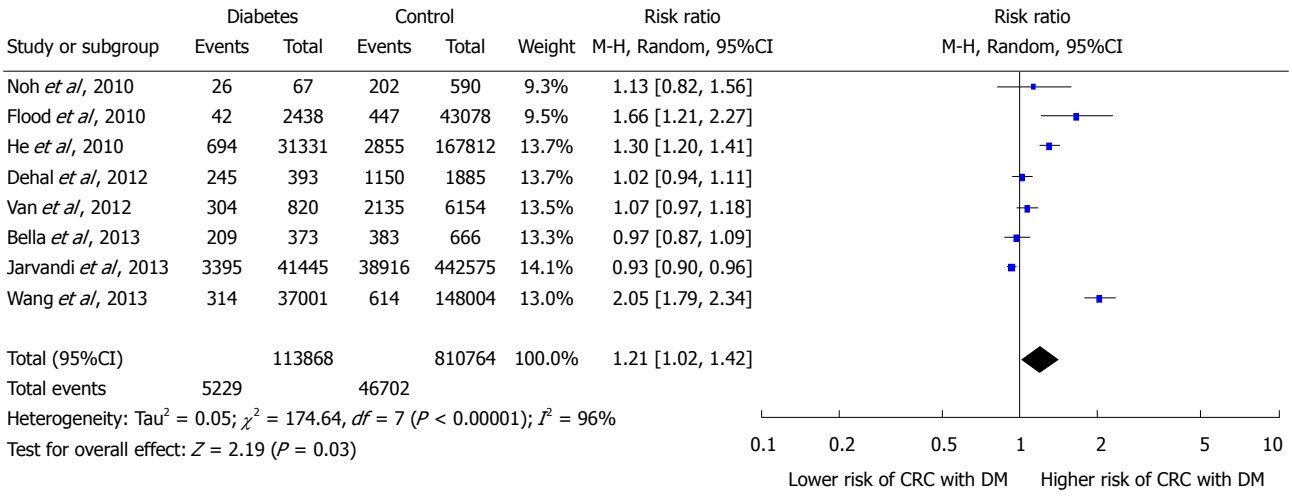

Figure 2 Forest plot showing the association between type 2 diabetes mellitus and colorectal cancer risk.

Figure 3, Forest plot of gender sub groups shows the consistent results as majority of the studies favors the right side and has greater than 1 RRs. The effect summary RR of 1.19 with (95%CI: 1.07-1.33) favor a positive relationship between DM and increased risk of CRC with significant heterogeneity (I^2 = 92% and *P*-value < 0.05). The *Z* test is significant at 5% level of significance for both sub groups showing significant risk of CRC with DM by random-effects model. Among both gender groups, women showed greater risk as their effect summary RR of 1.22 (95%CI: 1.01-49) with significant overall *Z* test at 5% level of significance was higher than the effect summary RR of 1.17 (95%CI: 1.00-1.37) of men showing insignificant *Z* test. However, both gender subgroups had considerable heterogeneity due to I^2 of 92% and 93% for men and women, respectively (Figure 3).

DISCUSSION

Literature review and analysis of the results of meta-analysis

The meta analysis showed a positive association of T2DM with the risk of CRC as shown by effect summary RR of 1.21 (95%CI: 1.02-1.42). Other studies have also demonstrated that CRC is more common in diabetics than in those without diabetes[7,17,18] and diabetic patients also have lower overall survival rates after CRC compared to non-diabetes, with 5-year survival of 35% and 48%, respectively[19,20].

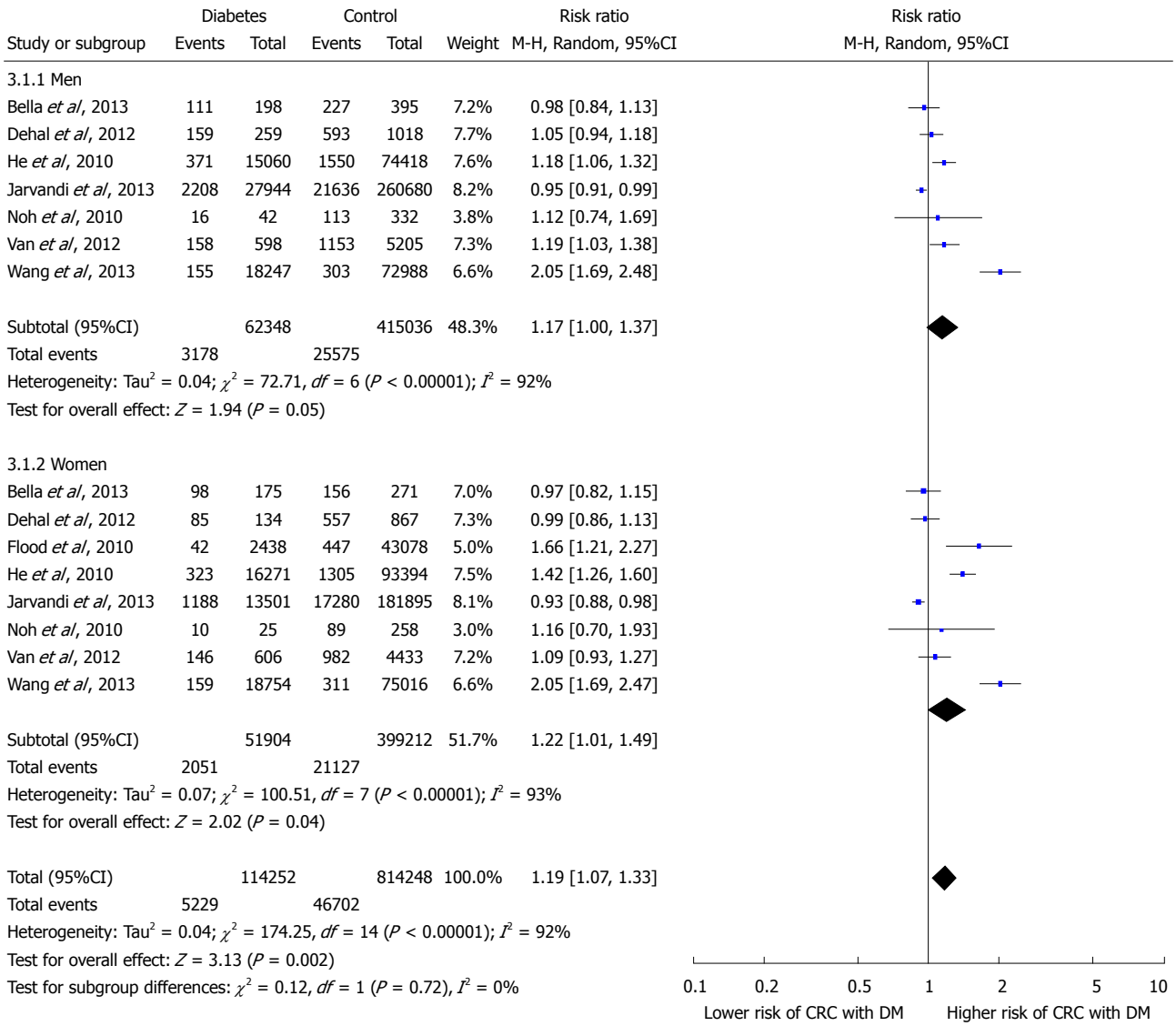

Figure 3 Forest plot illustrating the association between type 2 diabetes mellitus and colorectal cancer risk by gender.

A number of observational studies have described the association of T2DM with the risk of CRC[21-23]. A review of 97 prospective studies reported 123205 deaths among 820900 human subjects[24]. DM was associated with an increased risk of CRC (RR = 1.40; 95%CI: 1.20-1.63). Compared to people with fasting glucose levels < 5.6 mmol/L, the people with fasting glucose levels of 5.6-6.9 mmol/L had a 1.13-fold risk of death from any cancer. Participants with fasting glucose levels ⩾ 7.0 mmol/L showed a 1.39-fold risk of death from any cancer. European population-based or occupational cohorts involved in the DECODE (Diabetes Epidemiology: Collaborative Analysis of Diagnostic Criteria in Europe) study showed a significant increase in deaths from CRC in men with DM and prediabetes[25]. A meta-analysis of 15 studies including more than 2.5 million patients found about 30% increased relative risk of developing CRC in diabetic compared to nondiabetic people[26]. A study exploring the histopathological differences in CRC between populations with and without diabetes showed that diabetics had deeper tumor invasion, more lymphovascular invasion, and greater TNM staging [OR (95%CI): 2.06 (1.37-3.10), 2.52 (1.74-3.63), and 2.45 (1.70-3.52), respectively; $P < 0.001$][27]. This finding underpins the aggressive nature of CRC growths in the diabetic patients and demands appropriate measures for better control of DM.

New onset of DM is invariably considered as a marker of occult cancer, or of progression of a known disease (reverse causality: diabetes is a consequence of cancer)[28]. The pathogenesis of DM in the development of CRC has been elucidated in the literature. Low Vitamin D level[29], obesity, sedentary lifestyle, and a high fat diet are reported to be associated with an increased risk of CRC[30]. Since abdominal obesity and physical inactivity are strong independent determinants of insulin resistance and hyperinsulinemia, and high levels of insulin may stimulate the growth of colorectal tumors, hyperinsulinemia was considered

to mediate the effect of sedentary lifestyle on CRC risk. Hyperinsulinemia leads to CRC through a dose-dependent direct stimulation of cell growth and DNA synthesis in normal intestinal epithelial and CRC cells[31]. The intestinal endocrine L cells produce an incretin hormone, namely glucagon-like peptide-1, which stimulates insulin secretion in blood glucose dependent manner, pancreatic β cell proliferation and neogenesis[32]. Chronic hyperglycemia has been reported to induce an increasing production of reactive oxygen species, chronic oxidative stress[33], and marked activation of inflammatory pathways[34]. Inflammation is suggested to be one of the contributing mechanisms to the increased risk of cancerous growths[35].

The current meta-analysis showed that diabetic women had greater risk of CRC as their effect summary RR of 1.22 (95%CI: 1.01-49) with significant overall *Z* test at 5% level of significance was higher than the effect summary RR of 1.17 (95%CI: 1.00-1.37) of men. However, in their meta-analysis of 29 studies, Krämer *et al*[7] reported that overall estimates of RR were very similar amongst men (RR = 1.29; 95%CI: 1.19-1.140) and women (RR = 1.34; 95%CI: 1.22-1.47). Estimates of relative risk were very similar amongst men and women. Onitilo *et al*[36] examined the temporal relationship between CRC risk and DM using an electronic algorithm, clinical and lab data up to the onset of DM. The authors concluded that there was an increased risk of CRC in pre-diabetic men than women, and DM did not influence CRC risk in both genders after the clinical establishment of disease. In pre-diabetic men, CRC risk increased as time to DM onset decreased, indicating that the cumulative impacts of the pre-diabetes phase on colon cancer risk in men.

This meta-analysis has a limitation that the selected cohort studies might be prone to detection bias as the patients with diabetes are under continuous medical care, thus leading to high chances of CRC detection and early diagnosis.

In conclusion, this meta-analysis suggests that T2DM is associated with an increased risk of CRC. It is warranted to further investigate the underlying biological links between DM and CRC.

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COMMENTS

Background

As of 2010, more than 250 million people are suffering from type 2 diabetes mellitus (T2DM) worldwide; and this number is expected to reach 380 million in 20 years.

Research frontiers

This meta-analysis quantitatively assesses the results from published cohort studies to provide a more precise estimate of the association between T2DM as a possible predictor of the risk of colorectal cancer (CRC).

Innovations and breakthroughs

The effect summary RR of 1.19 with 95%CI of 1.07-1.33 indicate a positive relationship between DM and increased risk of CRC with significant heterogeneity (I^2 = 92% and *P*-value < 0.05).

Peer-review

Very good piece of work - methodology well done systematic review performed well; language very good. Topic of the review is important and innovative.

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META-ANALYSIS

Endoscopic submucosal dissection for early gastric cancer with undifferentiated-type histology: A meta-analysis

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Abstract

AIM: To evaluate the efficacy and safety of endoscopic submucosal dissection (ESD) for early gastric cancer (EGC) with undifferentiated-type histology.

METHODS: A systematic literature review was conducted using the core databases. Complete resection, curative resection, *en bloc* resection, recurrence and adverse event rate were extracted and analyzed. A random effect model was applied. The methodological quality of the enrolled studies was assessed using the Newcastle-Ottawa Scale. Publication bias was evaluated using a funnel plot, the trim and fill method, Egger's test, and a rank correlation test.

RESULTS: Fourteen retrospective studies between 2009 and 2014 were identified (972 EGC lesions with undifferentiated-type histology). The total *en bloc* and complete resection rates were estimated as 92.1% (95%CI: 87.4%-95.2%) and 77.5% (95%CI: 69.3%-84%), respectively. The total curative resection rate was 61.4% (95%CI: 44.5%-75.9%). The overall recurrence rate was 7.6% (95%CI: 3.4%-16%). Limited to histologically diagnosed expanded-criteria lesions, the *en bloc* and complete resection rates were 91.2% and 85.6%, respectively. The curative resection rate was 79.8%.

CONCLUSION: In this analysis, ESD is a technically feasible treatment modality for EGC with undifferentiated-type histology. Long-term studies are needed to confirm these therapeutic outcomes.

Key words: Carcinoma; Endoscopic submucosal dissection; Endoscopy; Gastric cancer; Meta-analysis

Core tip: Controversies regarding proposed expansions of the indication for endoscopic submucosal dissection (ESD)

for early gastric cancer (EGC) with undifferentiated-type histology still remain. In this meta-analysis, ESD is a technically feasible treatment modality for EGC with undifferentiated-type histology. However, cautious interpretation is needed because of heterogeneity among studies. Inconsistent implementation of indication, insufficient follow-up duration, and different outcome criteria are causes of heterogeneity. Further studies using common primary outcomes or large-scale, long-term studies will elucidate the feasibility of ESD for EGC with undifferentiated-type histology.

Bang CS, Baik GH, Shin IS, Kim JB, Suk KT, Yoon JH, Kim YS, Kim DJ, Shin WG, Kim KH, Kim HY, Lim H, Kang HS, Kim JH, Kim JB, Jung SW, Kae SH, Jang HJ, Choi MH. Endoscopic submucosal dissection for early gastric cancer with undifferentiated-type histology: A meta-analysis. *World J Gastroenterol* 2015; 21(19): 6032-6043 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6032.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6032

INTRODUCTION

Gastric cancer is a prevalent malignancy in East Asian countries[1]. With the widespread implementation of endoscopic screening programs in these countries, the proportion of patients with early gastric cancer (EGC) at the time of diagnosis has been increasing. Currently, endoscopic submucosal dissection (ESD) is the widely accepted treatment modality for a specific subset of EGC patients in South Korea and Japan[2,3]. However, the absolute indications for ESD for EGC have been criticized, because very strict criteria result in unnecessary operations[4].

Based on previous research, which stratified the risk of lymph node metastasis in patients with EGC, an expanded set of indications was proposed[5-7]. The proposed expanded criteria include the following: (1) differentiated-type mucosal adenocarcinoma without ulceration and lymphovascular invasion, irrespective of size; (2) differentiated-type mucosal adenocarcinoma 30 mm or smaller with ulceration and without lymphovascular invasion; (3) undifferentiated-type mucosal adenocarcinoma 20 mm or smaller without ulceration and lymphovascular invasion; and (4) differentiated-type adenocarcinoma 30 mm or smaller with minute submucosal invasion (SM1), but without lymphovascular invasion[3]. However, the results of clinical observations based on these expanded criteria have been conflicting, and endoscopic resection based on these indications is regarded as an investigational treatment[3].

EGC with undifferentiated-type histology generally refers to a poorly differentiated adenocarcinoma or signet ring cell carcinoma, although there are no such criteria in the WHO classification[8]. This group of cancers is included in the expanded indications in the Japanese guidelines based on clinical observations[3,5,9,10]. However, the results of clinical studies, including studies on EGC with undifferentiated-type histology, are conflicting. Thus, a meta-analysis was conducted to assess the feasibility of ESD for EGC patients with undifferentiated-type histology based on the expanded criteria.

MATERIALS AND METHODS

Literature search

MEDLINE (through PubMed), EMBASE, and the Cochrane Central Register of Controlled Trials (CENTRAL) in the Cochrane Library were searched using common keywords related to ESD for EGC with undifferentiated-type histology (from inception to April 2014). Medical Subject Headings (MeSH) terminology was used because all 3 databases permit searching using MeSH terminology. The keywords used included "gastric cancer", "endoscopic submucosal dissection", "ESD", "poorly differentiated", "signet ring cell carcinoma" or "undifferentiated" using Boolean operators. Only publications on human subjects were searched, and the bibliographies of relevant articles were also reviewed to identify additional studies. The language of publication was not restricted.

Selection criteria

Due to a lack of randomized-controlled studies relevant to this topic, we included non-randomized studies meeting both of the following criteria: (1) designed to evaluate ESD for EGC with undifferentiated-type histology in the target or control group; and (2) included at least one outcome (complete resection rate, curative resection rate, *en bloc* resection rate, recurrence rate or procedure-related adverse event rate) that enabled an evaluation of feasibility of ESD for EGC with undifferentiated-type histology. The exclusion criteria were as follows: (1) incomplete data; (2) review article; or (3) abstract only (study not published as full-text article).

Selection of relevant studies

Two of the authors (Bang CS and Baik GH) independently evaluated the eligibility of all studies retrieved from the databases based on the predetermined selection criteria. The abstracts of all identified studies were reviewed to exclude irrelevant articles. Full-text reviews were performed to determine whether the inclusion criteria were satisfied by the remaining studies. Disagreements between the two evaluators were resolved by discussion or consultation with a third author (Kim JH).

Assessment of methodological quality

The methodological quality of the enrolled studies was assessed using the Newcastle-Ottawa Scale. This tool comprises three parameters: the selection of the

study population, the comparability of the groups, and the ascertainment of the exposure or outcome. Each parameter consists of subcategorized questions: selection (n = 4), comparability (n = 1), and exposure or outcome (n = 3)[11,12]. The stars awarded for each item allow for a rapid visual assessment of the methodological quality of the studies. A study can be awarded a maximum of nine stars, indicating the highest quality. Two of the authors (Bang CS and Baik GH) independently evaluated the methodological quality of all the studies, and disagreements between the two evaluators were resolved by discussion or consultation with a third author (Kim DJ).

Main and modifier-based analyses

Two of the authors (Bang CS and Baik GH) independently extracted the outcomes of all the studies, and disagreements between the two evaluators were resolved by discussion or consultation with a third author (Kim JH). The primary outcomes were as follows: (1) *en bloc* resection rate: the proportion of cancers removed as a single piece without fragmentation: (2) complete resection rate: the proportion of cancer with no neoplastic components at the lateral or vertical margins on microscopic analysis, and without lymphovascular invasion; (3) curative resection rate: the proportion of cancers with 20 mm or less of intramucosal cancer without ulceration, without neoplastic components at the lateral or vertical margins, and without lymphovascular invasion; (4) recurrence rate: the proportion of cancers that reappeared at the site of the lesion (local recurrence) or synchronous, metachronous, or distant metastatic lesions, and (5) ESD adverse event rate: the proportion of cancers whose treatment resulted in procedure-related gastric hemorrhage or perforation. We also performed sensitivity analyses based on the indications for ESD (expanded *vs* beyond-expanded indication) and follow-up duration (long-term *vs* short-term follow-up). Both a cumulative analysis and a one-study-removed analysis were also performed.

Statistical analysis

Comprehensive Meta-Analysis (CMA) software (version 2.2.064, Borenstein M, Hedges L, Higgins J and Rothstein H. Englewood, NJ: Biostat; United States) was used for this meta-analysis. We calculated the pooled *en bloc* resection, complete resection, curative resection, recurrence and adverse event rates divided by gastric hemorrhage and perforation. To compare the efficacy of ESD according to treatment criteria (expanded *vs* beyond-expanded criteria), we calculated odds ratios (ORs) with 95% confidence intervals (CIs) using 2 × 2 tables from the original articles. Heterogeneity was tested using the I^2 test, which measures the percentage of total variation across studies[13]. I^2 was calculated as follows: I^2 (%) = 100 × (Q-df)/Q, where Q is Cochrane's heterogeneity statistic and df is the degrees of freedom. Negative values for I^2 were set to zero, and an I^2 value over 50% was considered to indicate substantial heterogeneity (range: 0%-100%)[14]. Pooled effect sizes with 95%CIs were calculated using a random effects model and the DerSimonian and Laird method due to methodological heterogeneity[15]. These results were confirmed again by the I^2 test. A fixed effects model using the inverse variance-weighted (Woolf's) method was used in the sensitivity analyses, including the cumulative and one-study-removed analyses, based on the assumption of a common effect size shared by the subgrouped studies[16,17]. Significance was set at P = 0.05 in both models. Publication bias was evaluated using Begg's funnel plot, Egger's test of the intercept, Duval and Tweedie's trim and fill, and Begg and Mazumdar's rank correlation test[18-22].

RESULTS

Identification of relevant studies

Figure 1 shows a flow diagram of how relevant studies were identified. A total of 170 articles were identified by a search of 3 core databases and a manual search of relevant bibliographies. In all, 28 duplicate studies and an additional 99 studies were excluded during the initial screening through a review of the titles and abstracts. The full texts of the remaining 43 studies were thoroughly reviewed. Among these studies, 29 were excluded from the final analysis. The reasons for study exclusion during the final review were as follows: review articles (n = 3), incomplete data (n = 3), or abstract only articles (n = 23). The remaining 14 non-randomized studies were included in the final analysis.

Characteristics of studies included in the final analysis

Among the 14 studies[23-36], we identified a total of 972 patients with EGC with undifferentiated-type histology. The clinical characteristics of the enrolled studies are shown in Tables 1-2 and Table 3. The enrolled studies were published between 2009 and 2014. All of the studies were conducted in Asia (10 studies in South Korea and 4 studies in Japan). Two studies were conducted in a multicenter setting[28,33], whereas the remaining studies were conducted in a single center setting. Twelve English and 2 Korean studies were selected. The duration of follow-up ranged from a median of 13.5 mo in one study to a mean of 101.9 mo in another. Ten studies reported *en bloc* resection rates, and 9 studies reported complete resection rates. Curative resection and recurrence rates were reported in 6 studies. The procedure-related adverse events included hemorrhage in 10 studies and perforation in 9 studies (Table 1).

In the evaluation of ESD based on the expanded criteria, we identified a total of 619 EGC patients with undifferentiated-type histology (11 studies). The clinical characteristics of the included studies are

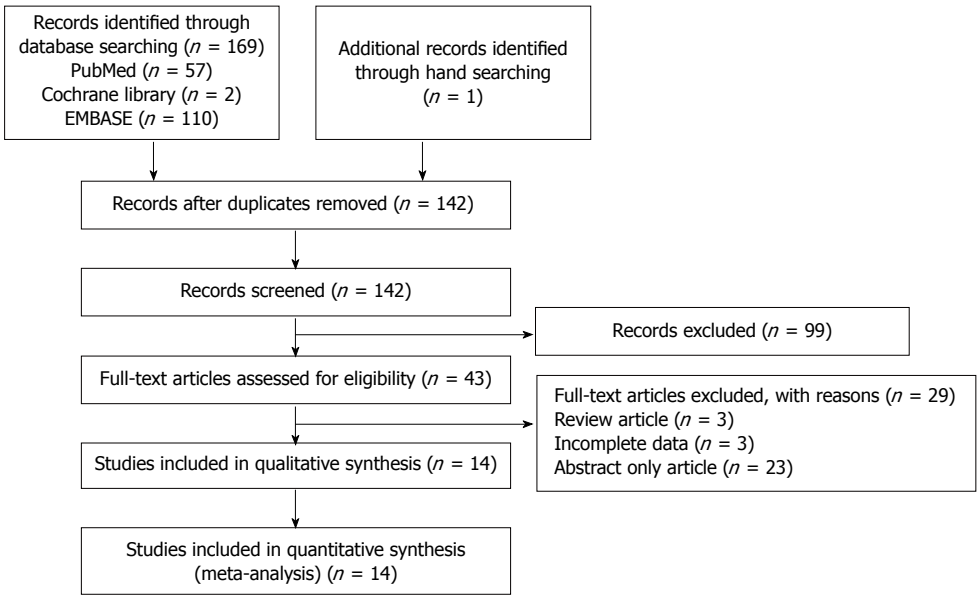

Figure 1 Flow diagram for identification of relevant studies.

Table 1 Clinical data of included studies

Ref.	Duration of follow up	Location, language	Complete resection	Curative resection	*En bloc* resection	Total recurrence	Adverse events	Total patients
Kim *et al*[23], 2009	Mean 17.1 ± 9.1 mo	South Korea (English)				1/32	Bleeding 6/32	32
Kang *et al*[24], 2010	Mean 16 mo	South Korea (English)	33/60		60/60	0/60	Bleeding 1/60 perforation 1/60	60
Lee *et al*[25], 2010	Median 13.5 mo	South Korea (English)		22/58[1]	48/58	0/16 (3 yr) 0/2 (5 yr)		58
Yamamoto *et al*[26], 2010		Japan (English)		46/58	57/58		Bleeding 5/58 perforation 2/58	58
Goh *et al*[27], 2011	Mean 19.39 ± 11.2 mo	South Korea (English)	10/18			3/14		18
Park *et al*[28], 2012	Mean 41 mo	South Korea, multicenter (2) (English)		23/55				55
Kamada *et al*[29], 2012	Mean 3.8 yr	Japan (English)	37/46		42/46	1/46[2]	Bleeding 2/46 perforation 2/46	46
Okada *et al*[30], 2012	Median 36 mo	Japan (English)		85/103	102/103		Bleeding 9/101 perforation 1/101	103
Park *et al*[31], 2013	Median 24.1 mo (absolute indication group), 30 mo (expanded indication group)	South Korea (English)	91/116		106/116		Bleeding 7/116 perforation 6/116	116
Choi *et al*[32], 2013	Mean 37.4 mo	South Korea (Korean)	66/82		72/82	3/82[3]		82
Kim *et al*[33], 2013	Median 34 mo	South Korea, multicenter (6) (English)	54/74	23/74	67/74	4/74	Bleeding 1/74 perforation 3/74	74
Abe *et al*[34], 2013	Median 76.4 mo	Japan (English)	88/97	62/97	96/97	2/79[4]	Bleeding 4/97 perforation 3/97 delayed perforation 1/97	97
Chung *et al*[35], 2014	Mean 41.7 ± 22.6 mo	South Korea (Korean)	58/76		64/76	9/64	Bleeding 4/76 perforation 0/76	76
Oka *et al*[36], 2014	Mean 101.9 ± 38.9 mo	Japan (English)	86/97	60/97			Bleeding 6/97 perforation 1/97	97

[1]Curative resection was defined as RM (-), LVI (-), and lesions not deeper than SM 500 um; [2]Seven patients who underwent operation after ESD were included; [3]Patients who underwent operation or APC ablation were included; [4]Nineteen patients who underwent operation after ESD were included. ESD: Endoscopic submucosal dissection.

shown in Table 2.

For a comparison of ESD based on the expanded *vs* beyond expanded criteria, we identified a total of 458 EGC patients (263 patients satisfying the expanded criteria *vs* 195 patients satisfying the beyond expanded criteria) with undifferentiated-type histology

Table 2 Clinical data of included studies (expanded indication)

Ref.	Complete resection	Curative resection	*En bloc* resection	Total recurrence	Total patients	Adverse events
Kang *et al*[24], 2010	17/18				18	
Lee *et al*[25], 2010	11/17				17	
Yamamoto *et al*[26], 2010	46/47[1]	46/47	47/47		47	Bleeding 5/47 perforation 2/47
Park *et al*[28], 2012		23/55			55	
Kamada *et al*[29], 2012	32/34				34	
Okada *et al*[30], 2012		85/103	102/103		103	
Park *et al*[31], 2013	91/116		106/116		116	Bleeding 7/116 perforation 6/116
Choi *et al*[32], 2013	66/82		72/82		82	
Kim *et al*[33], 2013	23/29	23/29	25/29		29	Bleeding 0/29 perforation 2/29
Chung *et al*[35], 2014	50/58		51/58		58	Bleeding 4/76 perforation 0/76
Oka *et al*[36], 2014	57/60				60	

[1]Complete *en bloc* resection.

Table 3 Clinical data of included studies (expanded indication *vs* beyond expanded indication)

Ref.	Location, language	Complete resection				*En bloc* resection		Recurrence	
		EI effective	EI total	Beyond EI effective	Beyond EI total	EI	Beyond EI	EI	Beyond EI
Kang *et al*[24], 2010	South Korea (English)	17	18	16	42	18/18	42/42	0/18	0/42
Lee *et al*[25], 2010	South Korea (English)	11	17	11	30				
Yamamoto *et al*[26] 2010	Japan (English)	46	47	6	11	47/47	10/11		
Kamada *et al*[29], 2012	Japan (English)	32	34	7	12			0/34	1/12
Kim *et al*[33], 2013	South Korea, multicenter (6) (English)	223	29	31	45			0/29	4/45
Chung *et al*[35], 2014	South Korea (Korean)	50	58	8	18	51/58	13/18		
Oka *et al*[36], 2014	Japan (English)	57	60	29	37				

(7 studies). The clinical characteristics of the included studies are shown in Table 3.

In terms of the methodological quality, the mean value of the awarded star was 7.6 [6 stars (1 study), 7 stars (5 studies), 8 stars (7 studies), and 9 stars (1 study) (Table 4)]. The majority of studies were classified as high quality, thus sensitivity analysis based on the methodological quality was not performed.

Overall efficacy and safety of ESD for EGC with undifferentiated-type histology

The overall efficacy of ESD for EGC with undifferentiated-type histology was evaluated using the *en bloc* resection, complete resection, curative resection, and recurrence rates. The total *en bloc* resection and complete resection rates were estimated as 92.1% (95%CI: 87.4%-95.2%, $P < 0.001$) and 77.5% (95%CI: 69.3%-84%, $P < 0.001$), respectively (Figure 2A, B).

The total curative resection rate was 61.4% (95%CI: 44.5%-75.9%, $P = 0.183$) (Figure 2C), and the overall recurrence rate was 7.6% (95%CI: 3.4%-16%, $P < 0.001$) (Figure 2D).

The overall safety of ESD for EGC with undifferentiated-type histology was evaluated according to procedure-related adverse events divided by gastric hemorrhage and perforation. The total procedure-related gastric hemorrhage and perforation rates were estimated as 6.5% (95%CI: 4.5%-9.4%, $P < 0.001$) and 3.3% (95%CI: 2.1%-5.0%, $P < 0.001$), respectively.

Overall efficacy and safety of ESD for EGC with undifferentiated-type histology based on expanded criteria

In the histologically diagnosed expanded-criteria lesions, the overall efficacy of ESD for EGC with undifferentiated-type histology was evaluated using the *en bloc* resection, complete resection, and curative resection rates. The total *en bloc* resection and complete resection rates were estimated as 91.2% (95%CI: 85.3%-94.8%, $P < 0.001$) and 85.6%

Table 4 Methodological quality of included studies measured by Newcastle-Ottawa scale

Ref.	Selection	Comparability	Exposure or outcome	Total
Kim *et al*[23], 2009	4	1	2	7
Kang *et al*[24], 2010	4	1	2	7
Lee *et al*[25], 2010	4	2	2	8
Yamamoto *et al*[26], 2010	4	1	3	8
Goh *et al*[27], 2011	3	1	2	6
Park *et al*[28], 2012	3	1	3	7
Kamada *et al*[29], 2012	4	1	3	8
Okada *et al*[30], 2012	4		3	7
Park *et al*[31], 2013	4	2	2	8
Choi *et al*[32], 2013	4	2	3	9
Kim *et al*[33], 2013	4	1	3	8
Abe *et al*[34], 2013	4	1	3	8
Chung *et al*[35], 2014	3	2	3	8
Oka *et al*[36], 2014	3	1	3	7

(95%CI: 78.5%-90.7%, $P < 0.001$), respectively. The total curative resection rate was 79.8% (95%CI: 51.4%-93.6%, $P = 0.041$).

The overall safety of ESD for EGC with undifferentiated-type histology based on expanded criteria was evaluated according to procedure-related adverse events divided by gastric hemorrhage and perforation. The total procedure-related gastric hemorrhage and perforation rates were estimated as 6.7% (95%CI: 4.1%-10.8%, $P < 0.001$) and 4.8% (95%CI: 2.6%-8.6%, $P < 0.001$), respectively.

Comparison of the efficacy of ESD for EGC with undifferentiated-type histology between expanded and beyond expanded criteria

To compare the efficacy of ESD between the expanded and beyond expanded criteria, ORs with 95%CIs for *en bloc* resection, complete resection, and recurrence were calculated. ESD based on the expanded criteria showed an OR of 3.475 (95%CI: 1.039-11.622, $P = 0.043$) for *en bloc* resection compared to ESD based on the beyond expanded criteria. ESD based on the expanded criteria showed an OR of 7.461 (95%CI: 3.027-18.394, $P < 0.001$) for complete resection compared to ESD based on the beyond expanded criteria. ESD based on the expanded criteria showed an OR of 0.134 (95%CI: 0.015-1.203, $P = 0.073$) for recurrence compared to ESD based on the beyond expanded criteria.

Sensitivity meta-analysis

The cumulative meta-analysis of the enrolled studies in the order of year published showed a constant but slightly increasing trend in *en bloc* resection rate. With regard to complete resection, the cumulative meta-analysis of the enrolled studies showed an increasing trend in complete resection rate. However, the curative resection rate showed a decreasing trend in the cumulative meta-analysis. In the evaluation of recurrence, the cumulative meta-analysis showed an increasing trend in the recurrence rate after ESD.

In the histologically diagnosed expanded-criteria lesions, the cumulative meta-analysis of the enrolled studies in the order of year published showed a constant but slightly decreasing trend in the *en bloc* resection rate. As for complete resection, the cumulative meta-analysis showed 2 outlier effect sizes[24,25]. Kang *et al*[24] showed the biggest effect size (complete resection rate: 17/18), and Lee *et al*[25] showed the smallest effect size (complete resection rate: 11/17). These 2 studies included the smallest numbers of patients of histologically diagnosed expanded-criteria lesions in the analysis (Table 2), whereas the other remaining studies showed relatively consistent effect sizes. The curative resection rate showed a decreasing trend in the cumulative meta-analysis of the enrolled studies.

The one-study-removed meta-analysis of the enrolled studies in the order of year published showed consistent results and no specific outlier for the *en bloc* resection rate. As for complete resection, the one-study-removed meta-analysis also showed consistent results. For the analysis of curative resection, the one-study-removed meta-analysis highlighted 2 influential studies[28,33]. These studies reported relatively lower curative resection rates; however, the methodological quality of these studies was not low (Table 4). Moreover, these 2 studies were performed in a multicenter setting. In the evaluation of recurrence, the one-study-removed analysis identified 3 influential studies[27,33,35]. Two studies[27,35] reported relatively higher recurrence rates, and 1 study[33] reported a relatively lower recurrence rate (Table 1). The follow-up duration was relatively short in the Goh *et al*[27]' s study (mean 19.39 ± 11.2 mo), and this study had the lowest methodological quality among the included studies (Tables 1 and 4). However, the follow-up durations in the studies by Chung *et al*[35] and Kim *et al*[33] were not in the short-term category (mean: 41.7 ± 22.6 mo), and the methodological quality was not low (Tables 1 and 4).

In the histologically diagnosed expanded-criteria lesions, the one-study-removed meta-analysis of the enrolled studies in the order of year published showed consistent results and no specific outlier for the *en bloc* resection rate. As for complete resection, the cumulative meta-analysis showed consistent results. For the analysis of curative resection, the one-study-removed meta-analysis identified 2 influential studies[28,30]. As in the total population analysis, Park *et al*[28] reported a relatively lower curative resection rate, whereas Okada *et al*[30] reported a relatively higher curative resection rate. The methodological quality of these studies was not low (Table 4).

To determine the total recurrence rate, a sensitivity analysis was performed by dividing the studies into a shorter follow-up duration group and a longer follow-up duration group. The distribution of the follow-up duration was as follows: mean 13.5, 16, 17.1, 19.39 mo, median 34 mo, and mean 41.7 mo. The studies

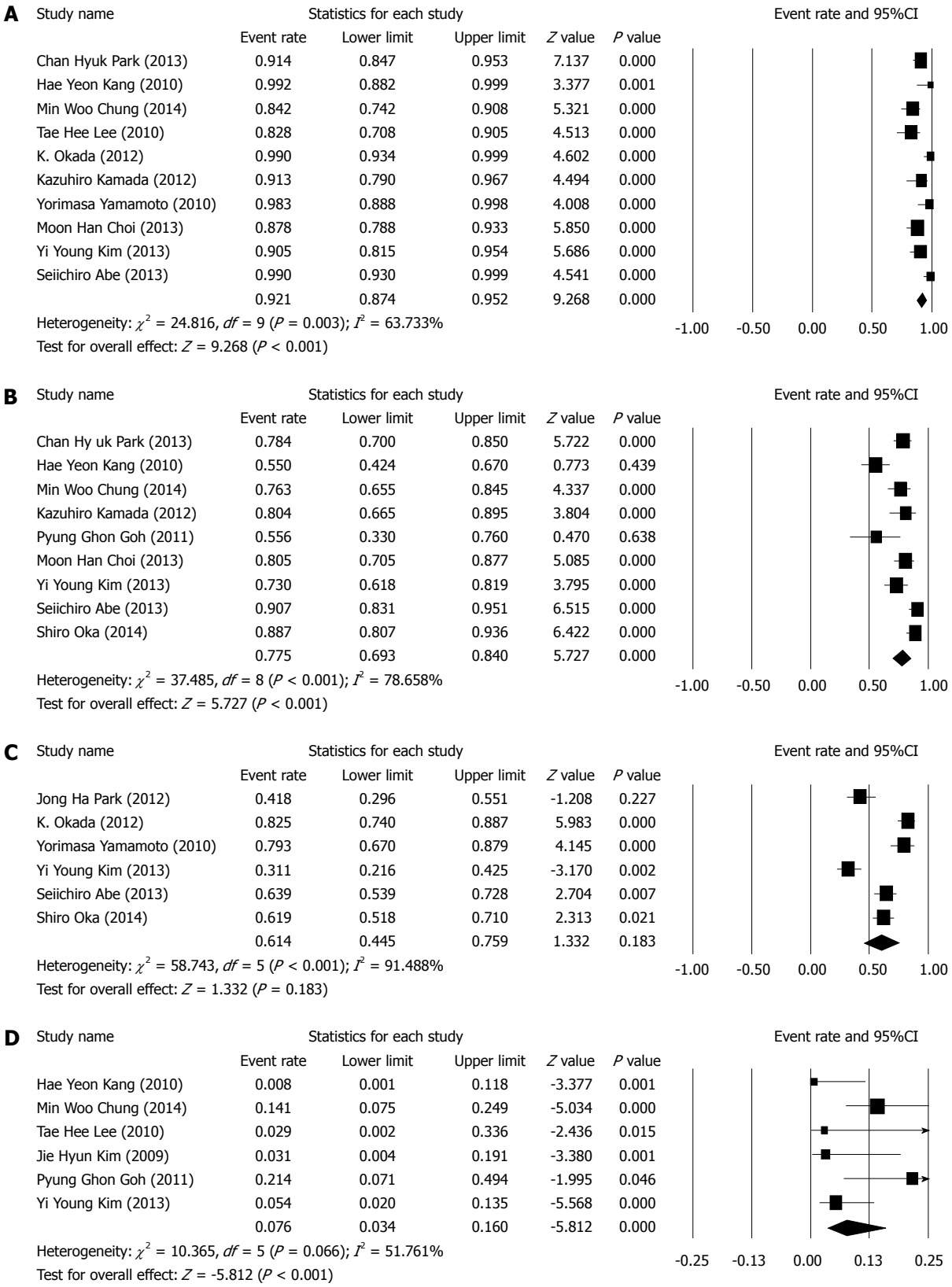

Figure 2 Enrolled studies. A: Total *en bloc* resection rate; B: Total complete resection rate; C: Total curative resection rate; D: Total recurrence rate. The size of each square is proportional to the study's weight. Diamond is the summary estimate from the pooled studies (random effect model).

with the former 4 follow-up duration times[23-25,27] were categorized into the shorter duration group, and the studies with the latter 2 follow-up duration times[33,35] were sorted into the longer duration group. An analysis of the studies in the shorter follow-up duration group showed a recurrence rate of 8.1% (95%CI:

0.033-0.185, $P < 0.001$). However, an analysis of the studies in the longer follow-up duration group showed a recurrence rate of 10.4% (95%CI: 0.061-0.171, $P < 0.001$).

Analysis of publication bias

A funnel plot for the enrolled studies is presented. For the studies of *en bloc* resection rate, the funnel plot is asymmetrical. Egger's regression test showed that the intercept was 3.334 [95%CI: 2.066-4.602, *t*-value: 6.064, $df = 8$, $P < 0.001$ (1-tailed) and $P < 0.001$ (2-tailed)]. A trim and fill analysis showed that 3 studies were missed or trimmed. The rank correlation test showed a Kendall's tau of 0.444 with a continuity correction [$P = 0.037$ (1-tailed) and $P = 0.074$ (2-tailed)].

For the studies of complete resection rate, the funnel plot is symmetrical. Egger's regression test showed that the intercept was 1.452 [95%CI: -7.706-10.611, *t*-value: 0.375, $df = 7$, $P = 0.359$ (1-tailed) and $P = 0.719$ (2-tailed)]. A trim and fill analysis showed that no study was missed or trimmed. The rank correlation test showed a Kendall's tau of 0.250 with a continuity correction [$P = 0.174$ (1-tailed) and $P = 0.348$ (2-tailed)].

For the studies of curative resection rate, the funnel plot is symmetrical. Egger's regression test showed that the intercept was 2.878 [95%CI: -26.503-32.259, *t*-value: 0.272, $df = 4$, $P = 0.400$ (1-tailed) and $P = 0.799$ (2-tailed)]. A trim and fill analysis showed that 1 study was missed or trimmed. The rank correlation test showed a Kendall's tau of 0.133 with a continuity correction ($P = 0.354$ (1-tailed) and $P = 0.707$ (2-tailed)).

For the studies of recurrence rate, the funnel plot is symmetrical. Egger's regression test showed that the intercept was -1.964 [95%CI: -4.832-0.905, *t*-value: 1.901, $df = 4$, $P = 0.065$ (1-tailed) and $P = 0.130$ (2-tailed)]. A trim and fill analysis showed that no study was missed or trimmed. The rank correlation test showed a Kendall's tau of -0.267 with a continuity correction [$P = 0.226$ (1-tailed) and $P = 0.452$ (2-tailed)].

For the studies of *en bloc* resection rate for EGC with expanded criteria, the funnel plot is asymmetrical. Egger's regression test showed that the intercept was 2.437 [95%CI: -0.299-5.173, *t*-value: 2.473, $df = 4$, $P = 0.034$ (1-tailed) and $P = 0.069$ (2-tailed)]. A trim and fill analysis showed that 1 study was missed or trimmed. The rank correlation test showed a Kendall's tau of 0.267 with a continuity correction [$P = 0.226$ (1-tailed) and $P = 0.452$ (2-tailed)].

For the studies of complete resection rate for EGC with expanded criteria, the funnel plot is asymmetrical. Egger's regression test showed that the intercept was 2.340 [95%CI: 0.164-4.515, *t*-value: 2.543, $df = 7$, $P = 0.019$ (1-tailed) and $P = 0.039$ (2-tailed)]. A trim and fill analysis showed that 3 studies were missed or trimmed. The rank correlation test showed a Kendall's tau of 0.417 with a continuity correction [$P = 0.059$ (1-tailed) and $P = 0.118$ (2-tailed)].

For the studies of curative resection rate for EGC with expanded criteria, the funnel plot is asymmetrical. Egger's regression test showed that the intercept was 3.814 [95%CI: -15.749-23.376, *t*-value: 0.839, $df = 4$, $P = 0.245$ (1-tailed) and $P = 0.490$ (2-tailed)]. A trim and fill analysis showed that 1 study was missed or trimmed. The rank correlation test showed a Kendall's tau of 0.000 with a continuity correction [$P = 0.500$ (1-tailed) and $P > 0.999$ (2-tailed)].

For the studies of complete resection rate by expanded criteria (*vs* beyond-expanded criteria), the funnel plot is asymmetrical. Egger's regression test showed that the intercept was 4.188 [95%CI: 0.779-7.598, *t*-value: 3.411, $df = 4$, $P = 0.014$ (1-tailed) and $P = 0.027$ (2-tailed)]. A trim and fill analysis showed that 3 studies were missed or trimmed. The rank correlation test showed a Kendall's tau of 0.800 with a continuity correction [$P = 0.012$ (1-tailed) and $P = 0.024$ (2-tailed)].

Overall, publication bias was detected in the analysis of *en bloc* resection rate for total EGC lesions. However, there was no evidence of publication bias in the analysis of total lesions, except for *en bloc* resection rate. In the histologically diagnosed expanded-criteria lesions, publication bias was detected in all of the analyses (*en bloc*, complete, and curative resection rates). The comparison of complete resection rate divided by expanded and beyond expanded criteria showed publication bias.

DISCUSSION

In this meta-analysis, ESD is a technically feasible treatment modality for the treatment of EGC with undifferentiated-type histology. The overall *en bloc* resection rate was 92.1%, and the overall complete resection rate was 77.5%. If limited to histologically diagnosed expanded criteria lesions, the overall complete resection rate increased (85.6%). In terms of the procedure-related adverse events, the reported gastric hemorrhage or perforation rate for the treatment of EGC with undifferentiated-type histology was not different from the rates reported in previous studies including intestinal type EGC[37]. This finding was confirmed again in the sensitivity analyses. The cumulative meta-analysis of the total *en bloc* and complete resection rates showed a recent increasing trend. The advancement of ESD instruments and technique seem to be the cause of technical feasibility for EGC with undifferentiated-type histology.

However, the therapeutic outcomes are not totally satisfactory. The overall curative resection rate was 61.4%, although it increased to 79.8% if limited to histologically diagnosed expanded-criteria lesions. This finding was confirmed again in the sensitivity analyses.

The one-study-removed meta-analysis showed that multicenter studies[28,33] reported relatively lower curative resection rates. Additionally, the overall recurrence rate was 7.6%, which is slightly higher than that of previous studies[35,38-40]. Some studies concluded that ESD for EGC with undifferentiated-type histology is a feasible treatment modality despite relatively lower complete or curative resection rates and a higher recurrence rate compared to other studies. However, there is no acceptable complete or curative resection rate standard for determining the feasibility of ESD. Moreover, all of the enrolled studies were performed retrospectively. Thus, selection bias could influence the therapeutic outcomes of ESD.

To obtain higher complete and curative resection rates based on this meta-analysis, performing ESD according to the expanded criteria rather than the beyond expanded criteria seems to be the appropriate approach for the treatment of undifferentiated-type EGC. However, the expanded criteria were developed based on retrospective studies of surgically treated EGC patients[3,5,7]. Discrepancies between pre- and post-ESD indication or pre- and post-ESD histology have been also reported[24,25,41]. ESD performed based on the expanded criteria could be found to have been based on the beyond expanded criteria after the procedure. A more serious problem is the difficulty in determining tumor extent and depth of invasion of EGC with undifferentiated-type histology. EGC with undifferentiated-type histology is known to extend laterally along the proliferative zone in the intermediate layer of mucosa and the development pattern from the intermediate layer type to the superficial type makes non-exposure to the surface mucosa[42]. The accuracy of EUS in the assessment of depth of invasion for EGC with undifferentiated-type histology is known to be declining compared to intestinal type EGC[43]. Accurately defining tumor extent and depth of invasion could be difficult for EGC with undifferentiated-type histology.

Another issue is the histologic heterogeneity (mixture of undifferentiated components with differentiated EGC). Neither the characteristics nor feasibility of ESD for this type of EGC have been settled[44-46]. As previously mentioned, the discrepancy between pre- and post-ESD histology could be a problem in the assessment of surgery indications or the risk of lymph node metastasis.

The majority of studies on EGC with undifferentiated-type histology do not report outcomes according to whether the tumors are signet ring cell carcinoma or poorly differentiated adenocarcinoma. Only 2 studies among the included articles performed separate analyses[23,32]. These studies commonly reported slightly better therapeutic outcomes (complete resection or *en bloc* resection rates) in signet ring cell carcinoma than in poorly differentiated adenocarcinoma or poorly differentiated adenocarcinoma with signet ring cell features, although the differences were statistically insignificant. However, more studies are needed to confirm these findings.

In addition to the results of this study, there is a fundamental criticism about the expanded criteria for ESD. As previously mentioned, there is a discrepancy in the term "EGC with undifferentiated-type histology" between the WHO classifications and the Japanese literature[3,8]. There is no such term in the WHO classification system. However, the term "undifferentiated-type EGC" is frequently used, and studies using this terminology are being published. Moreover, in terms of the therapeutic outcomes, curative resection was generally accepted as being complete resection satisfying the expanded criteria proposed by Japanese groups in the literature. However, analyzing results based on this definition could lead to misinterpretations. Curative resection implies neither a cure nor a low risk of recurrence. Furthermore, the expanded indication was developed based on a retrospective analysis of surgically resected EGCs. Regarding EGC tumor size in the expanded indication, some data on lymph node metastasis that are not completely consistent with Japanese studies have been reported[47-49]. Despite the criticism of the expanded indication for ESD to treat EGC, nearly all of the studies are being performed based on the definition proposed by Japanese groups. To solve these fundamental problems related to the indication for ESD, randomized or well-organized large-scale studies separately focused on size, depth of invasion, histologic type, and lymph node metastasis are needed.

This study is the first meta-analysis of the therapeutic outcomes of ESD for EGC with undifferentiated-type histology. A strength of this study is the rigorous search of the literature, which was not limited by language, although data from Western studies were lacking. Potential modifiers were detected when possible, and sensitivity analyses were performed to confirm the robustness of the results.

Despite these strengths, there are several limitations of the present study. First, there are no data on lymph node metastasis. Lymph node metastasis is one of the most important prognostic factors for EGC patients. The rate of lymph node metastasis is known to be approximately 2% in mucosal cancers and approximately 20% in submucosal invasive cancers[5,50]. The risk of lymph node metastasis is known to be higher in EGC with undifferentiated-type histology compared to intestinal type EGC due to lymphovascular invasion, which was estimated to be 14% in a study of post-gastrectomy patients[51]. Moreover, micrometastasis which is associated with worse disease-free survival was reported as 13.3% in the EGC with undifferentiated-type histology[52]. However, there is no definitive method to detect lymph node metastasis accurately before surgery. Second, there was substantial methodological heterogeneity between the included studies, which potentially affected the effect size estimates. The most noticeable

modifier was the heterogeneity in the reported outcomes and the inconsistent implementation of the indication. The reported outcomes for the *en bloc* resection, complete resection (R0 resection), curative resection, *en bloc* complete resection, and recurrence, rates were various and not consistent between the enrolled studies. Moreover, the outcomes were not defined in detail. For example, the recurrence rate was not divided according to local recurrence, synchronous or metachronous recurrence. The indication was also inconsistently implemented; thus, the beyond-expanded criteria were used for a substantial portion of the patients, despite the discrepancies between the pre- and post-ESD indications. The follow-up duration was another significant modifier. The sensitivity analysis of longer follow-up duration studies showed that the recurrence rate was higher than in shorter follow-up duration studies. These limitations are sources of heterogeneity and contributed to publication bias. Due to the lack of prospective or randomized studies on this topic, large-scale, well-organized, long-term follow-up studies are needed to elucidate the feasibility of ESD on EGC with undifferentiated-type histology. Prospective clinical trial by Japan Clinical Oncology Group completed recruiting patients with EGC with undifferentiated-type histology and outcomes are anticipated[53].

Based on this analysis, ESD is a technically feasible treatment modality for EGC with undifferentiated-type histology. However, cautious interpretation is needed because of heterogeneity among studies. Inconsistent implementation of the indication, insufficient follow-up duration, and differences in outcome measures are causes of heterogeneity. Further studies using common primary outcomes or large-scale, long-term studies will determine the feasibility of ESD for EGC with undifferentiated-type histology.

COMMENTS

Background

Endoscopic submucosal dissection (ESD) is the widely accepted treatment modality for a specific subset of early gastric cancer (EGC) patients in gastric cancer prevalent Asian countries. EGC with undifferentiated-type histology generally refers to a poorly differentiated adenocarcinoma or signet ring cell carcinoma, although there are no such criteria in the WHO classification. This group of cancers is included in the expanded indications in the Japanese guidelines based on clinical observations. However, the results of clinical studies, including studies on EGC with undifferentiated-type histology, are conflicting.

Research frontiers

Some studies concluded that ESD for EGC with undifferentiated-type histology is a feasible treatment modality despite relatively lower complete or curative resection rates and a higher recurrence rate compared to other studies. However, there is no acceptable complete or curative resection rate standard for determining the feasibility of ESD. Moreover, all of the enrolled studies were performed retrospectively. Thus, selection bias could influence the therapeutic outcomes of ESD.

Innovations and breakthroughs

From the fourteen retrospective studies, therapeutic outcomes were calculated. The total *en bloc* and complete resection rates were estimated as 92.1% (95%CI: 87.4%-95.2%) and 77.5% (95%CI: 69.3%-84%), respectively. The total curative resection rate was 61.4% (95%CI: 44.5%-75.9%). The overall recurrence rate was 7.6% (95%CI: 3.4%-16%). Limited to histologically diagnosed expanded-criteria lesions, the *en bloc* and complete resection rates were 91.2% and 85.6%, respectively. The curative resection rate was 79.8%.

Applications

In this analysis, ESD is a technically feasible treatment modality for EGC with undifferentiated-type histology. However, cautious interpretation is needed because of heterogeneity among studies. Inconsistent implementation of indication, insufficient follow-up duration, and different outcome criteria are causes of heterogeneity. Further studies using common primary outcomes or large-scale, long-term studies will elucidate the feasibility of ESD for EGC with undifferentiated-type histology.

Terminology

EGC: EGC is defined as gastric cancer that invades no more deeply than the submucosa, irrespective of lymph node metastasis. ESD: ESD has been developed for *en bloc* removal of large (usually more than 2 cm), flat GI tract lesions using specialized endoscopic knife to dissect lesions from the submucosa. It offers the potential to remove mucosal and submucosal tumors *en bloc*.

Peer-review

Authors used appropriate methods of analysis and elaborated this interesting meta-analysis on endoscopic submucosal dissection in the treatment of EGC with undifferentiated-type histology.

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META-ANALYSIS

Infliximab is superior to other biological agents for treatment of active ulcerative colitis: A meta-analysis

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Abstract

AIM: To compare the efficacy and safety of biological agents for the treatment of active ulcerative colitis (UC).

METHODS: PubMed, MEDLINE, EMBASE and the Cochrane library were searched to screen relevant articles from January 1996 to August 2014. The mixed treatment comparison meta-analysis within a Bayesian framework was performed using WinBUGS14 software. The proportions of patients reaching clinical response, clinical remission and mucosal healing in induction and maintenance phases were analyzed as efficacy indicators. Serious adverse events in maintenance phase were analyzed as safety indicators.

RESULTS: The meta-analysis results showed that biological agents achieved better clinical response, clinical remission and mucosal healing than placebo. Indirect comparison indicated that in induction phase, infliximab was more effective than adalimumab in inducing clinical response (OR = 0.41, 95%CI: 0.29-0.57), clinical remission (OR = 0.33, 95%CI: 0.19-0.56) and mucosal healing (OR = 0.33, 95%CI: 0.19-0.56), and golimumab in inducing clinical response (OR = 0.66, 95%CI: 0.39-2.33) and mucosal healing (OR = 2.15, 95%CI: 1.18-4.22). No significant difference was found between placebo and biological agents regarding their safety.

CONCLUSION: All biological agents were superior to placebo for UC treatment in both induction and maintenance phases with a similar safety profile, and infliximab had a better clinical effect than the other biological agents.

Key words: Biological agents; Drug safety; Efficacy; Meta-analysis; Ulcerative colitis

Core tip: Currently the selection of biological agents in ulcerative colitis (UC) therapy is still controversial. We performed this meta-analysis to compare the efficacy and safety of biological agents for the treatment of active UC, and finally found that all biological agents were superior to placebo for UC treatment in both induction and maintenance phases with a similar safety

profile, and infliximab had a better clinical effect than the other biological agents.

Mei WQ, Hu HZ, Liu Y, Li ZC, Wang WG. Infliximab is superior to other biological agents for treatment of active ulcerative colitis: A meta-analysis. *World J Gastroenterol* 2015; 21(19): 6044-6051 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6044.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6044

INTRODUCTION

Ulcerative colitis (UC) is a form of chronic inflammatory bowel disease (IBD) characterized by recurrent rectal bleeding, increased stool frequency and urgency, abdominal cramps and pain, and systemic symptoms (such as fever, anemia and weight loss)[1,2]. It is reported that the incidence of UC is 1.2-20.3 per 100000 person-years and its prevalence is 7.6-246.0 per 100000 persons[3]. Current options of treatment include aminosalicylates, corticosteroids, immunosuppressive medications such as azathioprine and 6-mercaptopurine, and biological agents including tumor necrosis factor-α (TNF-α) antibodies and integrin antagonists.

5-aminosalicylic acid (5-ASA) is the first-line medication used to induce and maintain remission in patients with mild-to-moderate active UC[4]. Patients who do not have an adequate response to 5-ASA are recommended to receive corticosteroid treatments[5]. Moreover, traditional immunosuppressive azathioprine (AZA) and 6-mercaptopurine are suggested to treat patients with moderate active UC who are not responsive to oral corticosteroids[6]. However, conventional treatments often lead to a series of adverse events and have a limited effect in long-term disease control.

Anti-TNF-α agents including infliximab, adalimumab and golimumab have been approved by United States Food and Drug Administration for the treatment of moderate-to-severe UC. All the 3 anti-TNF-α agents are demonstrated to be effective for the induction and maintenance of remission in moderate or severe UC. In addition, these agents can also induce mucosal healing and reduce glucocorticoid dependence[7]. Vedolizumab is a humanized immunoglobulin G1 monoclonal antibody to α4β7 integrin[8]. A phase 3 study investigating the efficacy and safety of vedolizumab in patients with moderate to severe active UC indicated that vedolizumab was significantly more effective in terms of clinical response and remission compared to placebo in both induction and maintenance phases[9].

Currently, the selection of biological agents in UC therapy is still controversial. Traditional methods cannot be applied for the comparison for lack of head-to-head studies comparing different biological agents. Therefore, we used a mixed treatment comparison (MTC) to compare the efficacy of biological agents, as MTC was available for indirect comparisons between drugs with different comparators[10,11].

MATERIALS AND METHODS

Search strategy and inclusion criteria

Four databases (PubMed, EMBASE, MEDLINE and the Cochrane library) were screened to obtain articles from January 1996 to August 2014 for inclusion in this study. The key words "ulcerative colitis"and ("infliximab" or "adalimumab" or "golimumab" or "vedolizumab") were used to search relevant articles. We included those studies meeting the following two criteria: (1) the study evaluated the efficacy of biological treatments using a random case-control design; and (2) trials had to be placebo controlled.

Data extraction and quality assessment

The following information was extracted from each study: the first author's name; the year of publication; the number of patients; the number of patients achieving clinical response; the number of patients achieving clinical remission; the number of patients achieving mucosal healing; the outcome of serious adverse events; endpoints; and study duration. The Jadad score was used to assess the quality of the included studies. Different doses of the same biological agent were regarded as separate interventions. Odds ratios (ORs) were used to measure the outcome of clinical response, clinical remission, mucosal healing and serious adverse events in induction and maintenance phases. Sandborn *et al*[12,13] and Feagan *et al*[14] presented induction phase results at week 6, and their studies were analyzed with trials presenting results at week 8[9]. Sandborn *et al*[12,13] presented maintenance phase results at week 54, and this study was analyzed with trials presenting results at week 52.

Statistical analysis

To evaluate the relative effectiveness of each biological agent, an MTC meta-analysis within a Bayesian framework was performed. For all Bayesian analyses, Markov-chain-Monte-Carlo methods were used[15]. A random effect model was used to estimate the ORs as the measurement of relative treatment effect. We carried out 60000 iterations. The first 10000 iterations were discarded after the burn-in period and estimates were based on the subsequent 50000 ones. Heterogeneity between studies was assessed by Cochrane Q statistics and I^2 test. A significant level of no less than 50% for I^2 test was considered as evidence of heterogeneity. A fixed effects model was used when there was no evidence of heterogeneity, otherwise a random effects model was chosen. Data analysis was performed using WinBUGS version 1.4.3 (MRC Biostatistics Unit, Cambridge, United Kingdom)

Table 1 Baseline characteristics of the included studies

Study	Age (yr)	Drug and dose	Case	Severity	Treatment	Duration (wk)
ACT 1 (Rutgeerts *et al*[16], Feagan *et al*[17], Sandborn *et al*[18])	41.4 ± 13.7	Placebo	121	Moderate to-severe active UC	Intravenous infusions at weeks 0, 2 and 6 and then every eight weeks or matching placebo	54
	42.4 ± 14.3	Infliximab 5 mg/kg	121			
	41.8 ± 14.9	Infliximab 10 mg/kg	122			
ACT 2 (Rutgeerts *et al*[16], Feagan *et al*[17], Sandborn *et al*[18])	39.3 ± 13.5	Placebo	123	Moderate-to-severe active UC	Intravenous infusions at weeks 0, 2 and 6 and then every eight weeks or matching placebo	30
	40.5 ± 13.1	Infliximab 5 mg/kg	121			
	40.3 ± 13.3	Infliximab 10 mg/kg	120			
Suzuki *et al*[19]	41.3 ± 13.6	Placebo	96	Moderate-to-severe active UC	Subcutaneous injections 160/80 mg at week 0, 80/40 mg at week 2 and then 40 mg beginning at week 4 every other week or matching placebo	52
	44.4 ± 15.0	Adalimumab 80/40 mg	87			
	42.5 ± 14.6	Adalimumab 160/80 mg	90			
ULTRA 2 (Sandborn *et al*[20])	41.3 ± 13.2	Placebo	246	Moderate-to-severe active UC	Subcutaneous injections 160 mg at week 0, 80 mg at week 2 and then 40 mg beginning at week 4 every other week or matching placebo	52
	39.6 ± 12.5	Adalimumab	248			8
Reinisch *et al*[21]	37.0 ± 9.0	Placebo	130	Moderate-to-severe active UC	Subcutaneous injections 160/80 mg at week 0, 80/40 mg at week 2 and then 40 mg beginning at week 4 every other week or matching placebo	
	40.0 ± 9.5	Adalimumab 80/40 mg	130			
	36.5 ± 9.5	Adalimumab 160/80 mg	130			6
PURSUIT-SC (Sandborn *et al*[12])	39.0 ± 13.0	Placebo	331	Moderate-to-severe active UC	Subcutaneous injections 400/200 mg at week 0 and 200/100 at week 2 or matching placebo	
	40.0 ± 13.5	Golimumab 200/100 mg	331			
	40.7 ± 13.7	Golimumab 400/200 mg	331			54
PURSUIT-M (Sandborn *et al*[13])	40.2 ± 14.1	Placebo	156	Moderate-to-severe active UC	Subcutaneous injections 100/50 mg every 4 wk or matching placebo	
	41.4 ± 13.8	Golimumab 50 mg	154			
	39.1 ± 13.1	Golimumab 100 mg	154			52
GEMINI 1 (Feagan *et al*[14], [9])	41.2 ± 12.5	Placebo	149	Moderate-to-severe active UC	Intravenous infusions every 4 wk or every 8 wk or matching placebo	
	40.1 ± 13.2	Vedolizumab 300 mg	746			

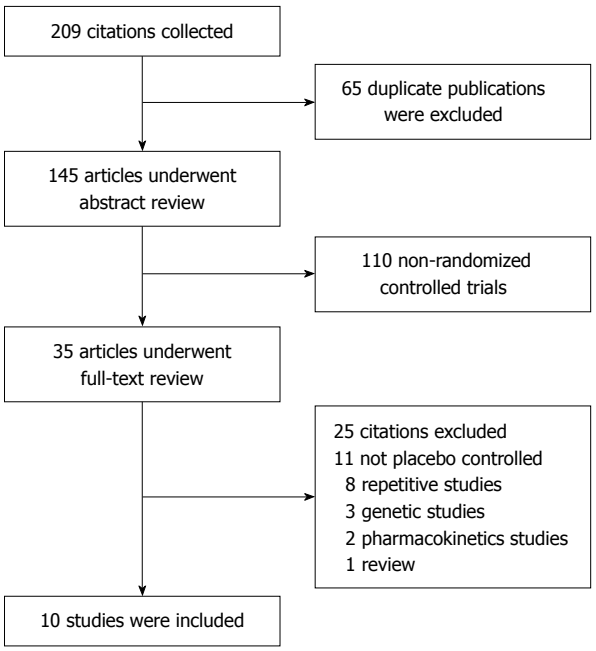

Figure 1 Flow diagram of the study selection.

and STATA12 (Stata Corp, College Station, Texas, United States). The statistical methods of this study were reviewed by Shanghai 2med Biotechnology Co., Ltd (Shanghai, China).

RESULTS

Search results and characteristics

A total of 209 articles were obtained *via* database searches; ten met the inclusion criteria for this study (Figure 1). A total of 4237 patients with moderate-to-severe active UC were involved. Among the UC patients, 484 were treated with infliximab; 685 with adalimumab; 970 with golimumab; 746 with vedolizumab; and 1352 with placebo. The information of these articles is summarized in Table 1.

Heterogeneity analysis

Before performing MTC meta-analysis, we analyzed the effect of single biological agent on response, remission, mucosal healing and serious adverse events compared to placebo. No heterogeneity was found between studies (Table 2).

Clinical response

Clinical response was defined as a decrease from baseline in the total Mayo score of at least 3 points and by at least 30%, with an accompanying decrease in the subscore for rectal bleeding of at least 1 point or an absolute subscore for rectal bleeding of 0 or 1. All biological agents were superior to placebo in both induction and maintenance phases (Figure 2). The results of MTC meta-analysis showed that in induction phase infliximab was more effective than adalimumab (OR = 0.41, 95%CI: 0.29-0.57) and golimumab (OR = 0.66, 95%CI: 0.44-0.97), while golimumab had a better effect than adalimumab (OR = 1.62, 95%CI: 1.13-2.33). In maintenance phase, vedolizumab was more effective than adalimumab (OR = 1.94, 95%CI: 1.11-3.44) and golimumab (OR = 1.85, 95%CI: 1.08-3.2). Forest plots are summarized in Figure 3.

Table 2 Heterogeneity analysis of the biological agents compared to placebo (%)

	Response		Remission		Mucosal healing		Serious adverse events
	Induction	Maintenance	Induction	Maintenance	Induction	Maintenance	Maintenance
Infliximab	0	0	47.4	0	0	0	0
Adalimumab	0	0	0	0	0	0	0
Golimumab	0	0	0	0	0	0	0
Vedolizumab	-	0	-	0	-	0	-

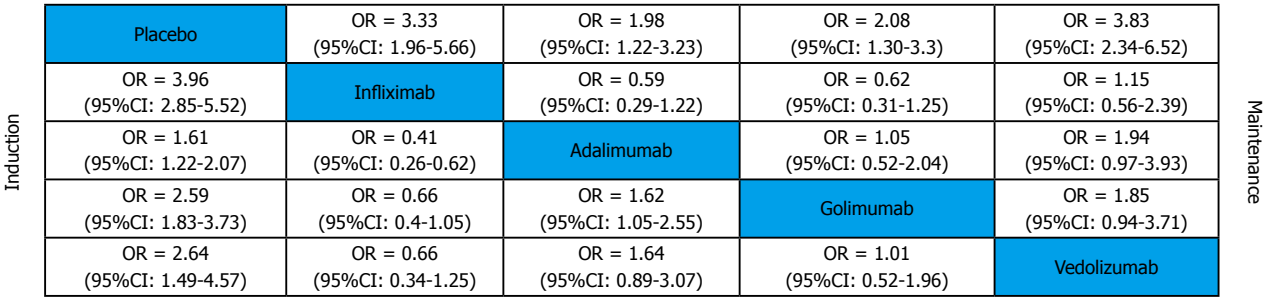

Figure 2 Comparison of biological agents for induction of clinical response in moderate to severe active ulcerative colitis.

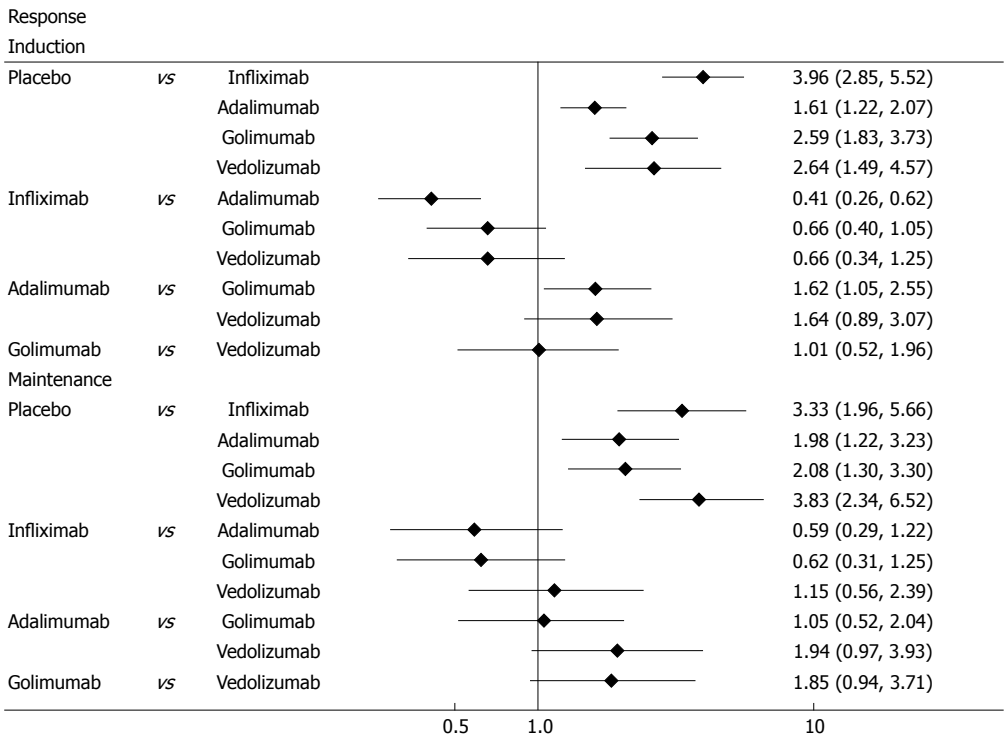

Figure 3 Forest plots of biological agents for induction of clinical response in moderate to severe active ulcerative colitis.

Clinical remission

Clinical remission was defined as a total Mayo score of 2 points or lower, with no individual subscore exceeding 1 point. All biological agents were better than placebo for clinical remission in induction and maintenance phases (Figure 4). In induction phase, adalimumab was less effective than infliximab (OR = 0.33, 95%CI: 0.19-0.56), golimumab (OR = 2.15, 95%CI: 1.18-4.22) and vedolizumab (OR = 2.49, 95%CI: 0.99-6.64). However, there was no significant difference between the biological agents in maintenance phase. Forest plots are summarized in Figure 5.

Mucosal healing

Mucosal healing was defined as an absolute subscore for endoscopy of 0 or 1. Biological agents were better than placebo for mucosal healing in induction and

Induction					Maintenance
Placebo	OR = 2.70 (95%CI: 0.86-8.43)	OR = 2.85 (95%CI: 0.93-9.47)	OR = 1.87 (95%CI: 0.59-5.79)	OR = 2.31 (95%CI: 0.71-7.04)	
OR = 4.48 (95%CI: 2.85-7.54)	Infliximab	OR = 1.05 (95%CI: 0.22-5.68)	OR = 0.69 (95%CI: 0.14-3.44)	OR = 0.85 (95%CI: 0.16-4.25)	
OR = 1.50 (95%CI: 0.93-2.37)	OR = 0.33 (95%CI: 0.16-0.62)	Adalimumab	OR = 0.66 (95%CI: 0.12-3.15)	OR = 0.81 (95%CI: 0.15-3.89)	
OR = 3.24 (95%CI: 1.72-6.28)	OR = 0.72 (95%CI: 0.32-1.60)	OR = 2.15 (95%CI: 1.02-5.03)	Golimumab	OR = 1.24 (95%CI: 0.24-5.99)	
OR = 3.72 (95%CI: 1.31-11.19)	OR = 0.83 (95%CI: 0.26-2.70)	OR = 2.49 (95%CI: 0.82-8.30)	OR = 1.15 (95%CI: 0.33-4.09)	Vedolizumab	

Figure 4 Comparison of biological agents for induction of clinical remission in moderate to severe active ulcerative colitis.

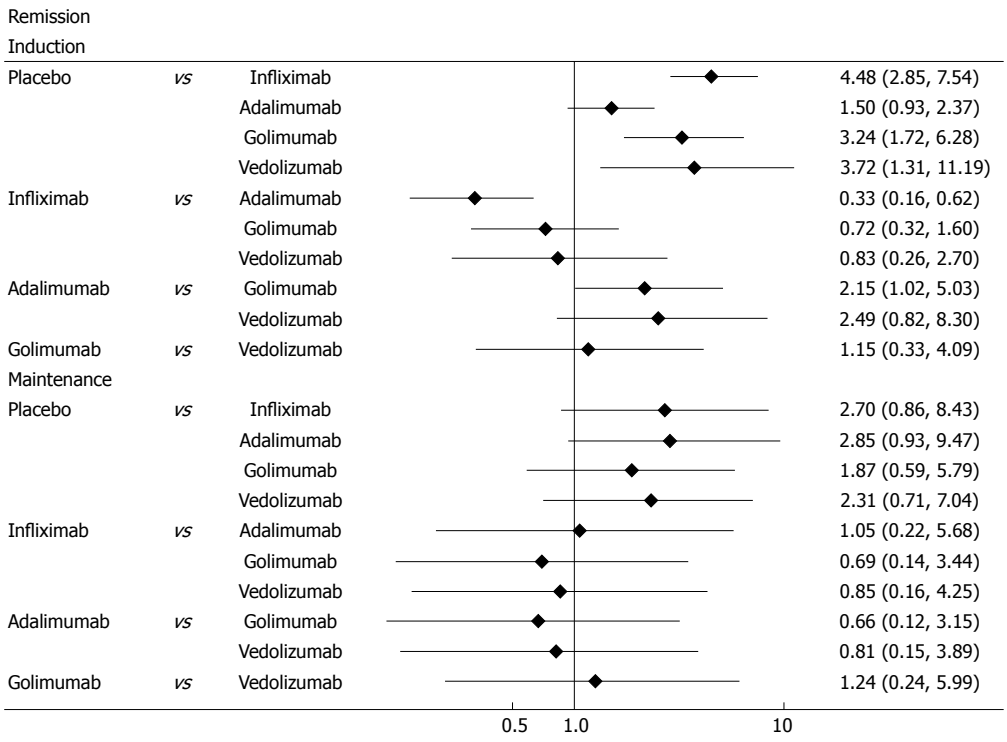

Figure 5 Forest plots of biological agents for induction of clinical remission in moderate to severe active ulcerative colitis.

Induction					Maintenance
Placebo	OR = 3.90 (95%CI: 1.29-12.17)	OR = 3.42 (95%CI: 1.18-11.03)	OR = 2.01 (95%CI: 0.67-5.96)	OR = 4.78 (95%CI: 1.56-14.47)	
OR = 3.24 (95%CI: 2.39-4.44)	Infliximab	OR = 0.88 (95%CI: 0.19-4.53)	OR = 0.51 (95%CI: 0.10-2.49)	OR = 1.22 (95%CI: 0.25-5.91)	
OR = 1.33 (95%CI: 1.02-1.74)	OR = 0.41 (95%CI: 0.27-0.62)	Adalimumab	OR = 0.59 (95%CI: 0.12-2.62)	OR = 1.40 (95%CI: 0.26-6.58)	
OR = 1.94 (95%CI: 1.37-2.77)	OR = 0.60 (95%CI: 0.37-0.95)	OR = 1.45 (95%CI: 0.94-2.26)	Golimumab	OR = 2.37 (95%CI: 0.49-11.38)	
OR = 2.10 (95%CI: 1.21-3.71)	OR = 0.65 (95%CI: 0.34-1.24)	OR = 1.58 (95%CI: 0.85-3.00)	OR = 1.09 (95%CI: 0.57-2.08)	Vedolizumab	

Figure 6 Comparison of biological agents for induction of mucosal healing in moderate to severe active ulcerative colitis.

maintenance phases (Figure 6). In induction phase, infliximab was more effective than adalimumab (OR = 0.41, 95%CI: 0.29-0.57) and golimumab (OR = 0.6, 95%CI: 0.41-0.87), while golimumab had a better effect than adalimumab (OR = 1.45, 95%CI: 1.02-2.09). However, no significant difference was found between the biological agents in maintenance phase. Forest plots are summarized in Figure 7.

Safety

This analysis used random trial data on serious adverse events from maintenance phase. The MTC

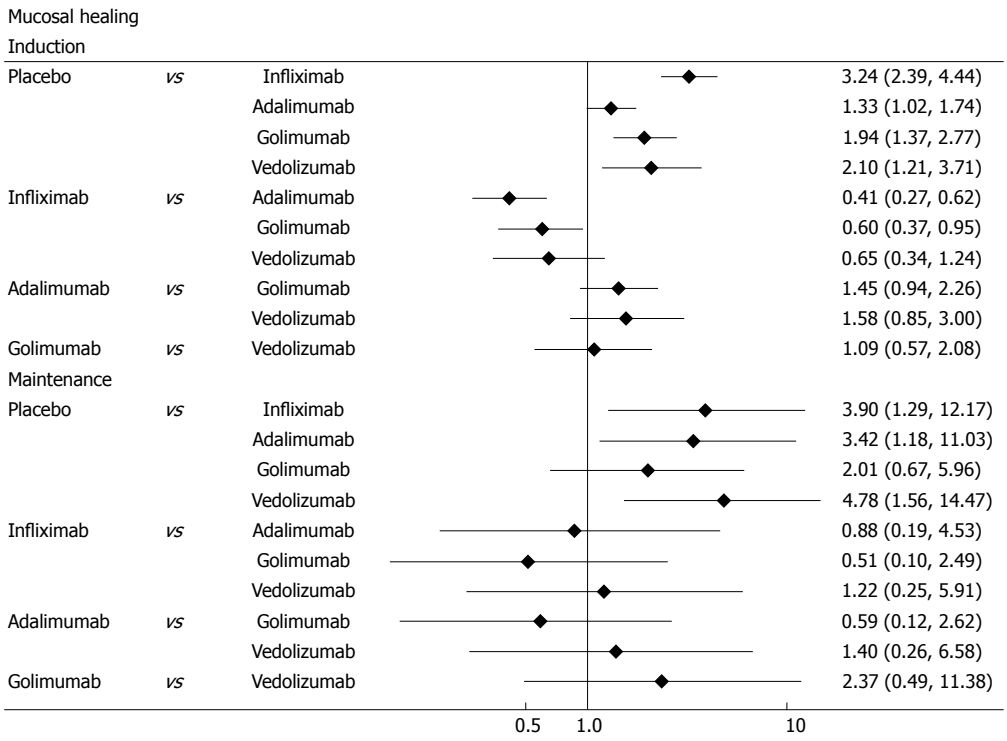

Figure 7 Forest plots of biological agents for induction of mucosal healing in moderate to severe active ulcerative colitis.

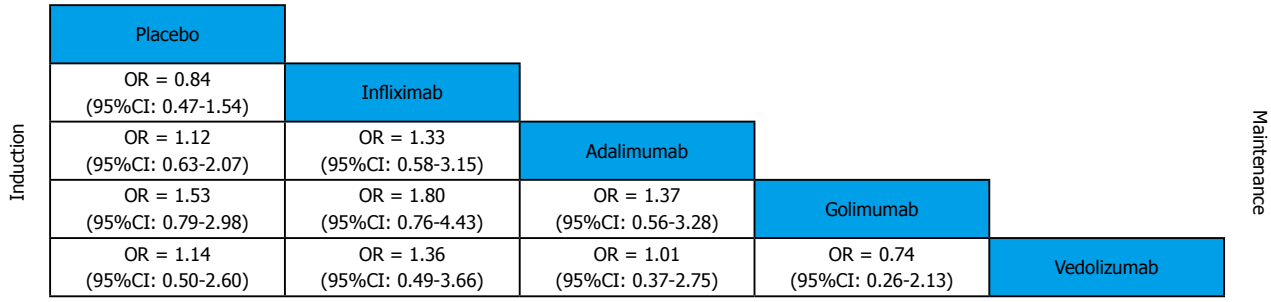

Figure 8 Comparison of serious adverse events of biological agents in moderate to severe active ulcerative colitis.

meta-analysis results showed that biological agents had a similar safety profile to placebo (Figure 8). Forest plots are summarized in Figure 9.

DISCUSSION

The appearance of biological agents dramatically changed the treatment landscape for UC. Biological agents have been used for the treatment of moderate to severe UC patients failing conventional treatment. Previous randomized controlled trials (RCTs) proved that biological agents were effective and safe for the treatment of UC in both induction and maintenance phases. Danese *et al*[3] compared the biological agents by performing a multiple-treatment meta-analysis. They illustrated that infliximab is more effective to induce clinical response and mucosal healing than adalimumab in induction phase[3]. However, there was still lack of head-to-head RCTs to compare the different treatment options for long-term efficacy and safety.

This meta-analysis assessing biological agents for the treatment of moderate to severe active UC included 9 RCTs, all of which were placebo controlled trials. No heterogeneity was found when assessing the effect of single biological agent. Meta-analysis results showed that all biological agents were effective for UC treatment in induction and maintenance phases. Indirect comparisons of induction studies indicated that infliximab had a more favorable clinical outcome than golimumab, vedolizumab and adalimumab, while adalimumab was less effective than the others. However, in maintenance phase, all biological agents had a similar effect without statistical difference. The incidence of serious adverse events was not different between the biological agents and placebo.

However, it should be noted that there were some limitations in our study. First, a potential weakness

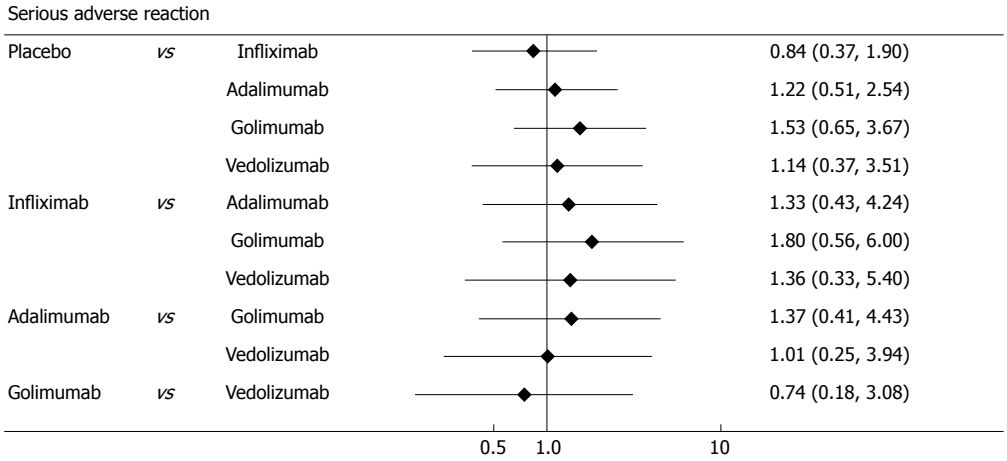

Figure 9 Forest plots of serious adverse events of biological agents in moderate to severe active ulcerative colitis.

of this meta-analysis was caused by the fact that the included trials were likely different in study design. For example, the studies by Sandborn *et al*[12,13] and Feagan *et al*[14] reported efficacy and safety results at week 6 while the others at week 8[9]. Patient characteristics such as previous treatments also varied slightly across studies. Second, the small sample size and lack of head-to-head trials may increase the uncertainty of the results. Finally, we could not assess the publication bias. Despite these limitations, we believe that our analysis could contribute to the evaluation of biological agents that might support clinical decision making.

In conclusion, the results of our meta-analysis suggested that all biological agents were superior to placebo in terms of clinical effects in both induction and maintenance phases. It was also showed that infliximab had a better clinical effect than the other biological agents. By analyzing the incidence of serious adverse events, it was found that biological agents had a similar safety profile to placebo. However, head-to-head comparisons, continuous data collection and benefit-risk assessment are needed to confirm our findings.

COMMENTS

Background

Ulcerative colitis (UC) is a form of chronic inflammatory bowel disease (IBD) characterized by recurrent rectal bleeding, increased stool frequency and urgency, abdominal cramps and pain, and systemic symptoms (such as fever, anemia and weight loss). Current options of treatment include aminosalicylates, corticosteroids, immunosuppressive medications and biological agents. However, conventional treatments often lead to a series of adverse events and have a limited effect in long-term disease control.

Research frontiers

Biological agents include tumor necrosis factor-α (TNF-α) antibodies and integrin antagonists. Anti-TNF-α agents including infliximab, adalimumab and golimumab have been approved by United States Food and Drug Administration for the treatment of moderate-to-severe UC. All the 3 anti-TNF-α agents are demonstrated to be effective for the induction and maintenance of remission in moderate or severe UC. In addition, these agents can also induce mucosal healing and reduce glucocorticoid dependence. Vedolizumab is a humanized immunoglobulin G1 monoclonal antibody to α4β7 integrin. A phase 3 study investigating the efficacy and safety of vedolizumab in patients with moderate to severe active UC indicated that vedolizumab was significantly more effective in terms of clinical response and remission compared to placebo in both induction and maintenance phases.

Innovations and breakthroughs

Previous studies have shown that biological agents were effective in treatment of UC. However, the selection of biological agents in UC therapy was still controversial. Traditional methods cannot be applied for the comparison for lack of head-to-head studies comparing different biological agents. Therefore the authors used a mixed treatment comparison (MTC) to compare the efficacy of biological agents, as MTC was available for indirect comparisons between drugs with different comparators.

Applications

The study results suggest that all biological agents were superior to placebo for UC treatment in both induction and maintenance phases with a similar safety profile, and infliximab had a better clinical effect than the other biological agents.

Terminology

UC is a form of chronic IBD characterized by recurrent rectal bleeding, increased stool frequency and urgency, abdominal cramps and pain, and systemic symptoms. Anti-TNF-α agents including infliximab, adalimumab and golimumab are monoclonal antibodies that bind to TNF-α with high affinity and specificity. Vedolizumab, a representative for integrin antagonists, is a humanized immunoglobulin G1 monoclonal antibody to α4β7 integrin.

Peer-review

This manuscript is a very interesting article.

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META-ANALYSIS

Associations between *CD24* gene polymorphisms and inflammatory bowel disease: A meta-analysis

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Author contributions: Huang XL and Xu DH contributed equally to this work; Huang XL and Xu DH performed the research; Wang GP and Zhang S were involved in editing the manuscript; Yu CG designed the study and revised the manuscript as the corresponding author.
Conflict-of-interest: We declare that there is no conflict of interests regarding the publication of this paper.

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Abstract

AIM: To evaluate the relationships between *CD24* gene polymorphisms and the risk of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD).

METHODS: The PubMed, Web of Science and Cochrane Library databases were searched (up to May 30, 2014). The search terms "CD24", "inflammatory bowel disease", "Crohn's disease", "Ulcerative colitis", "IBD", "CD" or "UC"; and "polymorphism", "mutation" or "variant" were used. Association studies were limited to the English language, but no limitations in terms of race, ethnicity or geographic area were employed. Stata SE12 software was used to calculate the pooled odds ratios (ORs) with 95% confidence intervals (CIs). $P < 0.05$ was considered statistically significant. The information was independently extracted from each eligible study by two investigators. Two common polymorphisms, C170T (rs8734) and TG1527del (rs3838646), in the *CD24* gene were assessed.

RESULTS: A total of three case-control studies including 2342 IBD patients and 1965 healthy controls were involved in this meta-analysis. The patients and controls were from Caucasian cohorts. The three articles included in this meta-analysis all conformed to Hardy-Weinberg equilibrium. This meta-analysis revealed that there were no significant associations between the two *CD24* polymorphisms and the risk for IBD (all $P > 0.05$). However, in a disease subgroup analysis, we found that the *CD24* C170T polymorphism was associated with an increased risk of UC in a dominant model (OR = 1.79, 95%CI: 1.15-2.77, $P = 0.009$) and an additive model (OR = 1.87, 95%CI: 1.19-2.93, $P = 0.007$), but this relationship was not present for CD. The CD24 TG1570*del* polymorphism was significantly associated with CD in the additive model (OR = 1.24, 95%CI: 1.01-1.52, $P = 0.037$).

CONCLUSION: Our findings provide evidence that the *CD24* C170T polymorphism might contribute to the susceptibility to UC, and the CD24 TG1527del polymorphism might be associated with the risk of CD.

Key words: CD24; Polymorphism; Inflammatory bowel disease; rs8734; rs3838646; Meta-analysis

Core tip: CD24 is a significant immune regulatory mediator of inflammatory bowel disease (IBD). Some recent studies have demonstrated that *CD24* gene polymorphisms are associated with the susceptibility to IBD, but the findings of other studies are contradictory. The present study sought to provide a more precise estimate of this potential association. A meta-analysis of Caucasian cohorts found that the *CD24* C170T polymorphism was associated with the susceptibility to UC and that the CD24 TG1527*del* polymorphism was associated with CD.

Huang XL, Xu DH, Wang GP, Zhang S, Yu CG. Associations between *CD24* gene Polymorphisms and inflammatory bowel disease: A meta-analysis. *World J Gastroenterol* 2015; 21(19): 6052-6059 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6052.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6052

INTRODUCTION

Inflammatory bowel disease (IBD) is a relapsing and chronic inflammatory disorder that is composed of two types of diseases, Crohn's disease (CD) and ulcerative colitis (UC). The incidence of IBD is increasing worldwide. IBD places a heavy burden on patients because it reduces life quality and the ability to work and increases disability[1]. The etiology of IBD is complicated and obscure but is primarily related to genetic, environmental, immune and infectious factors and interactions between these factors. Based on assessments of familial clustering and the high concordance in monozygotic twins, it is well established that a genetic component is implicated in the pathogenesis of IBD[2-4]. Recent studies have revealed that many gene variations are associated with the susceptibility to IBD, such as NOD2[5-7],ATG16L1[8,9], DLG5[10,11] and IL23R[12].

CD24 is a glycophosphatidylinositol (GPI)-anchored mucin-like cell surface glycoprotein that is expressed in a wide variety of cell types, including activated T cells[13], B cells[14], macrophages[15], and dendritic cells[16]. Human CD24 is encoded by a gene located on chromosome 6 and plays important roles in lymphocyte maturation[13,17-19], neuronal development[20], intercellular signal transmission and immune regulation. Some single nucleotide polymorphisms (SNPs) in the *CD24* gene have been shown to be associated with the susceptibilities to several chronic inflammatory and autoimmune diseases, such as multiple sclerosis (MS)[21,22], systemic lupus erythematous (SLE)[23,24], and others. There are some studies of the correlations between *CD24* SNPs and risk factors for IBD pathogenesis[25-27]. C170T (rs8734) and TG1527*del* (rs3838646) are two common CD24 genetic polymorphisms that are potentially related to IBD; however, the findings related to these polymorphisms are contradictory. To shed some light on the contradictory findings and provide a more precise estimate of the potential associations, we performed this meta-analysis to investigate whether the two *CD24* polymorphisms (C170T and TG1527del) contribute to the susceptibility to IBD.

MATERIALS AND METHODS

Literature search

We conducted a literature search for relevant studies on the relationships between polymorphisms of *CD24* and IBD risk in the PubMed, Web of Science, and Cochrane Library databases (up to May 30, 2014). The following search terms were used: "CD24"; and "inflammatory bowel disease", "Crohn's disease", "Ulcerative colitis", "IBD", "CD", or "UC"; and "polymorphism", "mutation", or "variant". The searched studies were limited to the English language.

Inclusion and exclusion criteria

Eligible studies were required to meet the following inclusion criteria: (1) case-control studies evaluating at least one polymorphism of the *CD24* gene; (2) studies containing original data; (3) studies with genotype or allelic distributions; (4) studies containing sufficient data to calculate odds ratios (ORs); and (5) studies in which the genotype distribution of the control population was in Hardy-Weinberg equilibrium (HWE). No limitations related to race, ethnicity or geographic area were utilized.

The exclusion criteria were as follows: (1) irrelevant and review articles; (2) studies containing overlapping data; (3) articles that did not provide detailed genotype data; (4) investigations of the associations of other genes with IBD or the relationships between *CD24* gene polymorphisms and other diseases; and (5) studies in which family members were studied because the analyses were based on linkage considerations.

Data extraction and synthesis

The following information was extracted from each eligible study independently by two investigators: first author's surname, year of publication, ethnicity of the study population, and the number of cases and controls for the *CD24* genotype. The allele and genotype frequencies of the *CD24* polymorphisms

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

First author	Year	Country	Ethnicity	Sample size			Polymorphisms	Genotype method
				UC	CD	Control		
Diaz-Gallo LM	2011	Spain	Caucasian	632	737	1257	P170 P1527	PCR
Van Limbergen	2013	Scottish	Caucasian	342	395	498	P1527	PCR
Lisiansky V	2014	Israel	Caucasian	174	62	210	P170 P1527	PCR-RFLP

UC: Ulcerative colitis; CD: Crohn's disease; RFLP: Restricted fragment length polymorphisms; PCR: Polymerase chain reaction.

Table 2 Distributions of two *CD24* genotypes and alleles among the inflammatory bowel disease patients and controls

Study	Arms	C170T						Arms	TG1570*del*					
		A	V	AA	AV	VV	HWE		TG	*del*	TGTG	TG*del*	*deldel*	HWE
Diaz-Gallo *et al*[26], 2011	CD (n = 366)	534	198	200	134	32		CD (n = 371)	662	80	301	60	10	
	UC (n = 322)	448	196	161	126	35		UC (n = 310)	580	40	270	40	0	
	Control (n = 628)	904	352	317	270	41	0.100	Control (n = 629)	1170	88	547	76	6	0.448
Lisiansky *et al*[25], 2014	CD (n = 31)	42	20	12	18	1		CD (n = 31)	57	5	26	5	0	
	UC (n = 87)	108	66	29	50	8		UC (n = 87)	165	9	78	9	0	
	Control (n = 105)	163	47	63	37	5	0.884	Control (n = 105)	198	12	93	12	0	0.534
Van Limbergen *et al*[27], 2013	CD (n = 395)	-	-	-	-	-			719	71	326	67	2	
	UC (n = 310)	-	-	-	-	-			623	61	283	57	2	
	Control (n = 498)	-	-	-	-	-			905	91	411	83	4	0.932

UC: Ulcerative colitis; CD: Crohn's disease; HWE: Hardy-Weinberg equilibrium.

were calculated from each article by the allele counting method. Disagreements were resolved by discussion.

Statistical analyses

Stata SE12 software was used to calculate the pooled ORs with 95% confidence intervals (CIs) based on the available data from each article. $P < 0.05$ was considered statistically significant. The allelic model (A *vs* V or TEL *vs* del), recessive model (AA *vs* AV + VV or TGTG *vs* TG*del* + *deldel*), dominant model (AA + AV *vs* VV or TGTG + TG*del vs deldel*), and additive model (AA *vs* VV or TGTG *vs deldel*) were estimated for genotype comparisons. Cochran's Q-statistic and the I^2 test were used to test the heterogeneity among the included studies, and $P < 0.1$ and $I^2 > 50\%$ suggested significant differences in study heterogeneity. When significant heterogeneity was observed across studies, the pooled results were based on random effects models. The χ^2 test was applied to assess whether the genotype distributions of the control populations conformed to HWE, and $P < 0.05$ was considered statistically significant. Begg's funnel plot and Egger's test were used to detect publication bias[28].

RESULTS

Literature search for eligible studies

Based on the research criteria, a total of 49 articles were identified, and 29 of these articles were excluded because they were not relevant to *CD24* SNPs and the risk for IBD. Eight repetitive studies and 9 reviews were also excluded. Ultimately 3 case-control studies consisting of 2342 IBD patients (UC = 1148, CD = 1194) and 1965 controls were included in our paper. These articles were conducted with Spanish, Scottish and Israeli Caucasians. The characteristics of the 3 studies are summarized in Table 1. The distributions of the *CD24* genotypes and alleles among the IBD patients and controls are listed in Table 2.

Association of the CD24 C170T polymorphism with IBD susceptibility

Two studies including 806 IBD patients (CD = 397, UC = 409) and 733 controls were selected in this meta-analysis. A statistical test suggested that heterogeneity was present (for the allele: I^2 = 87.7%, P = 0.004; for the recessive model: I^2 = 92.8%, $P < 0.001$; for the additive model: I^2 = 73.9%, P = 0.051); therefore, the random effects model was used in these model analyses. No significant associations between the CD24 C170T polymorphism and the IBD risk were revealed (V *vs* A: OR = 1.39, 95%CI: 0.73-2.64, P = 0.314; VV *vs* AA: OR=1.32, 95%CI: 0.40-4.39, P = 0.654; VV + VA *vs* AA: OR = 0.64, 95%CI: 0.21-1.90, P = 0.420; VV *vs* AA + VA: OR=0.94, 95%CI: 0.62-1.43, P = 0.777). Subgroup analyses indicated no modifying effects of the *CD24* C170T polymorphism on the risk of CD (V *vs* A: OR = 1.16, 95%CI: 0.69-1.93, P = 0.583; VV *vs* AA: OR = 1.23, 95%CI: 0.76-1.99, P = 0.405; VV + VA *vs* AA: OR = 0.76, 95%CI: 0.28-2.07, P = 0.594; VV *vs* AA +VA: OR = 1.32, 95%CI: 0.83-2.11, P = 0.245). However, we observed a significant association between the *CD24* C170T polymorphism and UC risk for the dominant model (OR = 1.79, 95%CI: 1.15-2.77, P = 0.009) and the additive model (OR = 1.87, 95%CI: 1.19-2.93, P = 0.007) (Figure 1, Table 3).

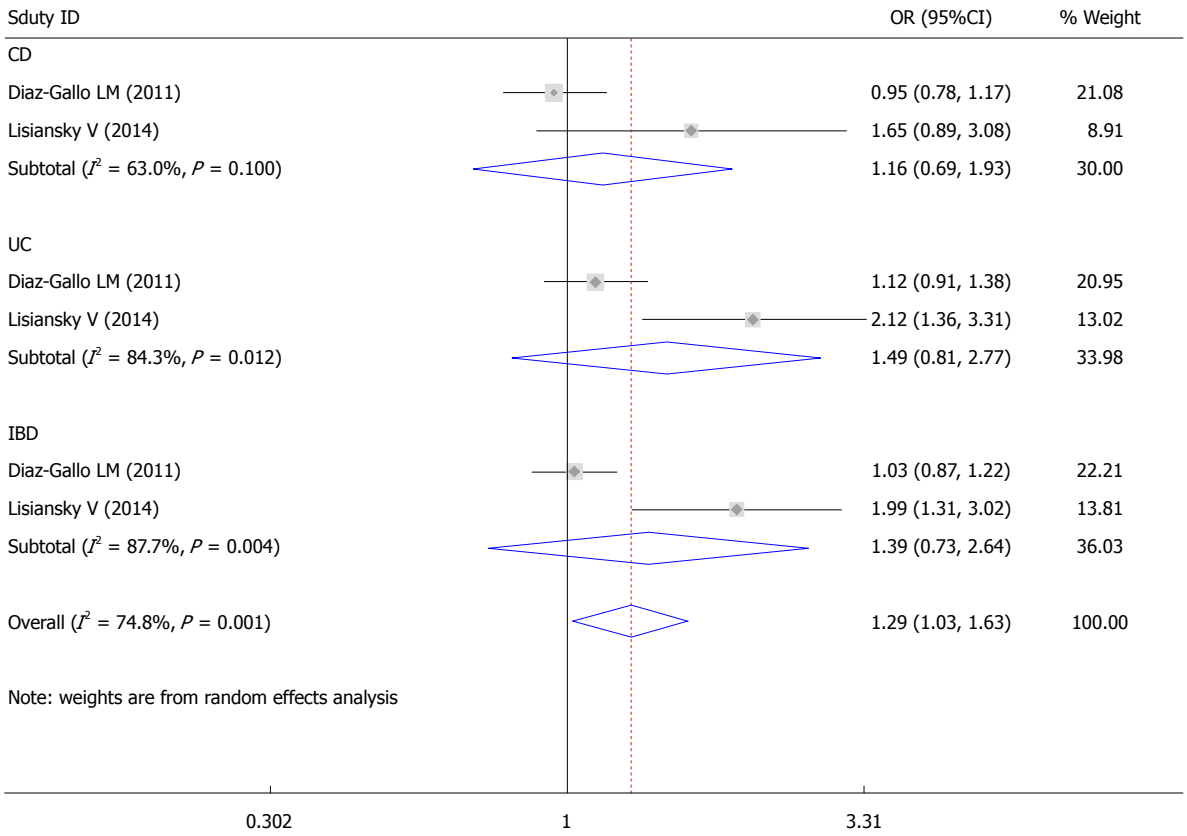

Figure 1 Pooled analysis of the variant allele *CD24*-C170T in the IBD, UC, and Crohn's disease subgroups. ORs and 95%CIs of the individual studies and pooled analyses of the associations between the *CD24* C170T C allele and the disease risks for Crohn's disease (CD) (OR = 1.16, 95%CI: 0.69-1.93), ulcerative colitis (UC) (OR = 1.49, 95%CI: 0.81-2.77), and inflammatory bowel disease (IBD) (OR = 1.39, 95%CI = 0.73-2.64). OR: Odds ratio.

Table 3 Meta-analysis of the associations between two promoter polymorphisms of *CD24* and inflammatory bowel disease

Polymorphism	Disease	Test of association			Test of heterogeneity		
		OR	95%CI	*P* value	Model	*P* value	I^2 (%)
P170V vs A	CD	1.16	0.69-1.93	0.583	R	0.100	63.0
	UC	1.49	0.81-2.77	0.203	R	0.012	84.3
	IBD	1.39	0.73-2.64	0.314	R	0.004	87.7
VV + AV *vs* AA	CD	0.76	0.28-2.07	0.594	R	0.019	81.9
(recessive)	UC	0.59	0.21-1.70	0.330	R	0.001	90.5
	IBD	0.64	0.21-1.90	0.420	R	< 0.001	92.8
VV *vs* AA + AV	CD	1.32	0.83-2.11	0.245	F	0.527	0.0
(dominant)	UC	1.79	1.15-2.77	0.009	F	0.816	0.0
	IBD	0.94	0.62-1.43	0.777	F	0.287	11.8
VV *vs* AA	CD	1.23	0.76-1.99	0.405	F	0.888	0.0
(additive)	UC	1.87	1.19-2.93	0.007	F	0.272	17.1
	IBD	1.32	0.40-4.39	0.654	R	0.051	73.9
P1527del *vs* TG	CD	1.24	1.01-1.52	0.037	F	0.101	56.5
	UC	0.95	0.76-1.19	0.649	F	0.967	0.0
	IBD	1.18	0.99-1.41	0.063	F	0.803	0.0
deldel + TGdel *vs* TGTG	CD	0.80	0.63-1.02	0.068	F	0.207	36.5
(recessive)	UC	1.02	0.79-1.33	0.870	F	0.980	0.0
	IBD	0.82	0.67-1.01	0.061	F	0.870	0.0
deldel *vs* TGTG + TGdel	CD	1.87	0.81-4.32	0.143	F	0.133	55.7
(dominant)	UC	0.40	0.10-1.63	0.201	F	0.345	0.0
	IBD	1.23	0.54-2.76	0.625	F	0.436	0.0
deldel *vs* TGTG	CD	1.93	0.84-4.47	0.123	F	0.121	58.4
(additive)	UC	0.40	0.10-1.64	0.203	F	0.348	0.0
	IBD	1.27	0.56-2.86	0.572	F	0.439	0.0

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

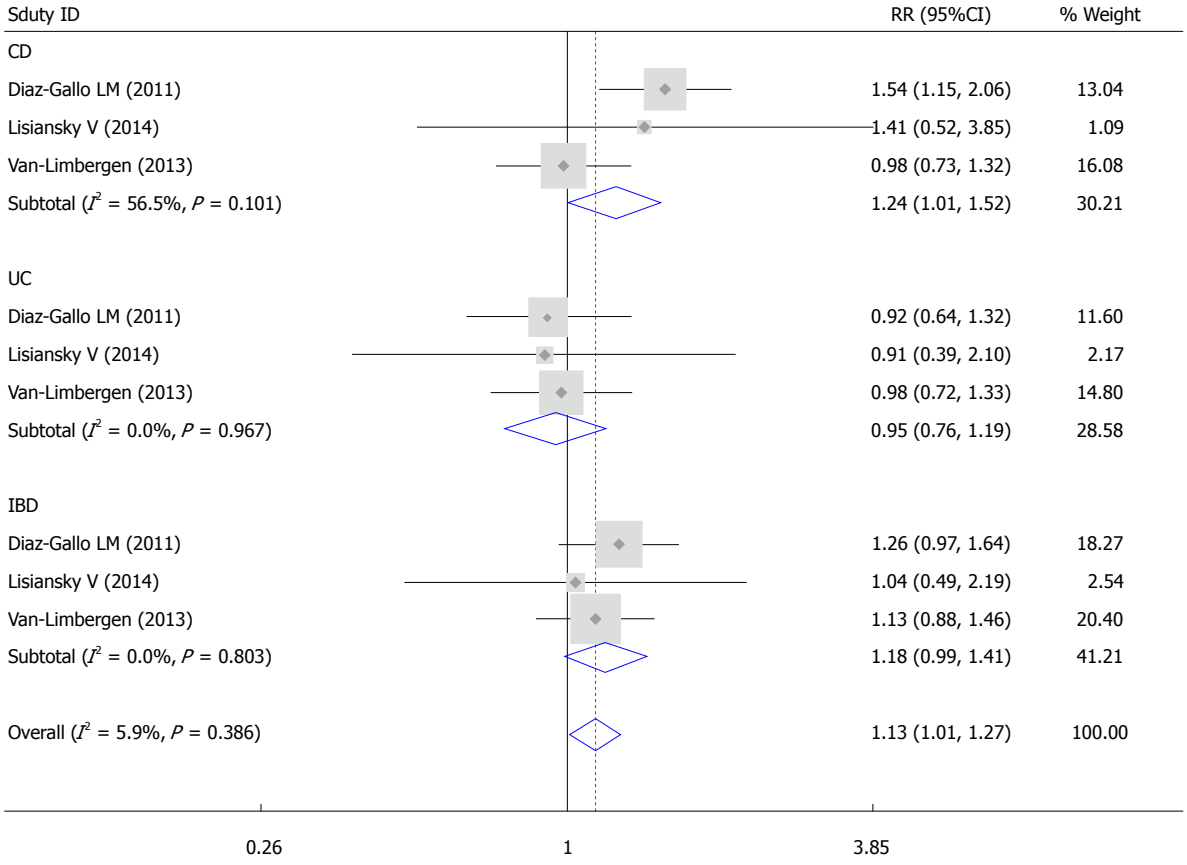

Figure 2 Pooled analyses of the variant allele CD24 TG1527del in the IBD, UC and Crohn's disease subgroups. ORs and 95% CIs of the individual studies and the pooled analyses of the associations between the *CD24* TG1527*del* allele and the disease risks for Crohn's disease (CD) (OR = 1.24, 95%CI: 1.01-1.52), ulcerative colitis (UC) (OR = 0.95, 95%CI: 0.76-1.19) and inflammatory bowel disease (IBD) (OR = 1.18, 95%CI: 0.99-1.41). OR: Odds ratio.

Table 4 Egger's tests for C170 T and TG1527*del* of *CD24* and the inflammatory bowel disease risk in all of the included studies

Contrast models	Coefficient	95%CI	SE	*t*	*P* value
C170T					
V *vs* A	3.902	0.912 to 6.891	1.077	3.62	0.022
VV +AV *vs* AA	-4.527	-5.832 to 3.075	1.969	-0.70	0.501
VV *vs* AA + AV	0.200	-7.276 to -1.779	0.990	-4.57	0.010
VV *vs* AA	1.399	-2.040 to 4.838	1.239	1.13	0.322
TG1527*del*					
del vs TG	-0.435	-2.669 to 1.799	0.945	-0.46	0.659
deldel + TG*del vs* TGTG	0.444	-1.833 to 2.721	0.963	0.64	0.659
deldel vs TGTG + TG*del*	-2.977	-4.820 to -1.134	0.664	-4.48	0.011
del*del vs* TGTG	-2.598	-4.249 to -0.947	0.595	-4.37	0.012

IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease.

Association of the TG1527del polymorphism with IBD susceptibility

Three studies consisting of 1625 IBD patients (UC = 759, CD = 866) and 1232 controls were included in this meta-analysis. A statistical test revealed that there was no heterogeneity between the studies (for the allele: I^2 = 0%, P = 0.803; for the recessive model: I^2 = 0%, P = 0.870; for the dominant model: I^2 = 0%, P = 0.436; for the additive model: I^2 = 0%, P = 0.439); hence, the fixed effect model was used. The results did not indicate any significant association of the allele in the overall sample or the subgroup analyses with the exception that the del allele was related to CD susceptibility (OR=1.24, 95%CI: 1.01-1.52, P = 0.037) (Figure 2, Table 3).

Sensitivity analysis and publication bias

A sensitivity analysis conducted *via* the omission of individual studies did not materially alter the pooled results (data not shown). Begg's funnel plots did not reveal obvious asymmetries for any of the comparison models. However, Egger's tests revealed that there were some publication biases for both of the genetic allele analyses (V *vs* A, P_{Egger} = 0.022; VV *vs* AA + AV, P_{Egger} = 0.010; *deldel vs* TGTG + TG*del*, P_{Egger} = 0.011; *deldel vs* TGTG, P_{Egger} = 0.012) (Table 4).

DISCUSSION

The etiology of IBD has not been completely elucidated, but the contributions of immunological and genetic factors have been demonstrated. IBD is believed to arise partly due to multiple genetic factors. Many reports have used the genome-wide association study (GWAS) approach to identify novel candidate

single nucleotide polymorphisms (SNPs) for IBD[12,29,30]. To date, 99 variants have been identified as associated with CD and/or UC. Among these, only 28 variants have been shown to overlap in their contributions to the susceptibilities to both diseases[31]. Some risk loci for CD have no reported effect on UC. Similarly, some risk loci for UC have been shown to have little effect on UC. Different mutations of the same gene might be associated with different IBD diseases. IBD is an autoimmune disease that is related to the innate and adaptive immune systems[32,33]. Evidence from animal models indicates that the failure to suppress immunity to the abundant intestinal foreign antigen load can cause inflammation[34]. Certain genetic variations and changes in the immune system might contribute to the development of IBD, such as those related to macrophage migration inhibitory factor (MIF)[35,36] and interleukin-10 (IL-10)[37].

CD24 has been reported to play an important role in the immune system and to be associated with autoimmune diseases, including IBD. CD24 has been shown to be a ligand for P-selectin and to play an important role in recruiting leukocytes to inflamed tissue. CD24 has also been implicated in the activation and differentiation of B lymphocytes[38], and it has been identified as an important mediator in a CD28-independent co-stimulatory pathway related to the activation of both CD4 and CD8 T cells[39]. Ahmed *et al*[40] found that *CD24* is upregulated by Wnt signaling in regenerating tissue in IBD and can confer enhanced colony forming abilities and enhanced cell motilities, which play an important roles in tissue healing. The association between *CD24* polymorphisms and the risk of IBD has been reported in recent studies[25,26]. The *CD24* gene contains multiple SNPs, such as the C170T (rs8734), TG1527del (rs3838646), A1626G (rs1058881) and A1056G (rs1058818) polymorphisms. Among these SNPs, C170T and TG1527del have received much attention. However, the existing data are contradictory. Van Limbergen *et al*[27] reported that the *CD24* TG1527*del* gene polymorphism is not an important determinant of genetic susceptibility to IBD. However, Diaz-Gallo *et al*[26] demonstrated that a TG1527del SNP is associated with an increased risk of CD but not UC. To better understand of the associations between these two polymorphisms and IBD, a meta-analysis with a larger sample and subgroup analyses is necessary.

The present study is the first meta-analysis that has attempted to determine the potential roles of *CD24* polymorphisms in IBD. Our results revealed a significant association between the *CD24* C170T polymorphism and UC risk in the dominant (OR = 1.79, 95%CI: 1.15-2.77, P = 0.009) and additive models (OR = 1.87, 95%CI: 1.19-2.93, P = 0.007), but the *CD24* C170T polymorphism was not related to the risk for CD. Moreover, our results indicated the *CD24* TG1570*del* polymorphism was significantly associated with CD in the additive model (OR = 1.27, 95%CI: 1.01-1.58, P = 0.037). These results support the hypothesis that CD and UC are related but different in terms of some immunologic mechanisms[41]. Similar results have been reported for other autoimmune diseases. Zhou *et al*[42] reported that the expression of *CD24* on the peripheral blood T cells of CD24V/V MS patients is higher than that of *CD24* A/A genotype patients. Sánchez *et al*[24] demonstrated that the frequency of the CD24V/V genotype in SLE patients was higher than that in the controls in a Spanish cohort. The TG1527*del* genetic variant has been reported to reduce the constitutive levels of *CD24* mRNA by more than two-fold and to reduce the risks for MS and SLE[43]. Diaz-Gallo[26] also observed that the TG1527*del* genetic variant is a risk factor for CD, specifically for CD with an age of diagnosis between 17 and 40 years. Van Limbergen *et al*[27] did not have any association between the *CD24* TG1527*del* SNP and CD susceptibility, possibly due to a low number of subjects.

However, our study has some limitations that should be considered. First, heterogeneities among the studies involving *CD24* C170T were present and might have partially influenced the results of this study. Therefore, additional details are needed to analyze the source of the heterogeneity. Second, the numbers of included studies and patients were limited. We are looking forward to the availability of additional relevant studies to help us to understand this problem. Third, only studies published in English were selected for this meta-analysis, and these studies do not include unpublished documents. Thus, a selection bias might have been present. Finally, some publication bias was present because the population of included studies was not uniform. Therefore, additional studies with the same patient inclusion criteria or studies that perform analyses stratified by age, gender and race will help to decrease the publication bias.

In conclusion, this study suggests that the *CD24* C170T polymorphism is associated with an increased risk of UC and that the *CD24* TG1527*del* polymorphism has some influence on CD risk. A large number of cases and controls are required to enable us to make a more precise risk estimate and minimize the bias in this meta-analysis.

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COMMENTS

Background

Inflammatory bowel disease (IBD) is a nonspecific chronic intestinal inflammatory disorder, and its etiology has not been completely elucidated. In the last three decades, the prevalence of IBD has increased both in developed and developing regions. However, increasing evidence indicates that environmental, genetic and immunological factors play important roles in the

pathogenesis of IBD.

Research frontiers

CD24 is a GPI-anchored mucin-like cell surface glycoprotein and plays an important role in the immune system. Some studies have reported that polymorphisms of *CD24* are associated with the pathogeneses of autoimmune diseases. Recently, some studies have indicated that *CD24* polymorphisms are related to IBD. However, contradictory findings exist.

Innovations and breakthroughs

This is a first meta-analysis to focus on the association between *CD24* polymorphisms and IBD. Based on this meta-analysis, the *CD24* C170T polymorphism plays a significant role in the risk of UC, and the *CD24* TG1527*del* polymorphism exerts some influence on the CD risk.

Applications

The different loci of *CD24* polymorphisms might be associated with the phenotypes of IBD. An exploration of the mechanisms might be useful for reducing the risk of IBD.

Terminology

Stata SE12 software was used to calculate the pooled odds ratios with 95% confidence intervals based on the available data from each article. Cochran's *Q*-statistic and the I^2 test were used to test the heterogeneity. Begg's funnel plots and Egger's tests were used to detect publication biases.

Peer-review

This is a well-performed meta-analysis of currently available studies about the associations between *CD24* gene polymorphisms and IBD risk. The authors found that the *CD24* polymorphisms C170T (rs8734) and TG1527*del* (rs3838646) are associated with the risk of UC and CD. This meta-analysis utilized appropriate methods for the literature search, data extraction and quality assessment of the literature.

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CASE REPORT

Fulminant ulcerative colitis in a healthy pregnant woman

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Author contributions: Orabona R designed the report; Orabona R, Salemme M and Manenti S collected the patient's clinical data; Orabona R, Valcamonico A, Tiberio GAM and Frusca T analyzed the data and wrote the paper.
Ethics approval: According to Italian regulations, ethical committee approval is not required for case reports analyzing anonymized routinely-collected data.
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Abstract

This case report concerns a 25-year-old patient with 6-7 bloody stools/d, abdominal pain, tachycardia, and weight loss occurring during the third trimester of pregnancy. Severe ulcerative colitis complicated by toxic megacolon and gravidic sepsis was diagnosed by clinical evaluation, colonoscopy, and rectal biopsy that were performed safely without risk for the mother or baby. The patient underwent a cesarean section at 28+6 wk gestation. The baby was transferred to the neonatal intensive care unit of our hospital and survived without complications. Fulminant colitis was managed conservatively by combined colonoscopic decompression and medical treatment. Although current European guidelines describe toxic megacolon as an indication for emergency surgery for both pregnant and non-pregnant women, thanks to careful monitoring, endoscopic decompression, and intensive medical therapy with nutritional support, we prevented the woman from having to undergo emergency pancolectomy. Our report seems to suggest that conservative management may be a helpful tool in preventing pancolectomy if the patient's condition improves quickly. Otherwise, surgery is mandatory.

Key words: Ulcerative colitis; Toxic megacolon; Pregnancy; Cesarean section; Colonoscopy

Core tip: Severe ulcerative colitis complicated by toxic megacolon is widely considered to be an indication for emergency surgery. We reported a case of acute colitis in a healthy pregnant woman conservatively treated by intensive monitoring combined with medical therapy and endoscopic decompression in order to prevent the mother from having to undergo pancolectomy and the neonate from suffering an adverse perinatal outcome.

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INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) which affects mainly young people in their reproductive period. A first disease attack of UC during pregnancy is uncommon, and is usually associated with an adverse outcome[1]. Antenatal disease progression correlates with activity at the onset of pregnancy: if UC is in remission at the time of conception, it will remain quiescent throughout pregnancy, while if it is active, it will progressively worsen, leading to an increased incidence of stillbirth, preterm delivery, or postpartum complications for both mother and neonate[2-6]. The management of UC during pregnancy is quite different from that in non-pregnant women because medical therapy, radiology, endoscopy, and surgery imply potential risks for the fetus[2,7]. This article describes an unusual case of UC which developed early in the third trimester of pregnancy and was managed by a multidisciplinary team.

CASE REPORT

In October 2013 (1st October), a 25-year-old woman gravida 1/para 0 at 27 + 3 wk gestation without previous known diseases, presented to a periphery hospital with 6 episodes of bloody diarrhea per day for 6 d. Over the subsequent 3 d, she failed to improve and was transferred to the Obstetrics Department of the University Hospital of Brescia, Italy after a diagnosis of preterm labor (3rd October). At admission she had no fever and was tachycardic (105 bpm). Initial laboratory investigations included hemoglobin concentration (8.8 g/dL), C-reactive protein (CRP, 178 mg/L), and erythrocyte sedimentation rate (ESR, 45 mm/h). Obstetric ultrasound showed fetal cardiac activity appropriate for gestational age biometry and normal amniotic fluid index. Corticosteroids for the induction of fetal lung maturation were administered, together with tocolysis with atosiban and antibiotic therapy (clindamycin from 4th to 8th October, then imipenem). Microbiological testing for infectious diarrhea including *Clostridium* difficile toxin, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Listeria monocytogenes*, and *Brucella melitensis* was performed, were all negative. Meanwhile, because of the appearance of fever and a rise of inflammation indexes with worsening diarrhea (7-8 episodes of bloody stool per day), antibiotic therapy was switched to amikacin, vancomycin, metronidazole, and meropenem. The patient was non-responsive to antibiotics and her clinical condition continued to deteriorate. On 9th October, the patient's bowels became closed to stool and gases. Computed tomography (Figure 1) revealed the presence of transverse colonic dilatation (9 cm), suggesting a diagnosis of toxic megacolon[8]. Hemocultures for aerobic and anaerobic bacteria were negative. Thereafter (10th October), the patient was transferred to the Intensive Care Unit with a diagnosis of severe sepsis (CRP 163 mg/L, procalcitonin 81.4 ng/mL). Serum albumin had fallen to 1.76 g/dL and albumin infusion was started. Endoscopic procedures were accurately planned in the surgery room and managed in conjunction with obstetricians and colorectal surgeons in order to eventually perform an emergency cesarean section (CS) and/or colectomy, if necessary. Presence of fetal heart activity was confirmed before sedation and after endoscopy. Colonoscopy showed complete substitution of the colorectal mucosa by a fibrinous layer (Figure 2). Ulcerative colitis was confirmed by histopathology (Figure 3). According to Truelove-Witts' criteria, a diagnosis of a severe form of the disease was formulated[9]. The patient's hemoglobin fell to 6.3 g/dL, and so she was given a blood transfusion. On 11th October (28 + 6 wk gestation), she underwent a CS. A male neonate was delivered weighing 1295 grams. He did not show any congenital anomalies. He was immediately intubated, transferred to the Neonatal Intensive Care Unit (NICU) and survived without complications. Immediately after delivery, a colorectal surgeon performed a bowel evaluation and definitively excluded colectomy. On 14th October, intravenous methyl-prednisolone 60 mg/24 h (then oral prednisone 50 mg once a day) and oral mesalazine 500 mg three times a day (then 1600 mg three times a day) were started. Another colonoscopic decompression was performed and then the patient was transferred to the Gastroenterology Unit (16th October), where she underwent her third endoscopic decompression. Oral azathioprine was undertaken. Gastroscopy and magnetic resonance enterography excluded a form of overlap between Crohn's disease and UC. Parenteral nutrition was stopped on 22nd November. The patient was discharged after 54 d of hospitalization, with follow-up being performed by the IBD Center of the Gastroenterology Unit. The baby was discharged five weeks later in good clinical condition.

DISCUSSION

On the basis of old reports and experiences, the course of UC during pregnancy correlates with the level of the disease activity at the time of conception. If conception occurs during a period of clinical remission, the disease will probably continue to be inactive throughout pregnancy[3,4]; while an active UC at conception will progressively worsen during pregnancy.

However, in our case report the patient presented with a first diagnosis of UC in the third trimester of

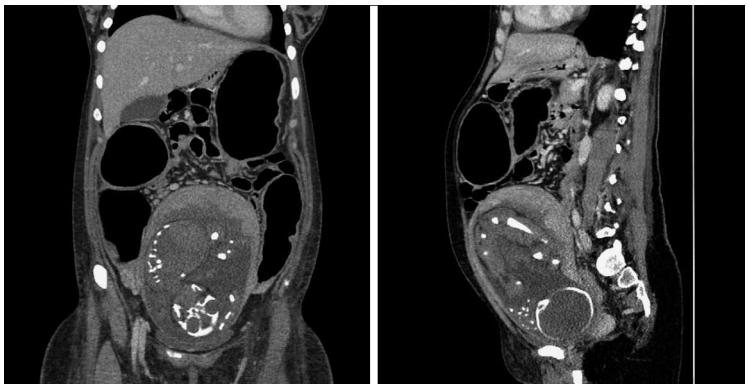

Figure 1 Abdominal computed tomography scan showing toxic megacolon during pregnancy. Ascending colon diameter was 7.6 cm, transverse colon 9.2 cm, and descending colon 5.7 cm, which was consistent with toxic megacolon. Bowel wall thickness is normal without signs of ischemic sufferance. No abdominal free gas or effusion.

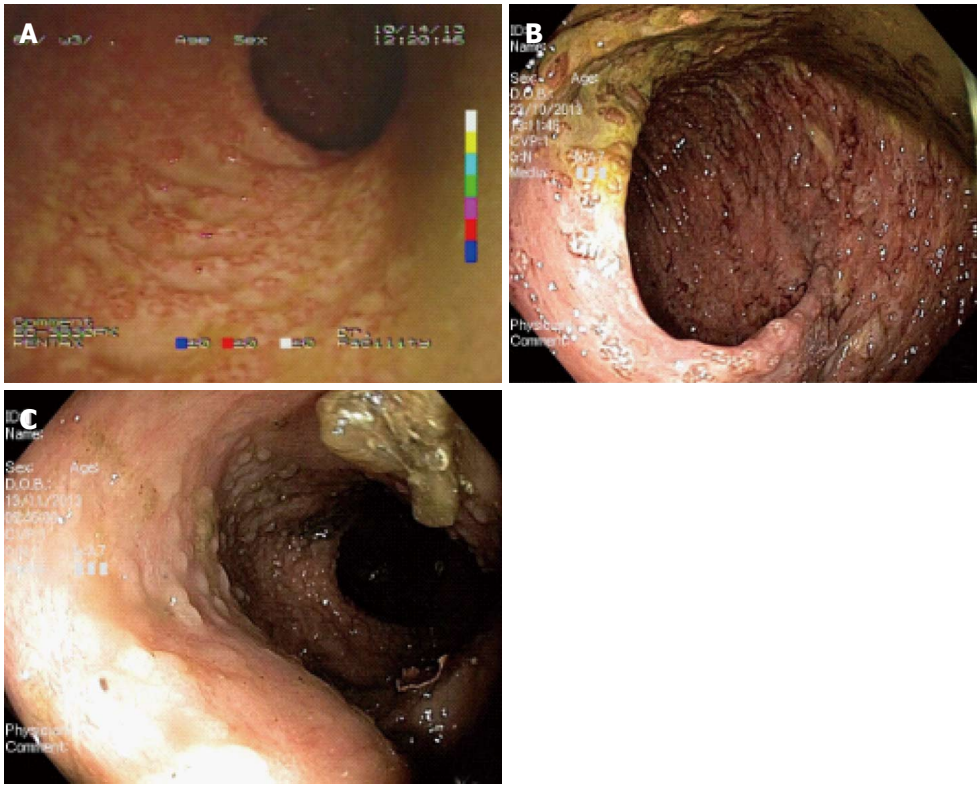

Figure 2 Colonoscopy in toxic megacolon at different times of pregnancy and therapy. A: Emergency colonoscopy performed during pregnancy before starting treatment: spontaneous bleeding and denudation of the mucosal surface, which is substituted by a fibrinous layer (descending colon); B: Colonoscopy after 9 d of treatment: mucosal hyperemia and erosions, fibrinous spots (cecum); C: Colonoscopy after one-month treatment: regenerative pseudopolyps of 2-4 mm (descending colon).

pregnancy. The onset of IBD in a pregnant woman is uncommon and is usually associated with a poor prognosis[1,10] because of an increased overall risk of an adverse neonatal outcome; the most frequently described are preterm delivery and low birth weight[5,11-13].

An accurate patient history is crucial, as are clinical and pertinent investigations to assess colitis activity. Endoscopic evaluation and rectal biopsy play an essential role in the diagnosis, management, prognosis, and surveillance of antenatal IBD, and can be performed safely without a significant increase in complications for the mother or fetus[14].

Concerning the method of delivery, most studies have also shown a significantly increased incidence of CS, but it is not consistently clear whether this is predominantly due to elective or emergency CS[5,12,13]. Our decision to perform a CS was taken not only due to the extreme early gestational age, but also in order to evaluate with colorectal surgeons a balanced view of the bowel and possible surgical interventions.

UC management in pregnant women implies special considerations concerning risks for the fetus. For example, most drugs necessary in such cases

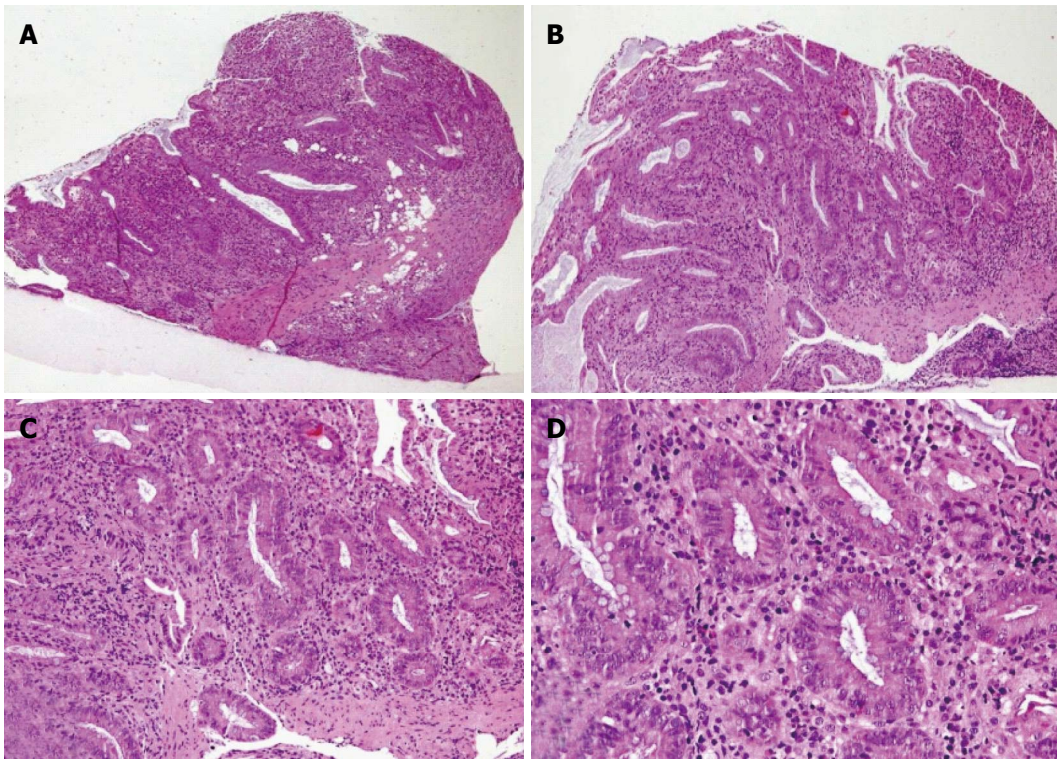

Figure 3 Histopathology of active ulcerative colitis. Colonic mucosa characterized by atrophic glands with architectural distortion, mucin depletion, intense inflammatory cell infiltrate of the lamina propria rich in eosinophils, basal plasma cells, crypt abscess, and surface ulceration (A, B: Hematoxylin-eosin staining, magnification × 4; C: Hematoxylin-eosin staining, magnification × 10; D: Hematoxylin-eosin staining, magnification × 20).

(*e.g.*, aminosalicylates agents and corticosteroids) are considered safe both for the mother and baby[2,14], but methotrexate and thalidomide are contraindicated[2]. Although there are limited data[15,16] on the safety of colonoscopy during pregnancy, it appears relatively safe, but requires several precautions concerning sedation and the left-sided position of the mother during the procedure, particularly in the third trimester. Obviously, it should be performed only when strongly indicated by an expert endoscopist after obstetric consultation[17]. In our case, the colonoscopy was performed not only for the diagnosis of UC complicated by toxic megacolon, but also to perform a colonoscopic decompression. Abdominal surgery, particularly when performed in the third trimester, is reported to increase the incidence of preterm labor and, consequently, implies a higher risk of adverse perinatal outcome[18]. According to guidelines, the indications for emergency surgery are the same for pregnant and non-pregnant women, including failed medical treatment[19] and complications such as toxic megacolon[2]. The principal advantages of a conservative management of toxic megacolon in pregnant women is to let the baby reach an appropriate gestational age to survive and, secondly, prevent the mother from having to undergo an emergency colectomy during gravidic sepsis. Although current guidelines describe toxic megacolon as an indication of emergency surgery[18], endoscopic decompression together with medical treatment and intensive monitoring should be a potentially successful management of severe UC complicated by toxic megacolon during pregnancy. Conversely, the presence of signs of acute abdomen, including perforation, abscess, ischemia, thrombosis (which were not present in our patient), and/or the lack of early response to conservative therapy, are indicators for surgery. Our report confirms the hypothesis that decisions regarding timing and indications for surgery are often challenging.

COMMENTS

Case characteristics

A 25-year-old woman gravida 1/para 0 with 6-7 bloody stools/d, abdominal pain, tachycardia, and weight loss occurring during the third trimester of pregnancy.

Clinical diagnosis

Bloody diarrhea, abdominal pain, hyporexia, apyrexy, and tachycardia.

Differential diagnosis

Infectious colitis (*Clostridium difficile*, *Cytomegalovirus*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Listeria monocytogenes*, and *Brucella melitensis*) and inflammatory bowel disease.

Laboratory diagnosis

8.8 g/dL hemoglobin concentration, 178 mg/L C-reactive protein, 45 mm/h erythrocyte sedimentation rate, and 81.4 ng/mL procalcitonin.

Imaging diagnosis

CT scan showed an ascending colon diameter of 7.6 cm, a transverse colon of 9.2 cm, and a descending colon of 5.7 cm, which was consistent with toxic megacolon. Bowel wall thickness was normal without signs of ischemic sufferance. No abdominal free gas or effusion was detected.

Pathological diagnosis

Colonoscopy and biopsy revealed severe ulcerative colitis in the active phase.

Treatment
The patient was treated with intravenous and oral corticosteroids, oral mesalazine, azathioprine, nutritional support, antibiotics, blood transfusion, potassium parenteral correction, and albumin infusion.

Related reports
New onset of ulcerative colitis during pregnancy is uncommon and associated with a poor prognosis.

Experiences and lessons
Although current guidelines describe toxic megacolon as an indication for emergency surgery, endoscopic decompression together with medical treatment and intensive monitoring should be lead to the potentially successful management of severe ulcerative colitis complicated by toxic megacolon during pregnancy.

Peer-review
This case report describes the onset of ulcerative colitis complicated by toxic megacolon in a 25-year-old pregnant patient. A cesarean section was performed and the colitis was managed conservatively.

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CASE REPORT

Fecal stream diversion and mucosal cytokine levels in collagenous colitis: A case report

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Abstract

In this case report, we examined the levels of cytokines expressed before and during fecal stream diversion and after intestinal continuity was restored in a patient with collagenous colitis. We report the case of a 46-year-old woman with chronic, active collagenous colitis who either failed to achieve clinical remission or experienced adverse effects with the following drugs: loperamide, cholestyramine, budesonide, methotrexate and adalimumab. Due to the intractable nature of the disease and because the patient was having up to 15 watery bowel movements per day, she underwent a temporary ileostomy. Colonic biopsies were analyzed for mucosal cytokine protein levels before and during fecal stream diversion and after intestinal continuity was restored. Mucosal protein levels of interleukin (IL)-1β, IL-2, IL-6, IL-12, IL-17 A, IL-23, TNF, IFN-γ, IL-4, IL-5, IL-10 and IL-13 were all higher during active disease and decreased to non-detectable or considerably lower levels during fecal stream diversion. One month after the restoration of bowel continuity, when the patient experienced a relapse of symptoms, IL-2, IL-23 and IL-21 levels were again increased. Our results indicate that fecal stream diversion in this patient suppressed the levels of all cytokines analyzed in colonic biopsies. With the recurrence of clinical symptoms and histological changes after bowel reconstruction, the levels of primarily proinflammatory cytokines increased. Our findings support the hypothesis that a luminal factor triggers the inflammation observed in collagenous colitis.

Key words: Microscopic colitis; Collagenous colitis; Luminex; Mucosal cytokines; Fecal stream diversion

Core tip: The pathophysiology of collagenous colitis remains poorly understood. We describe a patient with chronic, active collagenous colitis who either failed to achieve clinical remission or experienced adverse effects with all medications given; therefore the patient was treated with a temporary loop ileostomy. We analyzed cytokine protein production with Luminex assays in colonic biopsy tissues obtained before and during fecal stream diversion (FSD) and after intestinal continuity was restored. Because FSD leads to clinical and histological remission, this study protocol provided a unique opportunity to study cytokine dynamics during different stages of disease. We were thus able to demonstrate that FSD was followed by a decrease in the levels of nearly all cytokines and that the restoration of bowel continuity increased the levels of the proinflammatory cytokines interleukin IL-2, IL-21 and IL-23.

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INTRODUCTION

Collagenous colitis (CC) was first described in 1976 by Lindström *et al*[1] in rectal biopsies of a woman with chronic diarrhoea. In recent years, the incidence of CC has increased, and it is currently approximately 6 per 100000 inhabitants in Sweden[2]. The pathogenesis of the disease remains unknown, but it is considered to be multifactorial and to involve luminal agents that trigger a mucosal inflammatory reaction[3]. Furthermore, a mucosal barrier defect appears to be present in CC, resulting in an increased uptake of antigens and bacteria[4]. Little is known about the immunological mechanisms that drive the inflammation observed in CC, but recent studies have reported that a Th1 immunological reaction[5] or a mixed Th1/Th17 and Tc1/Tc17 reaction is present in microscopic colitis (MC)[6]. Medical treatment of CC is effective in the majority of patients, but in rare cases, if treatment fails, a temporary or permanent ileostomy (fecal stream diversion) may be necessary. Fecal stream diversion has been shown to lead to clinical and histological remission in patients with CC[7]; this phenomenon was first observed in patients with Crohn's disease[8]. It was also reported that mucosal barrier dysfunction and altered mucosal permeability normalized during fecal stream diversion in one patient with CC but reappeared with bowel continuity[9]. Thus, fecal stream diversion offers an opportunity to study the immunological processes in inflammatory bowel diseases such as CC. In this case report, we describe the dynamics of cytokine production before and during fecal stream diversion and after bowel reconstruction in a patient with CC.

CASE REPORT

Our case report involves a 46-year-old woman with a history of diarrhoea that began in 2006 after one week of antibiotic treatment with amoxicillin. Stool cultures were negative, and the patient received the diagnosis irritable bowel syndrome (IBS) without endoscopic evaluation. Her previous medical history included asthma and Raynaud's syndrome, for which she had been treated with verapamil on a continuous basis. In 2010, she was admitted to the gastroenterology clinic at the Linköping University Hospital because she was passing 10 watery stools per day and was consequently experiencing social disability as well. A colonoscopy was performed, and a diagnosis of CC was confirmed histologically.

She had already been given loperamide and cholestyramine for her IBS, with no symptomatic improvement. An induction treatment with 9 mg/d budesonide was initiated, which initially resulted in clinical improvement, but the patient's condition stopped responding to treatment after one month. Repeated stool samples ruled out infectious causes, a SeHCAT test ruled out bile salt malabsorption, and a gastroscopy with duodenal biopsies revealed no sign of celiac disease. Human immunodeficiency virus with secondary chronic diarrhoea and hyperthyroidism were also ruled out.

A new colonoscopy was performed, and biopsy tissue was collected; subsequent analysis of the biopsy tissue revealed no sign of lymphoma, although the tissue was positive for Congo red staining, confirming the presence of amyloidosis. Primary amyloidosis was ruled out, and the increased amyloid deposition observed in the colonic biopsy tissue was considered to be secondary to prolonged mucosal inflammation.

Therapy with subcutaneous injections of methotrexate was initiated while the patient was still receiving 6 mg of budesonide daily as maintenance therapy. After 6 wk on 15 mg/wk methotrexate and a further 6 wk on 25 mg/wk methotrexate, no symptomatic improvement was observed, and all medication was discontinued. As third-line therapy, adalimumab was administered at an initial dose of 160 mg/wk, followed by 80 mg/wk after 2 wk and finally 40 mg/wk after 2 more weeks. After induction therapy with adalimumab, the patient's symptoms improved significantly; the patient's mean stool frequency fell from an initial > 10 watery stools per day to 2.8 soft stools per day. Clinical remission was maintained for 2 mo after discontinuation of adalimumab before the patient

relapsed again. During relapse, she experienced fever, muscle and joint pain, and vomiting. Extensive blood analysis revealed elevated transaminase levels. Blood samples were tested for evidence of infection with CMV, EBV, HSV 1, HSV 2, rotavirus and parasites; the patient was positive for CMV-specific serum IgM, but CMV DNA was not detected in blood. An additional colonoscopy was performed, and biopsies were analyzed for the presence of CMV; all analyses were normal. At the same time, a consultation with a rheumatologist ruled out serum sickness and other potential underlying causes of her symptoms. No explanation of her symptoms was found, but it seemed unlikely that adalimumab had a causative role. Nevertheless, the decision was made not to continue with biological treatment. Because the patient continued to suffer from persisting, excessive joint pain, 30 mg of prednisolone daily was prescribed; her pain symptoms improved gradually, but her diarrhoea remained unchanged. Because all treatment regimens so far had failed and because the patient had developed severe anal skin ulcerations, a loop ileostomy was performed. Prednisolone treatment was tapered and stopped one month after surgery. Initially, there was a high flow of watery stool in the ileostomy, but normal ileac mucosa was found in biopsy tissue collected during an ileoscopy. After treatment with loperamide and a proton pump inhibitor, a normal flow was achieved. One year after the ileostomy, the patient experienced a herniation of the stoma, which caused stool leakage and consequent social and hygiene problems; thus, she requested bowel reconstruction. After bowel continuity was restored, her diarrhea reappeared immediately.

Methods

The patient gave written consent, and the regional ethical committee of Linkoping, Sweden consented to our obtaining extra mucosal biopsies for research purposes (Dnr 2012/216-31). A sigmoidoscopy with biopsies of the sigmoid colon was performed a week before and 4 mo after the ileostomy operation and 1 mo after bowel continuity was restored. The week before the operation, the patient was on 20 mg prednisolone per day (she had initially been receiving 30 mg prednisone, which was tapered down over a period of 2 mo before the first sigmoidoscopy); 4 mo after ileostomy and one month after bowel restoration, she was receiving no corticosteroid or other immunomodulating drug.

Protein extraction and cytokine analysis

Biopsy tissues were immediately placed in Allprotect and stored at -80 ℃ until use. The biopsy tissues were homogenized using a TissueLyser Ⅱ (Qiagen, Hilden, Germany) at 25 Hz for 5 × 1 min in radioimmunoprecipitation (RIPA) buffer containing 1 mmol/L Mini Protease Inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). The homogenization mixture was centrifuged for 5 min at 10000 rpm, and the supernatant was divided into aliquots and stored at -80 ℃ until further analysis.

Tissue levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-17A, IL-21, IL-23, IFN-γ and TNF proteins were analyzed using the xMAP technology developed by Luminex® (Austin, TX, United States). The concentrations were determined using the Milliplex® Map Kit (cat. no- #HCYTOMAG-60K and #HCYP2MAG-62K, Millipore, MA, United States) according to the manufacturer's instructions.

The assays were performed in duplicates. The levels of cytokines in samples were expressed as pg/mg tissue, based on a standard curve constructed using known amounts of each analyte (Millipore).

Table 1 Histological changes before, during and after fecal stream diversion

Ileostomy	IEL	Collagen layer thickness	Lamina propria infiltration	Epithelial degeneration
Before	58/100 enterocytes	10 μm	1	2
During	48/100 enterocytes	3 μm	1	2
After	108/100 enterocytes	25 μm	1	2

Values indicating the severity of changes were assigned subjectively. 1: Slight; 2: Moderate; 3: Severe.

Histology

All biopsies were analyzed by one pathologist with experience with microscopic colitis.

As shown in the Table 1 above, the first histological analysis performed before the ileostomy showed a picture of CC, but the classical signs of CC were not very apparent, most likely because the patient was taking 20 mg of prednisolone daily. The collagen layer thickness normalized during fecal diversion but increased to a clearly pathological level after intestinal reconstruction. The numbers of intraepithelial lymphocytes followed a similar pattern, with a considerable increase in their numbers observed after intestinal continuity was restored. However, the degree of lamina propria infiltration and epithelial degeneration remained unchanged throughout the study.

Cytokine concentrations before and during diversion and after restoration of bowel continuity

The mean ± SE weight of biopsy specimens used for cytokine quantification was 10 ± 0.98 mg. The levels of different cytokines in samples were expressed as pg/mg tissue.

Most tissue cytokine levels decreased substantially after fecal stream diversion (Figure 1). These decreases resulted in non-detectable concentrations for some cytokines, including IL-4, IL-10, and IL-1β. The levels of other cytokines, including IL-13, 1L-17 and TNF, decreased by 22%-40%, while the levels of IL-6, IL-

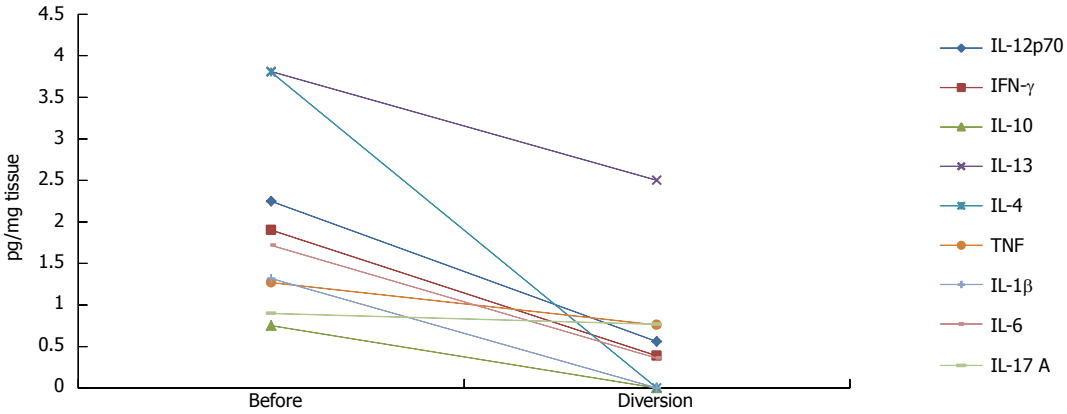

Figure 1 Cytokine levels before and during fecal stream diversion. Cytokine values are given in pg/mg tissue. IL: Interleukin; TNF: Tumor necrosis factor; IFN: Interferon.

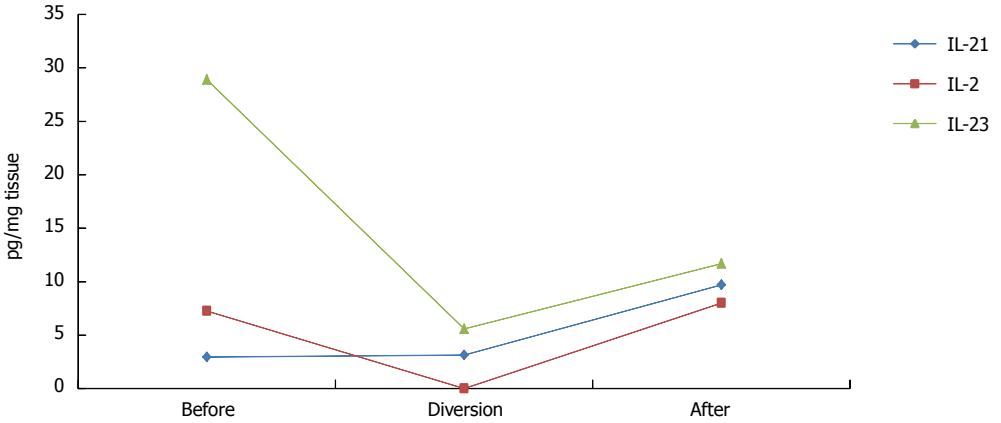

Figure 2 Cytokine levels before and during fecal stream diversion and after bowel reconstruction. Cytokine values are given in pg/mg tissue. IL: Interleukin.

12p70 and IFN-γ decreased by 75%-80%. IL-5 protein was not detectable at any time.

In comparison with all other measured cytokines (Figure 1), IL-21 was the only cytokine whose levels were unchanged during diversion (Figure 2). One month after intestinal continuity was restored; most cytokines remained at approximately the same low levels that were measured during fecal stream diversion (data not shown). However, IL-2, IL-21 and IL-23 exhibited a different trend; the levels of these cytokines increased after the fecal stream was re-established, as demonstrated in Figure 2.

DISCUSSION

Fecal stream diversion is known to cause clinical and histological remission in patients with CC[10]. Moreover, fecal stream diversion also appears to normalize mucosal barrier dysfunction in CC[4], but active disease unfortunately reoccurs after intestinal continuity is restored.

To our knowledge, this is the first case report to examine the dynamics of mucosal cytokine levels in a patient with collagenous colitis before and during fecal stream diversion and after bowel continuity was re-established. Fecal stream diversion in this patient offered an opportunity to investigate the levels of cytokines involved in immunological processes in the disease and the molecular basis of the effects of fecal stream diversion. We showed that diversion of the fecal stream led to a clear reduction in the protein levels of almost all analyzed cytokines and that the reoccurrence of symptoms and classical histological findings following the restoration of bowel continuity is accompanied by increases in mucosal IL-2, IL-21 and IL-23 levels. These results further demonstrate the importance of luminal agents as triggers of intestinal inflammation.

The role of fecal material in patients with Crohn's disease has been studied by Rutgeerts *et al*[8]. These investigators reported that inflammation in the neoterminal ileum in patients with Crohn's disease who underwent ileocecal resection was dependent on the fecal stream because inflammation did not appear in patients who were treated with a temporary proximal ileostomy. Harper *et al*[11] went a step further by either introducing either intestinal effluent or a sterile ultrafiltrate into the defunctioned colon of Crohn's disease patients who had undergone a split ileostomy. These investigators concluded that particles greater than 0.22 μm were the luminal triggers responsible for disease because the effluent caused a clinical response

as well as changes in laboratory values in comparison with the ultrafiltrate.

Few studies have analyzed cytokine profiles in MC, and those that do exist have focused primarily on mRNA and not protein levels. In the one study that analyzed both, Kumawat *et al*[6] found a Th1/Tc1 and Th17/Tc17 mRNA mucosal cytokine profile in both CC and LC, although the protein concentrations of these cytokines were not always increased[6]. Protein levels depend not only on mRNA levels but also on regulatory processes after mRNA is produced, *e.g.*, the protein degradation rate; these differences can explain the discrepancies found *in vivo*[12]. The relationship between mRNA and protein levels is complex, but protein levels may reflect biological activity and the cytokine milieu more accurately than mRNA levels. Tagkalidis *et al*[5] also described a Th1 reaction based on mucosal mRNA cytokine values; Th2 cytokines were difficult to detect, with the exception of IL-10, whose levels tended to be increased in MC patients compared with controls. Dey *et al*[13] described increased expression of TNF, IFN-γ and IL-8 mRNA in patients with lymphocytic colitis (LC), while IL-1β, IL-4, IL-10, IL-12 and IL-23 mRNA levels were not found to be significantly different in patients with LC compared with controls. These studies focused on patients with active MC and not on patients in remission. Only Tagkalidis *et al*[5] followed 6 of 18 patients and noted a decrease in IFN-γ mRNA levels over time; however, the dynamics of IL-10, TNF and IL-15 mRNA levels were not reported.

Although the patient in this study received 20 mg of prednisolone initially, all protein levels of cytokines were elevated during active disease compared to the period of fecal stream diversion. Surprisingly, little is known about the effect of prednisolone on cytokine levels in the intestinal mucosa *in vivo*, but in the bronchoalveolar lavage fluid of allergic patients, Liu *et al*[14] showed that treatment with prednisolone reduced mRNA and protein levels of IL-4, IL-5 and IL-2. Because we analyzed protein levels of cytokines, it is hard to compare our results with those of other studies; also, because we analyzed only one patient, our primary focus was to describe the dynamics of cytokine levels at different stages of treatment and disease. Most striking were the obvious increases in mucosal IL-2, IL-21 and IL-23 levels after intestinal continuity was restored, which paralleled a prompt relapse of symptoms and the reestablishment of the classical histological signs of CC.

The most pronounced changes were observed in the levels of IL-23 during the course of the study; IL-23 levels exhibited the largest increase among all cytokine levels one month after intestinal restoration. Studies in murine models have shown the importance of IL-23 in inducing and enhancing chronic intestinal inflammatory disease[15]. Furthermore, inhibition of the IL-23 p19 subunit has been shown to be effective both in preventing and treating active colitis in murine models[16]. This cytokine is thought to mediate communication between the innate and adaptive immune systems and is produced by activated dendritic cells and macrophages within hours after encountering a pathogen[17]. IL-23 also plays a role by inducing $CD4^+$ T cells to produce IL-17, and IL-17 levels are increased in another inflammatory bowel disease, Crohn's disease[18]. Kumawat *et al*[6] previously found colonic levels of both IL-17 and IL-23 mRNA to be increased in both CC and LC, while protein levels were not found to be markedly different from those in healthy controls[6]. These findings, together with our present results, indicate that IL-23 is involved in the inflammatory process.

The dynamics of IL-2 levels after bowel reconstruction resembled those of IL-23 but not to the same extent. IL-2 is a cytokine that has been studied extensively in the past 25 years. IL-2 levels have been found to be altered in autoimmune diseases; positive correlations between serum levels of IL-2 and both skin progression in scleroderma[19] and joint destruction in rheumatoid disease have been observed[20]. In gastrointestinal disorders, enhanced serum IL-2 is associated with advanced gastric cancer[21]. Using IL-2R-/- mice, Malek *et al*[22] showed that IL-2 and/or IL-2R act via stimulating the production of Tregs.

Finally, IL-21 is a cytokine known to be involved in a variety of tissues in many inflammatory diseases[23,24]. Although IL-21 is found in mucosal biopsies from healthy individuals, the expression of this cytokine is enhanced in the colonic mucosa of Crohn's patients[25]. It is thought to be produced by activated Th17 cells[26] and activated NKT cells[27]. In a murine experimental model of colitis, wild-type mice develop colitis when treated with dextran sulphate sodium and trinitrobenzene sulfonic acid, whereas IL-21-deficient mice do not[28]. A proposed mechanism of action of IL-21 in IBD is that colonic fibroblasts, when stimulated with IL-21, produce matrix metalloproteinases[29], which are involved in the epithelial damage that causes IBD[30].

In conclusion, our observations demonstrate that fecal stream diversion affects inflammation both histologically and on a molecular level by reducing the levels of cytokines involved in the inflammatory process in CC. Our results show that the levels of all cytokines, except IL-21, decreased during diversion and remained at lower levels after bowel reconstruction, with the exception of IL-2, IL-21 and IL-23, whose levels increased. These three cytokines are all known to be proinflammatory. Fecal stream diversion and bowel reconstruction provides a unique opportunity to investigate immunological processes involved in inflammatory bowel disorders.

COMMENTS

Case characteristics

The patient experienced diarrhea with 10 watery stools per day, which eventually caused anal skin ulceration and a decreased quality of life.

Clinical diagnosis

Collagenous colitis, a subtype of microscopic colitis.

Differential diagnosis

Repeated stool samples ruled out infectious causes. A SeHCAT investigation ruled out bile salt malabsorption, and gastroscopy with duodenal biopsies revealed no sign of celiac disease. Human immunodeficiency virus with secondary chronic diarrhea and hyperthyroidism were also ruled out by blood analyses. Finally, primary amyloidosis was ruled out with a biopsy of adipose tissue.

Laboratory diagnosis

Luminex assays were used to analyze cytokine levels, and while almost all cytokines levels decreased during ileostomy; only the levels of interleukin (IL)-21, IL-23 and IL-2 increased after intestinal continuity was restored.

Pathological diagnosis

Histological examination revealed classical findings of collagenous colitis.

Treatment

The patient was initially treated with budesonide, but the patient's symptoms stopped responding to treatment. Methotrexate had no effect on symptoms, and adalimumab did not have a long-lasting effect. Finally, a loop ileostomy was performed.

Related reports

Fecal stream diversion is known to induce remission in patients with collagenous colitis, but in this study, cytokine levels were measured throughout this process for the first time.

Term explanation

Collagenous colitis is an inflammatory intestinal disease that presents with non-bloody, watery diarrhea. Fecal stream diversion refers to the creation of a terminal ileostomy that excludes the colon from intestinal transit.

Experience and lessons

Collagenous colitis can be refractory to treatment, leading to severely impaired quality of life. IL-2, IL-21 and IL-23 appear to be involved in disease pathogenesis, and disease processes should be analyzed further in patients with this disease.

Peer-review

It is well written, comprehensively reviewed and potentially helpful in the understanding of basic mechanisms in inflammation of the bowel.

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CASE REPORT

Epstein Barr virus-positive mucocutaneous ulcer of the colon associated Hodgkin lymphoma in Crohn's disease

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Abstract

Epstein Barr virus (EBV) positive mucocutaneous ulcers (EBVMCU) form part of a spectrum of EBV-associated lymphoproliferative disease. They have been reported in the setting of immunosenescence and iatrogenic immunosuppression, affecting the oropharyngeal mucosa, skin and gastrointestinal tract (GIT). Case reports and series to date suggest a benign natural history responding to conservative management, particularly in the GIT. We report an unusual case of EBVMCU in the colon, arising in the setting of immunosuppression in the treatment of Crohn's disease, with progression to Hodgkin lymphoma 18 mo after cessation of infliximab. The patient presented with multiple areas of segmental colonic ulceration, histologically showing a polymorphous infiltrate with EBV positive Reed-Sternberg-like cells. A diagnosis of EBVMCU was made. The ulcers failed to regress upon cessation of infliximab and methotrexate for 18 mo. Following commencement of prednisolone for her Crohn's disease, the patient developed widespread Hodgkin lymphoma which ultimately presented as a life-threatening lower GIT bleed requiring emergency colectomy. This is the first report of progression of EBVMCU to Hodgkin lymphoma, in the setting of ongoing iatrogenic immunosuppression and inflammatory bowel disease.

Key words: Epstein Barr virus; Mucocutaneous ulcer; Hodgkin lymphoma; Inflammatory bowel disease; Crohn's disease

Core tip: This is the first reported case of Epstein Bar virus mucocutaneous ulcer (EBVMCU) affecting the gastrointestinal tract progressing to widespread Hodgkin lymphoma in the context of iatrogenic immunosuppression in the treatment of Crohns disease. EBVMCU is a newly recognised clinico-pathological condition that was previously thought to have a benign natural history. This case highlights the malignant potential of this disease entity even after withdrawal of immunosuppression.

Moran NR, Webster B, Lee KM, Trotman J, Kwan YL, Napoli J, Leong RW. Epstein Barr virus-positive mucocutaneous ulcer of the colon associated Hodgkin lymphoma in Crohn's disease. *World J Gastroenterol* 2015; 21(19): 6072-6076 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6072.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6072

INTRODUCTION

Epstein Barr virus (EBV) infection is a ubiquitous herpes virus. After primary infection at an early age, EBV establishes latent infection in B-cells. A higher EBV prevalence rate is found in immunosuppressed compared to healthy individuals[1]. EBV is able to elicit B-cell transformation and proliferation which is kept in check by T-cell immune surveillance[2,3]. Iatrogenic immunosuppression for autoimmune disorders and in the post-transplant setting as well as age-related immunosenescence can lead to the emergence of EBV-positive B-cell lymphoproliferative disorders (LPDs). In the setting of inflammatory bowel disease (IBD), the gastrointestinal mucosa has been identified as a site of EBV replication[2] and reactivation of EBV is more frequent among patients with IBD[4]. EBV has been linked with the development of lymphoma in the gastrointestinal tract (GIT)[5].

EBV positive mucocutaneous ulcers (EBVMCU) were first identified as a distinct clinico-pathological entity in 2010. Dojcinov *et al*[6] reported a study of 26 cases of ulcerative lesions arising in the skin, oropharynx and GIT in the context of immunosuppression, including age-related immunosenescence and iatrogenic immunosuppression for autoimmune diseases. These lesions displayed an indolent self-limited course, often regressing spontaneously or with reduction of immunosuppression, and with no reports of progression to disseminated disease. The entity shows an infiltrate of EBV^+ atypical large Hodgkin/Reed-Sternberg (HRS)-like cells with a polymorphous inflammatory background, mimicking classical Hodgkin lymphoma (cHL). Hence, a diagnosis of EBVMCU requires a combination of clinical, morphologic and immunophenotypic parameters. Since that classification, EBVMCU have been increasingly reported in the literature particularly relating to iatrogenic immunosuppression, with methotrexate[7,8] azathioprine[6,9,10] and ciclosporin[6] therapy.

Reports of EBVMCU affecting the GIT have been limited. Case reports to date suggest an indolent course with regression following withdrawal of immunosuppression[7,9]. Dojcinov *et al*[6] described four cases involving the gastrointestinal tract, all of which achieved complete remission with either no intervention, or with reduction of immunosuppression. In one case no intervention was required, whereas in the other cases a reduction of immunosuppression induced regression of the EBVMCU. There was no malignant progression and no need for treatment cessation or further intervention. This is the first case report of an IBD-associated EBVMCU with progression to cHL.

CASE REPORT

We present an unusual case of primary colorectal Hodgkin Lymphoma in a 53-year-old woman with a six year history of histologically confirmed Crohn's disease (CD). She had glandular fever in her teenage years and no other significant past medical history. There was no personal or family history of primary immunodeficiency. Initial CD treatment was with aminosalicylates and corticosteroids but escalated to immunomodulators for recurrent need for steroids. Due to azathioprine intolerance, methotrexate was substituted. She had primary non-response to induction and six months maintenance of adalimumab, and induction and maintenance infliximab 5 mg/kg every 8 wk was commenced together with methotrexate. One year later surveillance colonoscopy revealed ulceration at the splenic flexure, sigmoid colon and rectum. Colonoscopic biopsies showed a polymorphous infiltrate in the lamina propria containing large atypical HRS-like cells in a background of lymphocytes, histiocytes, plasma cells, neutrophils and eosinophils (Figure 1). The polyclonal atypical cells showed a classic Hodgkin lymphoma like immunophenotype with positivity for CD30, weak positivity for PAX5, strong positivity for MUM-1 and variable positivity for CD20, CD15, and OCT2. The atypical cells were positive for EBV-encoded small RNAs (EBER) by *in situ* hybridisation (ISH) (Figure 2). The morphological appearance, immunohistochemical profile and clinical context were consistent with EBVMCU without clonal proliferation. Methotrexate and infliximab were discontinued and repeat colonoscopy at 2, 6, 12 and 18 mo after cessation showed persistence of the colorectal ulcers (Figure 3). Subsequent colonoscopic biopsies with reduced immunosuppression still showed persistence of the ulcers with similar histological findings. Surgical resection was strongly considered if it were not for the benign course of the condition in small case series, the patient's refusal for ileostomy and the distal location of one EBVMCU which was unable to be easily resectable with primary intestinal anastomosis.

The patient was completely off immunosuppression for 18 mo and was only taking 5-aminosalicylates. CD control was sub-optimal requiring recommencement of prednisolone. The ulcers showed some improvement following treatment with prednisolone 40 mg daily. Extensive multi-disciplinary discussion reaffirmed the diagnosis of an EBVMCU given the superficial localised nature of the ulceration and the presence of atypical EBV-positive lymphoid infiltrate and absence of clonal expansion. Within two months of commencing prednisolone, the patient had presented with fever and nausea. A computed tomography scan revealed multiple circumscribed liver lesions. Biopsies of the

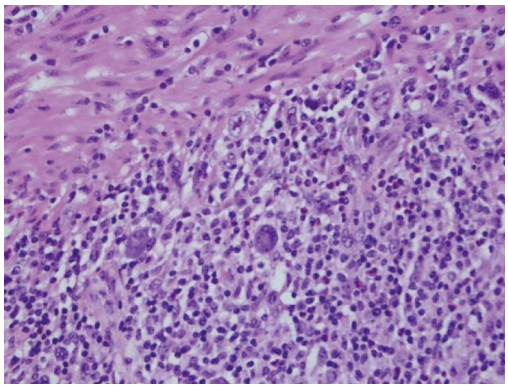

Figure 1 Hematoxylin and eosin staining of the colectomy specimen (magnification × 40). Scattered Hodgkin/Reed-Sternberg-like cells are present in a polymorphous background of lymphocytes, histiocytes and eosinophils. Muscularis propria is seen in the upper left of the field.

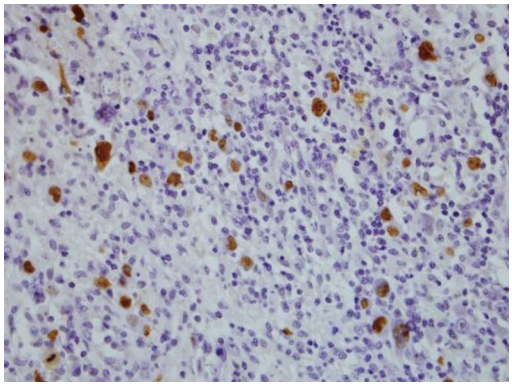

Figure 2 Epstein Barr virus-encoded small RNAs *in situ* hybridisation of the colectomy specimen (magnification × 40). The Hodgkin/Reed-Sternberg-like cells show strong nuclear staining, indicating Epstein-Barr virus positivity.

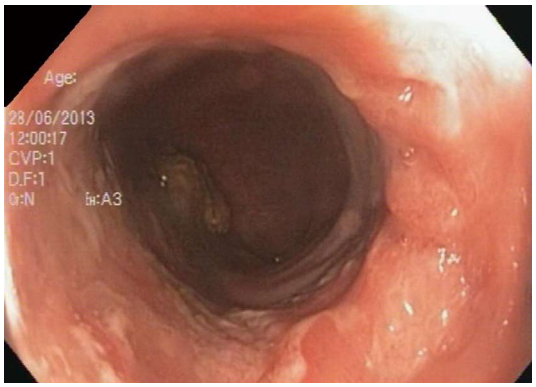

Figure 3 Persistence of ulceration in the sigmoid colon 18 mo post methotrexate and infliximab cessation.

splenic flexure and sigmoid colon ulceration remained consistent with EBVMCU. Ultrasound-guided cores biopsies of the liver lesions showed a polymorphous inflammatory infiltrate with HRS cells which were CD30, CD15, MUM1 and weakly PAX5 positive; negative for CD45, OCT2 and BOB1; and EBER ISH positive; an immunophenotype indistinguishable from cHL.

Soon after, the patient presented with life-threatening rectal haemorrhage requiring an emergency colectomy with end ileostomy. Macroscopically, multiple large transmural colonic ulcers were present in the colon, measuring up to 15 cm in size and extending through the muscularis propria. Mesenteric lymphadenopathy was present. Microscopically, there was a transmural involvement of the bowel wall and local lymph nodes by an identical process to that in the liver, features consistent with a diagnosis of Hodgkin lymphoma (HL), mixed cellularity. Urgent chemotherapy with adriamycin, bleomycin, vinblastine and dacarbazine was commenced. PET negative status was achieved after two months of chemotherapy and the patient remained in complete remission after six cycles.

DISCUSSION

EBVMCU has been described as indolent in its clinical behaviour, in most cases responding to withdrawal of immunosuppression. This is the first reported case of gastrointestinal tract EBVMCU progressing to classical Hodgkin Lymphoma despite cessation of infliximab and methotrexate some 18 mo previously.

The GIT is a common extranodal primary site of lymphoma especially B-cell non-Hodgkin lymphomas. Primary GIT cHL is rare, representing only a minority of primary GIT lymphomas and < 0.5% of all cHL[11,12]. There are few reports of EBVMCU involving the GIT and specifically the colon. However, due to the need for clinicopathological correlation to diagnose EBVMCU, there is the possibility that cases in the literature diagnosed as cHL or other LPDs represent genuine cases of EBVMCU. In this case report, the finding of EBVMCU which failed to progress with cessation of immunosuppression and subsequent development of cHL when prednisolone was reinstated is suggestive of EBVMCU as a precursor of cHL. Although the histomorphologic and immunophenotypic characteristics of the HRS-like cells from the colonic ulcers were indistinguishable from cHL, EBVMCU were initially diagnosed instead of cHL because she remained haematologically well throughout the persistence of the ulcers. It was only upon reinstatement of the immunosuppression and evidence of progression in the form of liver, transmural colonic and nodal involvement by the HRS-like cells that cHL was diagnosed. In our opinion, this highlights the point that EBVMCU should be a clinico-pathological diagnosis. It is known that molecular studies for immunoglobulin heavy chain (*IgH*) gene rearrangement on microdissected HRS cells were performed on a case consistent with EBVMCU and a case of systemic disease[13]. Monoclonal *IgH* rearrangement was found in the case of systemic disease whilst the localised disease was polyclonal. This is suggestive that EBVMCU represents an early polyclonal EBV-driven LPD which may show molecular

progression to cHL, analogous to post-transplant LPDs, in which polymorphic B-cell proliferations may regress upon removal of immunosuppression, or progress to lymphoma[14].

There is significant concern regarding the risk of developing lymphoma associated with immunosuppressive therapy in IBD. Population based studies have demonstrated an increased risk of lymphoma in patients treated with thiopurines[15], and more recently a meta-analysis confirmed a higher lymphoma risk in patients treated with thiopurines which reduces with discontinuation of therapy[16]. The refurbish study demonstrated an increased incidence of non-Hodgkin lymphoma with combination tumor necrosis factor (TNF)-α and thiopurine use but not in TNF-α monotherapy[17]. Cases of EBV-associated colonic B-cell lymphoma following treatment with infliximab for IBD have however been reported[5], as well as regression of colonic lymphoma following infliximab and thiopurine withdrawal[18].

Our patient only had azathioprine temporarily and infliximab for 12 mo prior to its cessation. Failure of the EBV MCU to regress despite cessation of immunosuppression may also be secondary to the inherent immunodysregulation present in some patients with IBD[19,20]. This constitutional failure of immune-surveillance may predispose patients with IBD to the emergence of EBV driven LPDs.

The natural history of EBVMCU in the setting of immunosuppression in IBD is poorly understood. What is apparent, however, is that EBVMCU can fail to regress despite cessation of immunosuppressive therapy and in fact progress towards cHL. Failure of EBVMCU to regress after cessation of immunosuppression for over 12 mo may an indication for localised intestinal resection, if amenable.

COMMENTS

Case characteristics

Colonoscopy revealed ulceration at the splenic flexure, sigmoid colon and rectum which histological analysis of biopsies confirmed as Epstein Barr virus positive mucocutaneous ulcers (EBVMCU).

Clinical diagnosis

EBVMCU developed in the context of immunosuppression for the treatment of Crohn's disease. These were initially considered benign given the available evidence in the literature. The patient main remained asymptomatic.

Laboratory diagnosis

Clinico-pathological diagnosis.

Imaging diagnosis

Abdomen computed tomography revealed multiple circumscribed liver lesions suspicious for metastasis 18 mo following withdrawal of immunosuppression.

Pathological diagnosis

Histological analysis revealed atypical cells showing a classic Hodgkin lymphoma like immunophenotype with positivity for CD30, weak positivity for PAX5, strong positivity for MUM-1 and variable positivity for CD20, CD15, and OCT2 - consistent with a diagnosis of EBVMCU.

Treatment

Previous reports suggested resolution of EBVMCU on withdrawal of immunosuppressive therapy - however in this case the EBVMCU progressed to Hodgkin Lymphoma despite treatment cessation 18 mo previously.

Related reports

There are limited reports of EBVMCU in the gastrointestinal tract. Previously reported cases have demonstrated a benign natural history, with regression following cessation of immunosuppressive therapy.

Experiences and lessons

This case reports the important finding of potential malignant progression in Epstein Barr virus mucocutaneous ulcers in the setting of iatrogenic immunosuppression.

Peer-review

The rectal circumferential ulcer was too distal to allow sufficient margin for an end-to-end anastomosis of low anterior resection. It was visualised by the author and on further discussion with the patient she was not prepared for the quality-of-life consequence of an AP resection. The authors had considered surgery and even tattooed the lesions with India Ink endoscopically in preparation for guiding surgical resection. However, the patient did not provide consent in light of cases of EBVMCU demonstrating a benign outcome.

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CASE REPORT

Surgical repair of intractable chylous ascites following laparoscopic anterior resection

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Author contributions: Lee MR performed the operation; Ha GW collected case data; Ha GW wrote the manuscript; Lee MR proofread and revised the manuscript; all authors approved the version to be published.

Ethics approval: The study was reviewed and approved as exemption by the Chonbuk National University Hospital Institutional Review Board.

Informed consent: Study participant provided informed written consent prior to study enrollment.

Conflict-of-interest: There are no potential conflicts of interest in this manuscript.

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Abstract

Chylous ascites is the accumulation of a milk-like peritoneal fluid rich in triglycerides and it is an unusual complication following surgical treatment of colorectal cancer. Conservative management is usually sufficient in patients with chylous ascites after surgery. However, we describe a patient with intractable chylous ascites after laparoscopic anterior resection for sigmoid colon cancer who failed initial conservative treatment. This patient was successfully managed by surgery.

Key words: Sigmoid colon cancer; Laparoscopy; Anterior resection; Chylous ascites; Surgical repair

Core tip: Chylous ascites is unusual following surgical treatment of colorectal cancer. Postoperative chylous ascites is always difficult to manage, due to the consequences of the primary surgery and the constant loss of lymph. Although conservative management is usually sufficient in patients with chylous ascites after surgery, we describe a patient who experienced intractable chylous ascites after laparoscopic anterior resection for sigmoid colon cancer. This patient was successfully managed by surgery.

Ha GW, Lee MR. Surgical repair of intractable chylous ascites following laparoscopic anterior resection. *World J Gastroenterol* 2015; 21(19): 6077-6081 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6077.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6077

INTRODUCTION

Chylous ascites is the accumulation of a milk-like peritoneal fluid rich in triglycerides, due to the presence of lymph in the abdominal cavity. Chylous ascites develops in patients with disruption of the lymphatic system due to traumatic injury or obstruction, which may result from malignancy, liver cirrhosis, infectious etiologies, congenital disorders, and inflammatory diseases[1].

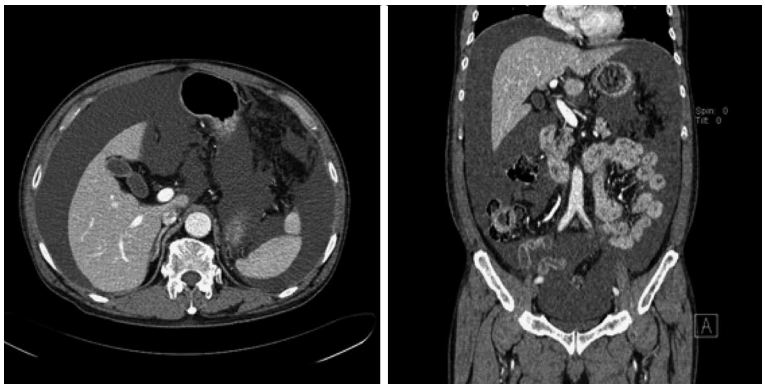

Figure 1 Abdominal computed tomography images showed a large volume of ascites.

Chylous ascites is unusual following surgical treatment of colorectal cancer. Postoperative chylous ascites is always difficult to manage, due to the consequences of the primary surgery and the constant loss of lymph. Management of chylous ascites may include repeat palliative paracentesis, dietary measures, total parenteral nutrition (TPN) therapy and surgical repair of the fistula[2].

We describe a patient who experienced intractable chylous ascites after laparoscopic anterior resection for sigmoid colon cancer. This patient did not show improvements following primary conservative treatment, including TPN, administration of somatostatin and dietary modification, but was subsequently successfully managed surgically.

CASE REPORT

In April 2014, a 65-year-old man underwent laparoscopic anterior resection with lymph node dissection up to the level of the inferior mesenteric artery for sigmoid colon adenocarcinoma. The procedure was uneventful. The histopathologic stage was T3N1a. Of the 17 lymph nodes obtained, one was positive for malignancy. The initial postoperative period was unremarkable, except for small amounts of milky, odorless fluid. The volume of drainage decreased and the drain was removed on the fifth postoperative day. The patient was discharged on the seventh postoperative day without any complications.

Two weeks later, he complained of increased abdominal girth after discharge from the hospital. On physical examination, he had a moderately distended abdomen with dullness to percussion. Abdominal computed tomography with intravenous (IV) contrast showed a large volume of ascites (Figure 1). The patient was rehospitalized and underwent abdominal percutaneous drainage, which resulted in the removal of 2500 mL of milky white, odorless fluid. Laboratory analysis of the fluid revealed a triglyceride level of 457 mg/dL, albumin 2.3 g/dL, lactic dehydrogenase 112 IU/L, and protein 3.2 g/dL, consistent with chylous ascites. Cytology was negative for malignancy and cultures were negative. All other laboratory tests were nonspecific with a serum albumin level of 2.6 g/dL. The patient was put on a low fat diet and medium chain triglyceride supplementation. After one week, the volume of chylous ascites still drained over 1500 mL per day. At that time, oral feeding was discontinued, and the patient was started on TPN and subcutaneous somatostatin injections, which were continued for four weeks. The volume of drainage had decreased to 300 mL per day, and oral feeding was restarted. This increased the volume of drainage to 1500-3300 mL per day of chylous ascites. Moreover, the poor nutritional status of the patient, with a weight loss of approximately 10 kg, and his desire for a definitive solution led to a plan for surgical treatment. Preoperative lymphoscintigraphy was performed but no specific site of extravasation was identified (Figure 2).

Explorative laparotomy was elected. The patient ingested a high fat food four hours before the operation to facilitate visualization of the lymphatic fistula. A streak of clear chyle was immediately visible. The fistula, considered a branch of left lumbar trunk, was found to stem from a 1 mm hole in one of the lymphatic channels on the left side of the ligated inferior mesenteric artery (Figure 3). The tract was sutured and no additional sites of leakage were found in the abdomen. Ten days after the surgery, the patient was discharged. After three months, there has been no evidence of ascites (Figure 4).

DISCUSSION

Postoperative chylous ascites is an infrequent condition usually resulting from operative trauma caused by the unrecognized interruption of lymphatic channels[2]. Chylous ascites cannot always be avoided due to the inconsistent anatomy of lymphatic structures. However, meticulous ligation and clipping, or coagulation of lymphatic tissue near the vessel origins, can minimize its occurrence.

The incidence of chylous ascites after laparoscopic anterior resection is not clear. Only two studies have

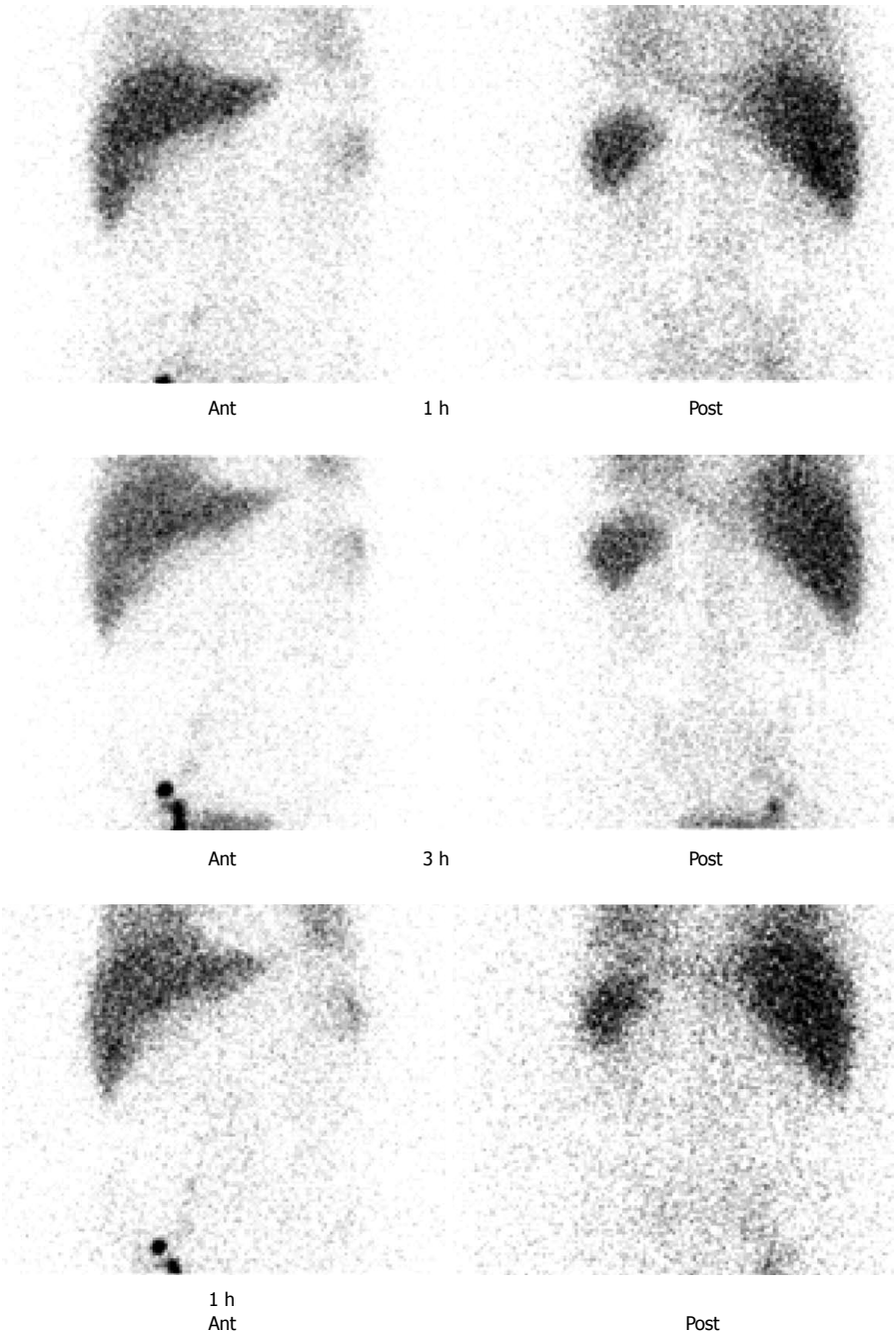

Figure 2 Preoperative lymphoscintigraphy showed no evidence of lymphatic leakage in abdominal cavity.

reported cases of this complication, with all patients treated conservatively[3,4]. Conservative management of chylous ascites can include dietary modifications, use of TPN, administration of somatostatin or octreotide, and use of diuretics to reduce lymph formation[5]. Dietary modifications include a high protein, low fat, medium chain triglyceride diet, or interruption of oral feeding. Conservative management is usually successful, making initial, conservative management reasonable in patients with this complication.

Surgical repair may be effective in patients who fail conservative management. However, surgical repair may not be successful and may even cause additional trauma. The timing of surgical repair remains unclear. Surgical and traumatic causes of chylous ascites may be explored early. Congenital and malignant causes may be given longer trials of conservative management. The decision for surgical repair should be tailored to the particular situation of the patient. Patients should be managed conservatively for approximately 6-8 wk before conservative management is judged to have failed[2,5].

If surgical repair of chylous ascites is elected, it is important to have knowledge about the anatomy of lymphatic drainage. For example, in the left colon, lymph from the descending, iliac, and pelvic parts of the colon passes to the intermediate groups of inferior mesenteric glands, with most subsequently passing

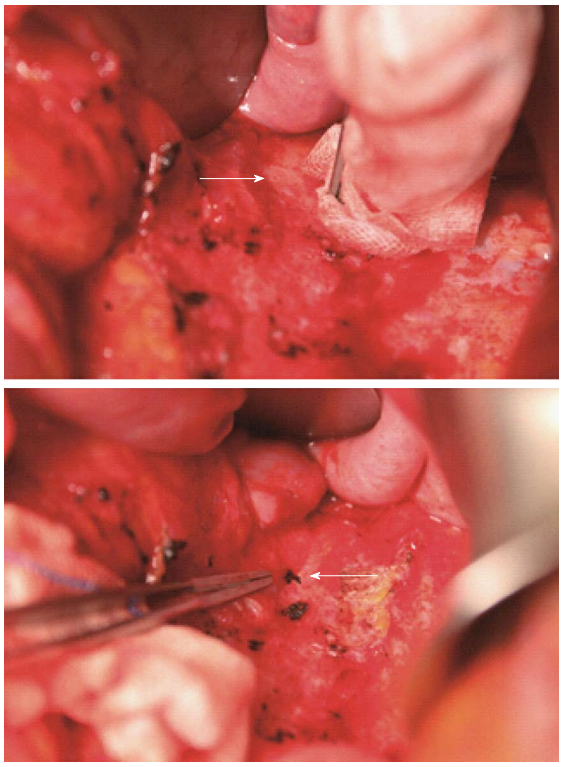

Figure 3 Fistula was found to stem from a 1 mm hole on the left side of the ligated inferior mesenteric artery, and the tract was sutured (white arrows).

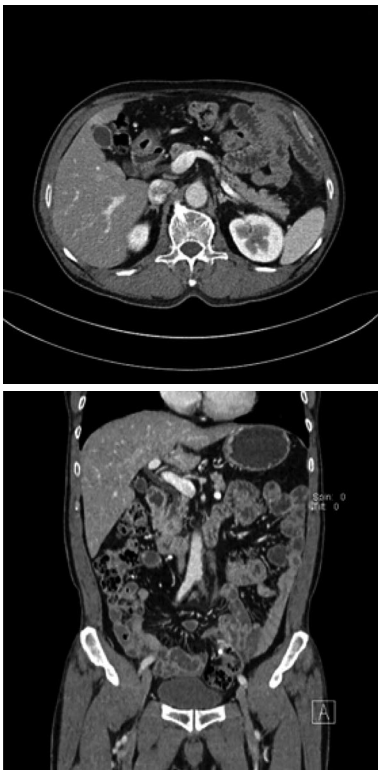

Figure 4 Postoperative abdominal computed tomography images showed no evidence of ascites.

to the lumbar glands. The main glands of the inferior mesenteric group receive lymph from the intermediate left colic glands and transmit it to the lumbar glands. Lymph then passes through the lumbar lymph trunks to the cisterna chyli. Based on anatomic knowledge, we were easily able to locate the fistula tract in our patient, finding it on the left side of the ligated inferior mesenteric artery. It was considered a branch of the left lumbar lymphatic trunk.

In conclusion, we have described a patient with a lymphatic fistula that appeared through an abdominal drainage after laparoscopic anterior resection. Meticulous ligation and clipping or coagulation of lymphatic tissue near the major vessel origins is important in preventing this complication. Although most patients with chylous ascites may be successfully treated conservatively, those who fail conservative management can undergo surgical repair and it may be performed effectively.

COMMENTS

Case characteristics

The patient who had undergone laparoscopic anterior resection for sigmoid colon cancer revisited two weeks after discharge from hospital presenting with abdominal distention.

Clinical diagnosis

On physical examination, he had a moderately distended abdomen with dullness to percussion.

Differential diagnosis

Intraabdominal abscess caused by delayed anastomotic microperforation.

Laboratory diagnosis

Laboratory analysis of the ascitic fluid revealed a triglyceride level of 457 mg/dL, albumin 2.3 g/dL, and protein 3.2 g/dL, and all other laboratory tests were nonspecific with a serum albumin level of 2.6 g/dL.

Imaging diagnosis

Abdominal computed tomography showed a large volume of ascites.

Treatment

This patient was successfully managed by surgical repair of the lymphatic fistula.

Related reports

Surgical repair of intractable chylous ascites after surgery has been reported rarely.

Experiences and lessons

A patient with intractable chylous ascites following surgery can undergo surgical repair and it may be performed effectively.

Peer-review

The authors have described intractable chylous ascites after laparoscopic anterior resection who failed initial conservative treatment. This patient was successfully managed by surgical repair.

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CASE REPORT

Severe Henoch-Schönlein purpura with infliximab for ulcerative colitis

Yang Song, Yan-Hong Shi, Chong He, Chang-Qin Liu, Jun-Shan Wang, Yu-Jie Zhao, Yan-Min Guo, Rui-Jin Wu, Xiao-Yue Feng, Zhan-Ju Liu

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Author contributions: Song Y and Shi YH contributed equally to this study; Liu ZJ and Liu CQ designed the study; Wang JS, Wu RJ and Zhao YJ collected the patients' clinical data; He C, Guo YM and Feng XY performed the experiments; Song Y and Shi YH analyzed the data and wrote the manuscript.

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Ethics approval: The study was reviewed and approved by the Hospital and University Review Committees for Medical Research.

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

Conflict-of-interest: The authors declared no conflict interests.

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Abstract

Infliximab (IFX) is an anti-tumor necrosis factor chimeric antibody that is effective for treatment of autoimmune disorders such as Crohn's disease and ulcerative colitis (UC). IFX is well tolerated with a low incidence of adverse effects such as infections, skin reactions, autoimmunity, and malignancy. Dermatological manifestations can appear as infusion reaction, vasculitis, cutaneous infections, psoriasis, eczema, and skin cancer. Here, we present an unusual case of extensive and sporadic subcutaneous ecchymosis in a 69-year-old woman with severe UC, partial colectomy and cecostomy, following her initial dose of IFX. The reaction occurred during infliximab infusion, and withdrawal of IFX led to gradual alleviation of her symptoms. We concluded that Henoch-Schönlein purpura, a kind of leukocytoclastic vasculitis, might have contributed to the development of the bruising. Although the precise mechanisms of the vasculitis are still controversial, such a case highlights the importance of subcutaneous adverse effects in the management of UC with IFX.

Key words: Henoch-Schönlein purpura; Infliximab; Vasculitis; Subcutaneous ecchymosis; Ulcerative colitis

Core tip: Infliximab (IFX) is effective in the management of ulcerative colitis (UC). Henoch-Schönlein purpura (HSP) appears as a dermatological adverse effect of IFX. However, extensive ecchymosis in old UC patients is rare. This report describes a case of HSP reaction, which resolved upon IFX withdrawal.

Song Y, Shi YH, He C, Liu CQ, Wang JS, Zhao YJ, Guo YM,

Wu RJ, Feng XY, Liu ZJ. Severe Henoch-Schönlein purpura with infliximab for ulcerative colitis. *World J Gastroenterol* 2015; 21(19): 6082-6087 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6082.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6082

INTRODUCTION

Ulcerative colitis (UC) is a chronic autoimmune disorder of the colonic mucosa, which generally affects the rectum and may extend proximally to other parts of the colon, with continuous alternating relapse and remission. The crude annual incidence of UC per 100000 is up to 2.22 and has increased rapidly in recent years[1]. Traditional therapies include aminosalicylates, corticosteroids, and immunosuppressive medications such as azathioprine and 6-mercaptopurine. Approximately 9.8% of patients required colectomy under conventional medication[2]. Infliximab (IFX), a monoclonal antibody against tumor necrosis factor (TNF), is effective in UC patients[3-5]. Clinical remission of refractory UC patients under IFX treatment was 53% and 47% at 6 and 12 mo, respectively[6]. For long-term efficacy, 46% of patients achieved sustained clinical response at median follow-up of 41.5 mo[7]. The cumulative incidence of colectomy was 10% for IFX, while 17% of patients under placebo treatment needed colectomy after 54 wk[8]. Therefore, IFX is an effective medication to avoid colectomy and sustain long-term remission from refractory UC. However, significant, but rare, sometimes fatal drug-induced adverse effects have been reported in association with IFX, including infections, skin reactions, immunogenicity, malignancy, neurological complications, hepatotoxicity, congestive heart failure and hematological side effects[9].

Cutaneous complications can appear as infusion and injection site reactions, papulopustular eruptions, vasculitis, cutaneous infections and malignant neoplasms, and autoimmune skin disorders[10-12]. Henoch-Schönlein purpura (HSP), an acute vasculitic syndrome, is a rare side effect of anti-TNF medication and it is a complication associated with several anti-TNF agents, such as etanercept for psoriasis and rheumatoid arthritis[13,14], adalimumab for Crohn's disease[15,16], and IFX for herpes zoster infection in UC[17]. HSP is more prevalent in children, while no study has described the development of severe HSP in elderly patients, which is rare in clinical situations. Here, we present an unusual case of IFX-induced subcutaneous ecchymosis during intravenous treatment in a 69-year-old woman with severe UC, partial colectomy and cecostomy.

CASE REPORT

A 69-year-old woman, previously diagnosed with UC, with partial colectomy and cecostomy, was admitted to the Department of Gastroenterology of Shanghai 10th People's Hospital, China, complaining of lower abdominal pain and hematochezia for 8 mo.

In September 2008, she was admitted to a local hospital for the first time with frequent hematochezia and abdominal pain. Colonoscopy at that time showed multiple ulcers in the ascending colon. A specimen biopsy of inflammatory regions revealed mucosal congestion, infiltration of massive lymphocytes, plasmocytes and neutrophils in the hepatic and splenic flexure of the transverse colon. Polypoid hyperplasia was also seen in part of the colon. Thus, she was diagnosed with UC and received mesalamine (Salofalk; Dr. Falk Pharma GmbH, Germany) at a dose of 1.0 g three times daily for 4 years until now, but her symptoms of hematochezia and abdominal pain were not alleviated completely.

In August 2012, the patient did not respond well to previous medication and still had persistent abdominal pain and hematochezia. Therefore, she received partial colectomy at the local hospital, including surgical removal of the transverse colon, and partial removal of the ascending and descending colon. Cecostomy was also performed. Five months later, ileostomy was performed with surgical removal of the terminal ileum. Eight months later, she complained of abdominal pain and frequent hematochezia about twice daily. Colonoscopy revealed mucosal congestion and edema, as well as multiple ulcerations. Thus, she received enemas of mesalamine (4.0 g qd; Salofalk), diosmectite (6.0 g qd; Smecta; Beaufour-Ipsen Pharmaceutical Co. Ltd., Tianjin, China) and ethamsylate (0.5 g qd; Tianjin Jinyao Amino Acid Co. Ltd., Tianjin, China) but hematochezia persisted.

The patient was admitted to the Department of Gastroenterology in Shanghai 10th People's Hospital on July 30, 2013 for further treatment. She still had hematochezia, about twice daily. Her medical history included partial colectomy and cecostomy as described previously. She denied a history of bleeding or coagulation disorders, high blood pressure, or diabetes. Family history revealed nothing for both her parents and children. Allergic history was negative for any specific medication or food. She was married and gave birth to a daughter and a son at the age of 23 and 25 years, respectively. Routine blood tests showed a leukocyte count of 6.10 × 10^9/L (normal range: 3.5 × 10^9-9.5 × 10^9/L), with 72.5% neutrophils (normal range: 40%-75%), 18.5% lymphocytes (normal range: 20%-50%) and 7.5% monocytes (normal range: 3%-10%). Hemocytological analysis showed a hemoglobin level of 99 g/L (normal range: 130-175 g/L), erythrocyte count of 3.25 × 10^{12}/L (normal range: 4.3 × 10^{12}-5.8 × 10^{12}/L), platelet count of 291 × 10^9/L (normal range: 100 × 10^9-300 × 10^9/L), and erythrocyte sedimentation rate (ESR) of 45 mm/h (normal range: 0-15 mm/h). The level of C-reactive

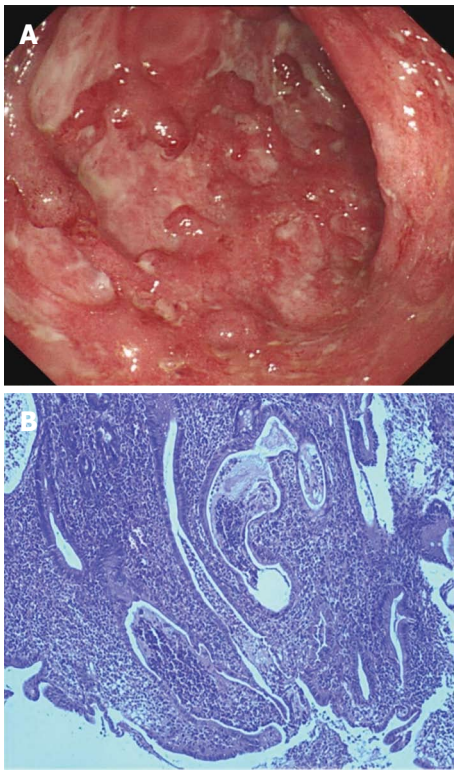

Figure 1 Colonoscopy and histological finding in a ulcerative colitis patient. A: Colonoscopy demonstrated severe mucosal congestion and edema, multiple hemorrhage and ulcerations in the descending colon; B: Histological section revealed extensive infiltration of immune cells, cryptitis and glandular distortion in the inflamed colon.

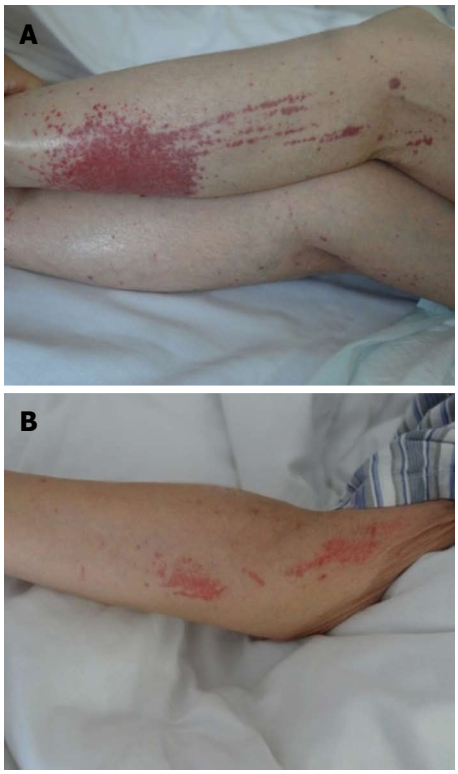

Figure 2 Subcutaneous ecchymosis on the left upper and lower extremities. A: Extensive bruising on the left lower extremity; B: Sporadic purpura on the left upper extremity.

protein (CRP) was 3.3 mg/L (normal range: 0-8.2 mg/L). Coagulation parameters were within normal ranges. All autoimmune antibodies, including antinuclear antibody, anti-smooth muscle antibody, anti-SSA antibody, anti-SSB antibody, perinuclear antineutrophil cytoplasmic antibody, anti-Scl-70 antibody, anti-Jo-1 antibody, anti-PM-Scl antibody, anti-double-stranded DNA antibody, anti-nucleosome antibody, anti-histone antibody, and anti-mitochondrial M2 antibody, were negative at the time of examination. Renal and liver function tests were unremarkable. Stool microbial cultures including bacteria [*Clostridium difficile* (*C. difficile*), *Salmonella*, *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*)] and viruses (cytomegalovirus and human immunodeficiency virus) were also negative. Bacterial toxins from *C. difficile*, *E. coli* O157, *Salmonella* and *S. aureus*, as well as lipopolysaccharide and peptidoglycan in serum were undetectable. T-SPOT.TB test for *Mycobacterium tuberculosis* infection was negative. Computed tomography enterography indicated inhomogeneous colonic mural thickening enhancement and stratification. Colonoscopy demonstrated severe inflammation in the intestinal lumen 50 cm from the anus, mucosal congestion and edema, and multiple hemorrhage and ulceration, with purulent adhesions (Figure 1A). Histological biopsy revealed extensive infiltration of immune cells, cryptitis, and glandular distortion in the intestine (Figure 1B). Thus, diagnosis of UC was confirmed.

She received mesalamine enema (4.0 g qd) and started intravenous IFX (200 mg; Cilag AG, Schaffhausen, Switzerland) for improvement of her symptoms. During IFX infusion, the patient had extensive subcutaneous ecchymosis on her left lower extremity, with diameters of 4 cm (Figure 2A). Sporadic bruising was also present on her upper extremities, with diameters of 1 cm (Figure 2B). All ecchymoses were painless and faded under pressure. Physical examination showed a small amount of purulent fluids around the fistula and drainage was unobstructed. There was slight tenderness in the abdomen, with no rebound pain. IFX was discontinued on the suspicion that it might have been responsible for the bruising. Emergency laboratory analyses revealed a leukocyte count of 4.06 × 10^9/L (normal range: 3.5 × 10^9-9.5 × 10^9/L), with 80.5% neutrophils (normal range: 40%-75%), 11.1% lymphocytes (normal range: 20%-50%) and 7.8% monocytes (normal range: 3%-10%). Hemocytological analysis demonstrated a platelet count of 249 × 10^9/L (normal range: 100 × 10^9-300 × 10^9/L), and hemoglobin of 99 g/L (normal range: 130-175 g/L). CRP was 20.3 mg/L (normal range: 0-8.2 mg/L), and D-dimer was 3.11 mg/L (normal range: 0-0.55 mg/L). Electrolytes results indicated 2.9 mmol/L potassium (normal range: 3.5-5.1 mmol/L), 135 mmol/L sodium (normal range: 136-145 mmol/L), 2.04 mmol/L sodium

(normal range: 2.10-2.55 mmol/L), and 0.66 mmol/L magnesium (normal range: 0.7-1.0 mmol/L). Urinalysis showed proteinuria and microscopic hematuria. Coagulation index demonstrated prothrombin time of 11 s (normal range: 9.5-14.5 s), activated partial thromboplastin time of 28.4 s (normal range: 23-36 s), international normalized ratio of 0.92 (normal range: 1.1-1.6), thrombin time of 20.6 s (normal range: 12-18 s), and fibrinogen of 3.10 g/L (normal range: 1.8-3.5 g/L). Liver function tests and serum amylase were unremarkable. She had not taken other drugs, over-the-counter medications, or herbal products. She was just undergoing UC diet therapy and denied a history of recent trauma. Upon a suggestion from the hematologist and dermatologist, she was prescribed with vitamin C tablets (0.1 g qd; Beijing Double-Crane Pharmaceutical Co. Ltd.), carbazochrome tablets (2.5 mg tid; Yabang Pharmaceutical Co. Ltd., Jiangsu, China), compound glycyrrhizin tablets (0.5 g tid; Minophagen Pharmaceutical Co. Ltd., Japan) and calamine lotion (twice daily; Shanghai Winguide Huangpu Pharmaceutical Co. Ltd. China) for treatment of the bruising. Such bruising did not vanish until 7 d later after discontinuation of IFX.

On August 23, 2013, the patient was admitted again for hematochezia and planned to continue a second round of IFX administration. About 40 min after infusion of 200 mg IFX, the patient complained of pruritus all over her body and sporadic erythema could be seen, especially distributed on her extremities. Dyspnea, abdominal pain, nausea or other remarkable symptoms were not observed. Physical examination showed blood pressure (106/70 mmHg), heart rates (70 beats/min), respiratory rate (14/min) were within the normal ranges. Emergency laboratory results, including liver and renal function tests, bleeding time and blood-clotting tests, and electrolytes were all normal. ESR was 37 mm/h (normal range: 0-15 mm/h). Urinalysis showed proteinuria and microscopic hematuria. IFX infusion was immediately discontinued temporarily. She was prescribed with promethazine (25 mg; Phenergan; Shanghai Harvest Pharmaceutical Co. Ltd., China) intramuscularly and 10% calcium gluconate (10 mL; Tianjin Jinyao Amino Acid Co. Ltd.) intravenously for further treatment of her skin reactions. Her symptoms alleviated partially and spontaneously 7 d later.

DISCUSSION

Anti-TNF agents have revolutionized the therapy of several immune-mediated diseases, including corticosteroid-dependent fistulizing Crohn's disease and refractory UC. However, adverse effects may occur during IFX management. They include infections, dermatological reactions, immunogenicity, neurological complications, malignancy, hepatotoxicity, congestive heart failure and hematological side effects. In some cases, these reactions may cause drug discontinuation, thus making it harder to treat UC[9,15]. Thus, adverse effects of anti-TNF agents can't be neglected in clinical situations.

One of the dermatological manifestations is HSP, which is an acute leukocytoclastic vasculitis characterized by IgA immune complexes in small vessels, with extensive or sporadic purpura especially on the lower extremities and buttocks as a characteristic clinical manifestation. The incidence of vasculitis during administration of TNF antagonists ranges from 0.02% to 3.9%[18,19]. It is reported more frequently in children than adults. HSP has been described as a significant adverse effect with commonly used anti-TNF medications, including etanercept[13,14], adalimumab[15,16], and IFX[17]. Aside from skin features, renal dysfunction such as crescentic glomerulonephritis can be involved[20]. In some serious cases, gastrointestinal impairment, such as intestinal bleeding and perforation, as well as peripheral nervous system injury has been reported[21,22]. Usually, serum anti-neutrophil cytoplasmic antibodies are undetectable before the initiation of anti-TNF medication, but are positive in some patients during vasculitis. The prognosis is more severe in adults with more complications than children.

Michel *et al*[23] identified six diagnostic criteria for HSP, including palpable purpura not related to thrombocytopenia, bowel angina, gastrointestinal bleeding, hematuria, age < 20 years, and no history of medication at the onset of disease. A patient is diagnosed with HSP if ⩾ 3 criteria are met. The possible explanation for our case is the development of HSP. In our study, the patient was an old woman with a medical history of UC for 5 years. She was diagnosed with UC in 2008 and underwent partial colectomy and cecostomy in 2012, but her symptoms recurred 8 mo later. In July 2013, the patient was admitted to our department and started IFX administration, during which she developed extensive and sporadic ecchymosis. Urinalysis revealed hematochezia and microscopic hematuria. Although hematochezia could be a clinical characterization of UC, and it is doubtful whether hematochezia is caused by UC or IFX-induced HSP, mesalamine had been used before IFX to control her symptoms of UC. Besides, hematochezia deteriorated after IFX administration, further indicating that IFX might have contributed to the exacerbation. She complained of slight abdominal tenderness. No abnormalities were found in her platelet counts, bleeding time or coagulation function tests. All clinical hallmarks coincided with HSP. A specimen biopsy of bruising skin lesions was warranted for confirmation of the diagnosis. HSP is more prevalent in pediatric patients, and is rare in elderly patients, with complex medical histories and weak general condition, such as the present case. In previous studies, bruising was sporadically distributed on the extremities. In our case, ecchymosis was extensive which was rare in clinical situations. Such extensive ecchymosis indicated

vasculitis could be more severe.

Although the precise mechanisms of purpural vasculitis are still controversial, current hypotheses focus on a type Ⅲ hypersensitivity reaction triggered by release of anti-IFX autoimmune antibodies from capillaries, possible cytokine imbalance due to blockage of TNF and its regulation of CD23 on T-cell-activated B cells, and a shift from T helper (Th)1 to Th2 response[24-26].

In conclusion, in agreement with previous studies[15,27,28], our study highlights the importance of subcutaneous adverse manifestations during IFX management, especially those whose clinical symptoms are not typical enough to make an accurate diagnosis. Thus, prior to biological therapies with anti-TNF agents, certain risk analyses should be assessed and patients need to be carefully selected for optimal anti-TNF agents.

COMMENTS

Case characteristics

A 69-year-old female patient with ulcerative colitis (UC) developed severe Henoch-Schönlein purpura (HSP) during infliximab (IFX) administration.

Clinical diagnosis

Subcutaneous purpura on her extremities, severe hematochezia and slight tenderness in the abdomen were observed.

Differential diagnosis

Cutaneous infections, psoriasis, eczema, and other dermatological disorders.

Laboratory diagnosis

All laboratory results coincided with the diagnosis of UC and no abnormalities were found in her platelet counts, bleeding-time and coagulation function tests.

Imaging diagnosis

Computed tomography enterography indicated inhomogeneous colonic mural thickening enhancement and stratification.

Pathological diagnosis

Histological biopsy revealed extensive infiltration of immune cells, cryptitis, and glandular distortion in the intestine, while pathological examination of the ecchymosis was not performed.

Treatment

She was prescribed vitamin C tablets, carbazochrome tablets, compound glycyrrhizin tablets, and calamine lotion for treatment of the bruising.

Related reports

HSP has been described as a significant adverse effect with commonly used anti-tumor necrosis factor (TNF) agents, including etanercept, adalimumab, and IFX in young patients.

Term explanation

HSP is a type of vasculitis characterized by IgA immune complexes in the blood vessels and is regarded as a cutaneous side effect of anti-TNF agents such as IFX.

Experiences and lessons

The authors should take this adverse effect HSP into consideration during IFX administration for refractory UC.

Peer-review

This case report focused on HSP, which is a cutaneous complication during IFX treatment. In our case, a 69-years-old patient developed severe HSP, which is rare in clinical situations. Clinical points of diagnosis and treatment are addressed. A specimen biopsy of bruising skin lesions was warranted.

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CASE REPORT

Primary hepatic angiosarcoma: A report of two cases and literature review

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Abstract

Primary hepatic angiosarcoma (PHA) is a rare malignancy that carries a poor prognosis. Of 1500 patients who underwent hepatectomy for primary hepatic tumors between 1994 and 2013 at our center, two patients were pathologically diagnosed with PHA. Clinical characteristics, treatment modalities, and outcomes of the two patients were collected and analyzed. Both patients underwent hepatectomy and had a postoperative survival time of 8 and 16 mo, respectively. A search of PubMed yielded eight references reporting 35 cases of PHA published between 2004 and 2013. On the basis of the presented cases and review of the literature, we endorse complete surgical resection as the mainstay definitive treatment of PHA, with adjuvant postoperative chemotherapy potentially improving survival. Palliative chemotherapy is an option in advanced hepatic angiosarcoma.

Key words: Diagnosis; Hemangiosarcoma; Therapy; Surgery; Liver

Core tip: Primary hepatic angiosarcoma (PHA) is a rare malignancy with poor prognosis. Two cases of PHA undergoing surgical resection at our center are presented in this paper. A literature review including 35 cases of PHA is discussed. Preliminary experience suggests that complete surgical resection is the definitive treatment for PHA and adjuvant chemotherapy after surgery might improve survival.

Zhu YP, Chen YM, Matro E, Chen RB, Jiang ZN, Mou YP, Hu HJ, Huang CJ, Wang GY. Primary hepatic angiosarcoma: A report of two cases and literature review. *World J Gastroenterol* 2015; 21(19): 6088-6096 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i19/6088.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i19.6088

INTRODUCTION

Primary hepatic angiosarcoma (PHA) is a rare liver tumor, comprising only 0.1%-2% of all primary liver malignancies[1,2]. PHA was first reported by Block[3] in 1974. Since then, there has been a relative paucity of data published regarding the diagnosis, management, and prognosis of this tumor. Although it is known that PHA is a highly malignant and rapidly progressing tumor of endothelial origin, many physicians remain unfamiliar with the clinical features of this disease. Of 1500 patients who underwent hepatectomy for primary hepatic tumors from 1994 to 2013 at our center, only two cases were pathologically diagnosed as PHA. This paper reports the characteristics, treatment modalities, and outcomes of these two patients with PHA. We also conducted a literature review of PHA using the PubMed database, with hepatic angiosarcoma, hepatic hemangiosarcoma, liver angiosarcoma, and liver hemangiosarcoma as search terms. We evaluated 35 cases of PHA from eight studies published between 2004 and 2013 (Table 1).

CASE REPORT

Case 1

A 58-year-old man was referred to our hospital from a local clinic on December 25, 2013, complaining of abdominal pain with radiation to the right shoulder and back for 2 wk. During that period, he also had intermittent fever with chills. His past medical history included cholangiolithiasis and cholecystolithiasis over 30 years ago, and his surgical history included cholecystectomy and hepatectomy of the left lateral lobe 30 years ago. He reported possible occupational exposure to vinyl chloride because he worked with paint-related chemicals for 10 years. The patient denied any history of hepatitis, diabetes mellitus, or cancer. Physical examination revealed slight tenderness of the right upper abdominal quadrant. Percussion elicited pain over the liver area. Laboratory examinations on admission revealed: white blood cell (WBC) count 9.6 × 10^9/L, hemoglobin 12.9 g/dL, platelet count 248 × 10^9/L, alanine aminotransferase (ALT) 64 U/L, aspartate transaminase (AST) 59 U/L, alkaline phosphatase (ALP) 460 U/L, γ-glutamyl transpeptidase (GGT) 329 U/L, albumin 30.5 g/L, total bilirubin (TBIL) 14.6 μmol/L, direct bilirubin (DBIL) 7.1 μmol/L, prothrombin time (PT) 15.4 s, and international normalized ratio (INR) 1.23. Serum tumor markers, including α-fetoprotein (AFP), carcinoembryonic antigen (CEA), and carbohydrate 125 (CA125), were within normal ranges, apart from carbohydrate antigen 19-9 (CA19-9), which was slightly elevated at 58.74 IU/mL. Serology for hepatitis A, B and C tested negative. Abdominal T1-weighted magnetic resonance imaging (MRI) revealed a heterogeneous low-intensity mass about 4.3 cm × 6.2 cm, and high signal intensity was evident on T2-weighted imaging. The focal areas of higher signal intensity on both images indicated hemorrhage (Figure 1). To obtain a definitive diagnosis, computed tomography (CT)-guided fine-needle aspiration (FNA) biopsy was performed, and suggested a diagnosis of spindle cell tumor. The patient underwent hepatectomy of the right posterior lobe (segments Ⅵ and Ⅶ), had an uneventful postoperative course, and was discharged 16 d after surgery. Specimen cross-sectioning revealed a clearly demarcated yellowish-white lesion measuring 6.3 cm × 4.5 cm (Figure 2). Histology revealed spindle-shaped neoplastic cells exhibiting marked nuclear pleomorphism arranged in a whorled pattern, and present in fascicular clusters (Figure 3A). In addition to these solid areas, necrotic and hemorrhagic regions were also noted (Figure 3B). Immunohistochemical examination revealed staining positive for CD31 (Figure 3C) and Ki-67 (positive rate of 40%-50%), characteristic of tumor cells. Staining for CD34 and S-100 were negative. The histopathological diagnosis was consistent with PHA.

At follow-up 1 mo after surgery, the patient was in good condition. To suppress possible local recurrence or tumor metastasis, transcatheter arterial chemoembolization (TACE) was performed 1 mo after hepatectomy and the patient was initiated on a regimen of cisplatin (50 mg), mitomycin (10 mg), and pirarubicin (50 mg). No significant tumor staining foci were seen at that time. However, the patient was readmitted to our hospital due to fever and right upper abdominal pain 3 mo after surgery. Contrast-enhanced CT revealed emerging lesions in the right liver close to the incisal margin and enlarged retroperitoneal lymph nodes; both of which suggested tumor recurrence (Figure 4). The patient refused further treatment, and was reported to survive for 8 mo after hepatectomy by telephone follow-up.

Case 2

A 58-year-old woman was admitted to our hospital on November 5, 2007 due to recurrent upper abdominal pain for the past 4 years, along with fatigue and weight loss. Her past history was unremarkable except for hypertension. She denied any carcinogen exposure. Physical examination revealed a tender abdominal mass in the right upper abdomen. Complete blood count revealed: WBC count 4.8 × 10^9/L, hemoglobin 8.7 g/dL, and platelet count 237 × 10^9/L. Liver function test showed moderate elevation of ALP (129

Table 1 Summary of patients with primary hepatic angiosarcoma

Ref.	*n*	Age (yr) (median)	Sex (M/F)	Clinical manifestations	Tumor characteristics (*n*)	Extrahepatic metastasis	Tumor rupture	Treatment	Surgical margin	Survival time
Kim *et al*[10]	5	41-69 (45)	5/0	Right upper abdominal pain, leg edema, hemoptysis, ascites, chest discomfort, DOE	Multiple nodules (5)	Spleen (2), lung (1), pericardium (1), bone (1)	1	Palliative chemotherapy (4)	/	86 d (m)[1]
Yin *et al*[11]	7	34-71 (52)	6/1	Abdominal pain, jaundice, ascites, anemia, chest pain, edema, weight loss	NS	Spleen (1), lung (2), bone (1)	None	Hepatectomy + chemotherapy (3), chemotherapy (2)	NS	89.9 d (m)[1] (13-238 d), of hepatectomy + chemotherapy: 94 d (m)[1]
López *et al*[36]	2	20, 31	2/0	Acute liver failure	Diffuse micronodules (1), multiple nodules (1)	NS	None	Transplantation + chemotherapy (1), Conservative therapy (1)	/	2 mo, 17 d
Egea Valenzuela *et al*[15]	2	65, 73	2/0	Jaundice, abdominal distension, bleeding tendency	Multiple nodules (2)	None	None	Conservative therapy	/	36 h, several days
Matthaei *et al*[13]	5	20-80 (56)	3/2	NS	Dominant mass (5)	Lung (3), peritoneum (1)	None	Hepatectomy	R0 (4), R (1)	30 mo (m)[1], 1 alive with recurrence (R0), 1 no evidence of recurrence (R0)
Yang *et al*[37]	1	70	1/0	Asymptomatic	Multiple nodules	None	None	Conservative therapy	/	6 mo
Park *et al*[12]	6	29-80 (59.5)	5/1	Abdominal pain, fever, abdominal distension, hematuria, anemia	Dominant mass and/or intrahepatic nodules(6)	Spleen (1)	3	TACE (4), TAE (2)	/	3.5 mo (m)[1]
Bruegel *et al*[14]	7	51-78 (65)	4/3	Abdominal pain, weight loss, fatigue, jaundice	Dominant mass (1), multiple nodules (6)	Spleen (1), bone (3), lung (2), cerebellum (1) retroperitoneum (2)	1	Hepatectomy + chemotherapy(1), hepatectomy (1), chemotherapy(4)	NS	16 mo (m)[1]

[1]Represents median survival time. DOE: Dyspnea on exertion; NS: Not specified; R0: No residual tumor; R1: Microscopic residual tumor; M: Male; F: Female.

U/L) and GGT (67 U/L), while ALT, AST, ALB, TBIL and DBIL were within normal ranges. AFP, CEA, CA125 and CA19-9 were also within normal ranges. Serology for hepatitis A, B and C was negative. Contrast-enhanced CT revealed a mass in the right hepatic lobe measuring about 13 cm × 9 cm. The mass was heterogeneously hypoattenuated with non-homogeneous enhancement in both arterial and venous phases. Vascular imaging with contrast suggested a possible diagnosis of PHA (Figure 5). Right hemihepatectomy was performed and the surgical procedure was unremarkable. The patient recovered uneventfully and was discharged 11 d after surgery. Although the margins of the specimen were clear-cut, cross-sectional histology demonstrated a red-gray lesion measuring 13 cm × 9.5 cm, with large necrotic areas. Vascular channels were formed by atypical cells, which exhibited marked nuclear pleomorphism, and were filled with erythrocytes (Figure 6A). Neoplastic cells infiltrated the adjacent liver parenchyma (Figure 6B). Immunohistochemically, the specimen was positive for vascular endothelial markers (CD31 and CD34) (Figure 6C), and negative for cytokeratin, CD68 and S100. A diagnosis of PHA was established based upon the aforementioned pathological findings. The patient was available for follow-up and she survived for 16 mo after the operation.

DISCUSSION

PHA, arising from endothelial and fibroblastic tissues, accounts for only 0.1%-2% of primary hepatic tumors[1,2], and is rarely seen in practice. It usually develops during the sixth and seventh decades of

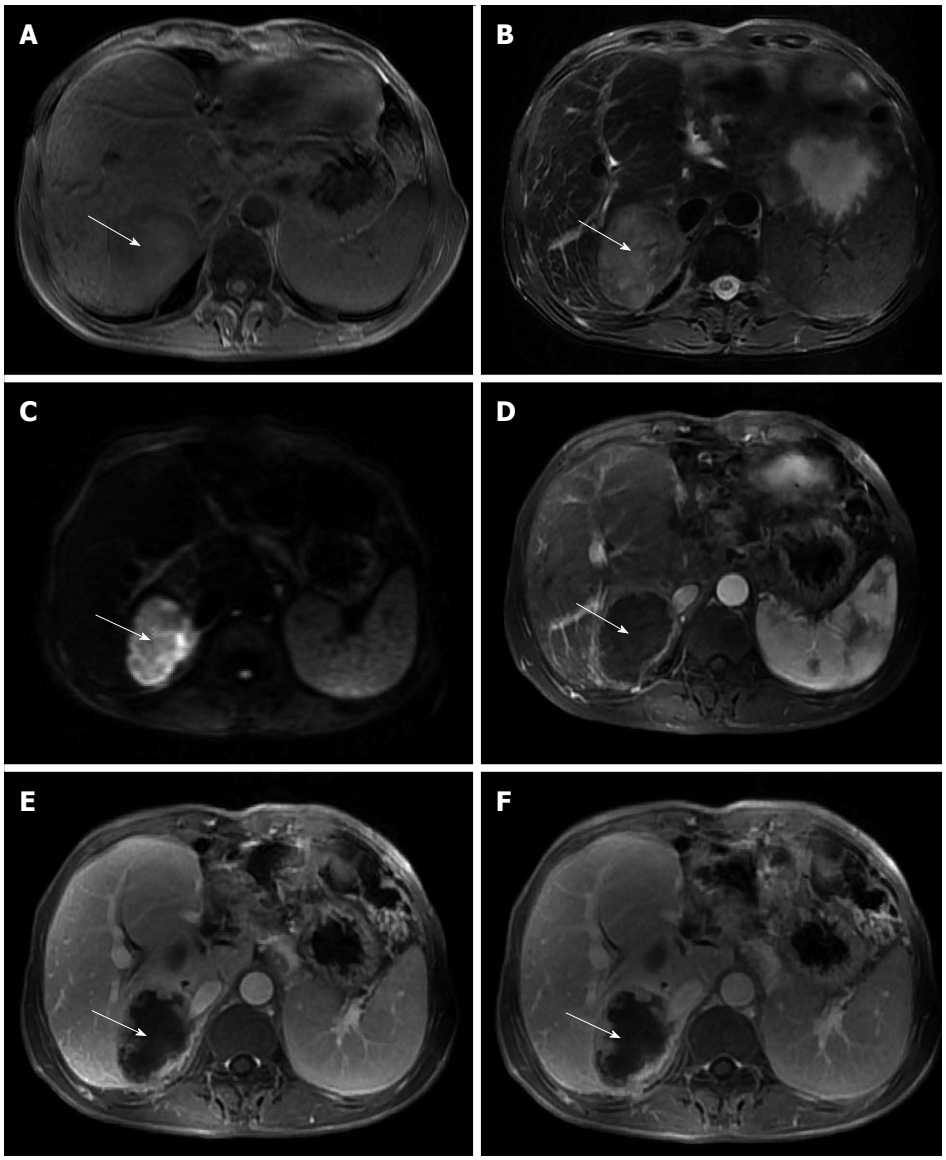

Figure 1 Magnetic resonance imaging. A: Markedly heterogeneous low signal intensity mass (white arrow) in the right lobe of the liver on T1-weighted imaging, with focal areas of high signal intensity that indicate fresh hemorrhage; B: Heterogeneous high signal intensity mass (white arrow) in the right lobe of the liver on T2-weighted imaging, with focal areas of higher signal intensity that indicate necrosis or hemorrhage; C: Mass (white arrow) shows high signal intensity on diffusion-weighted imaging that reveals restricted diffusion; D: Mass (white arrow) has a ring-like enhancement in the arterial phase; E: Progressive enhancement of the ring (white arrow) in the portal phase, revealing nodular enhanced foci within the ring; F: Progressive enhancement of the ring (white arrow) and nodular enhanced foci within the ring in the delayed phase.

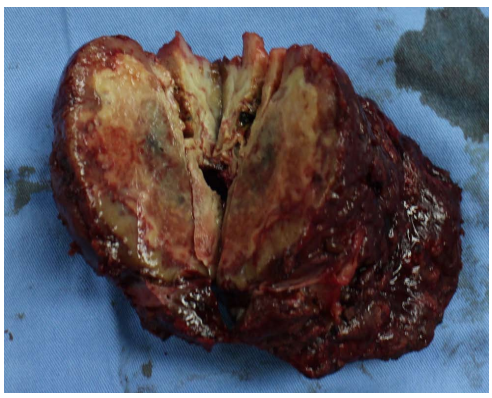

Figure 2 Cross-section of the specimen showing a yellow-white lesion 6.3 cm × 4.5 cm.

life and shows an obvious male predominance with a male to female ratio of 3:1[4]. According to an epidemiological study in the United States, vinyl chloride monomer exposure, use of thorotrast in angiography, exposure to inorganic arsenic, and treatment with androgenic anabolic steroids are associated with development of PHA in 25% of all cases. The remaining studied cases were of uncertain etiology[5].

Symptoms of PHA are variable and mimic chronic liver disease. They include abdominal pain, anorexia, fatigue, weight loss, fever, and low back pain, and may even be asymptomatic[2,6,7]. As symptoms tend to be nonspecific, most patients are diagnosed at an advanced stage. Reported physical findings of

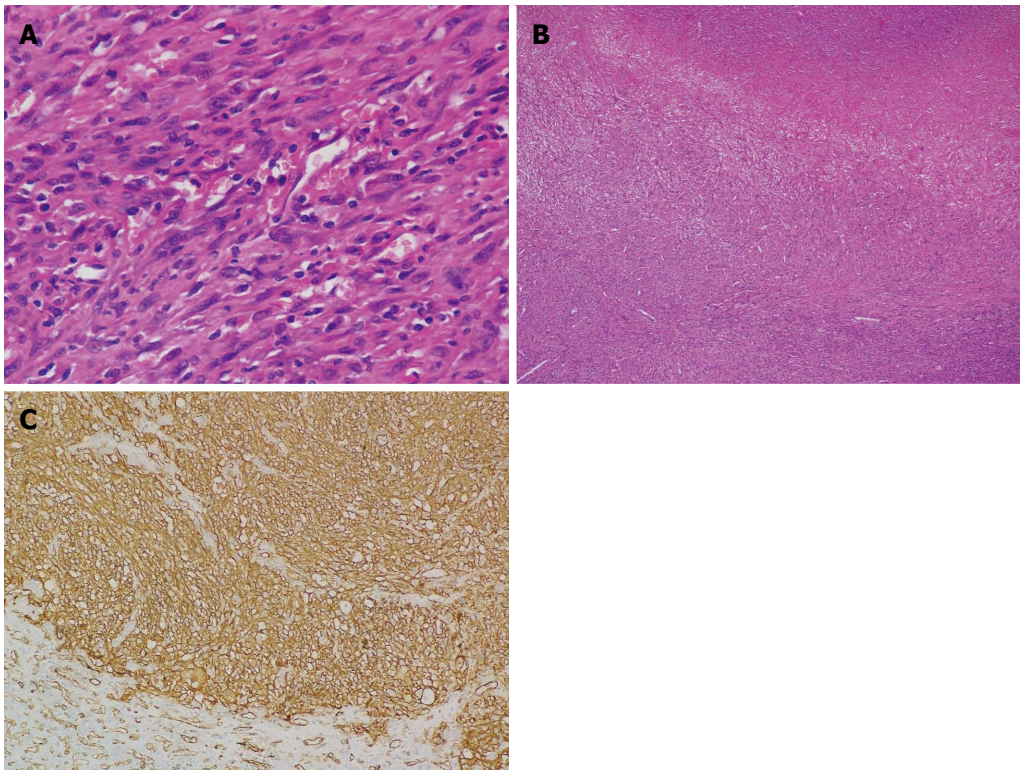

Figure 3 Histological examination. A: Spindle-shaped neoplastic cells exhibiting marked nuclear pleomorphism with whorled shape and fascicular clusters (HE stain; magnification × 400); B: Necrotic areas seen between the clusters of neoplastic cells (HE stain; magnification × 40); C: Immunohistochemical examination reveals positive staining of CD31 (magnification × 200). HE: Hematoxylin and eosin.

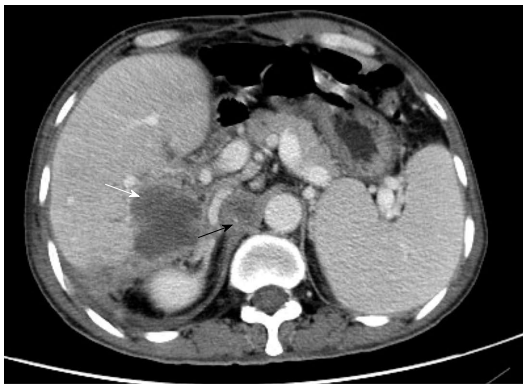

Figure 4 Computed tomography imaging. Emerging lesions in the right liver (white arrow) and enlarged retroperitoneal lymph nodes (black arrow) suggested postoperative tumor recurrence.

PHA include jaundice, ascites, hepatomegaly, and splenomegaly[2,4,8]. About 15%-27% of PHA patients present with hemoperitoneum, due to spontaneous tumor rupture; a potentially fatal complication[2,9]. Acute hepatic failure, as a primary symptom in PHA patients, has also been reported[7,8]. In our literature review, PHA was documented to metastasize to the lungs (8 cases), spleen (5 cases), bone (5 cases), peritoneum (3 case) and cerebellum (1 case)[10-14]. Sometimes, patients presented with symptoms due to a metastatic lesion, such as chest pain in a case with lung metastasis.

Laboratory tests of PHA patients often show moderate elevation of liver enzymes, such as ALT, AST, and most commonly, ALP, which suggests a cholestatic manifestation[2,15]. Thrombocytopenia and anemia are relatively common hematological findings. The former, along with the vascular nature of tumor, may contribute to hepatic rupture. Tumor markers such as AFP, CEA, CA19-9, and CA125 are always within normal ranges or only mildly elevated[4,16].

The absence of specific clinical manifestations and laboratory findings in PHA reasonably emphasizes the diagnostic value of imaging studies. From a morphological perspective, PHA appears as multiple nodules, a dominant mass, or a mixed pattern of a dominant mass and multiple nodules, and rarely, manifests as an infiltrative, micronodular subtype[17,18]. Conventional ultrasound examination has been reported as a nonspecific imaging modality and small lesions often appear isoechoic and clearly demarcated, while large lesions appear hypoechoic and poorly demarcated. However, specific characteristics are revealed under contrast-enhanced ultrasound (CEUS), with a central unenhanced area and peripheral irregular enhancement in the arterial and portal phases, and complete late-phase wash-out[19]. PHA presents differently on CT imaging according to its various morphological patterns. Necrosis and hemorrhage, which occur frequently in the solid portion of PHA, both contribute to the complexity of imaging. PHA lesions are generally hypoattenuating, sometimes with hyperattenuating foci on unenhanced CT. On

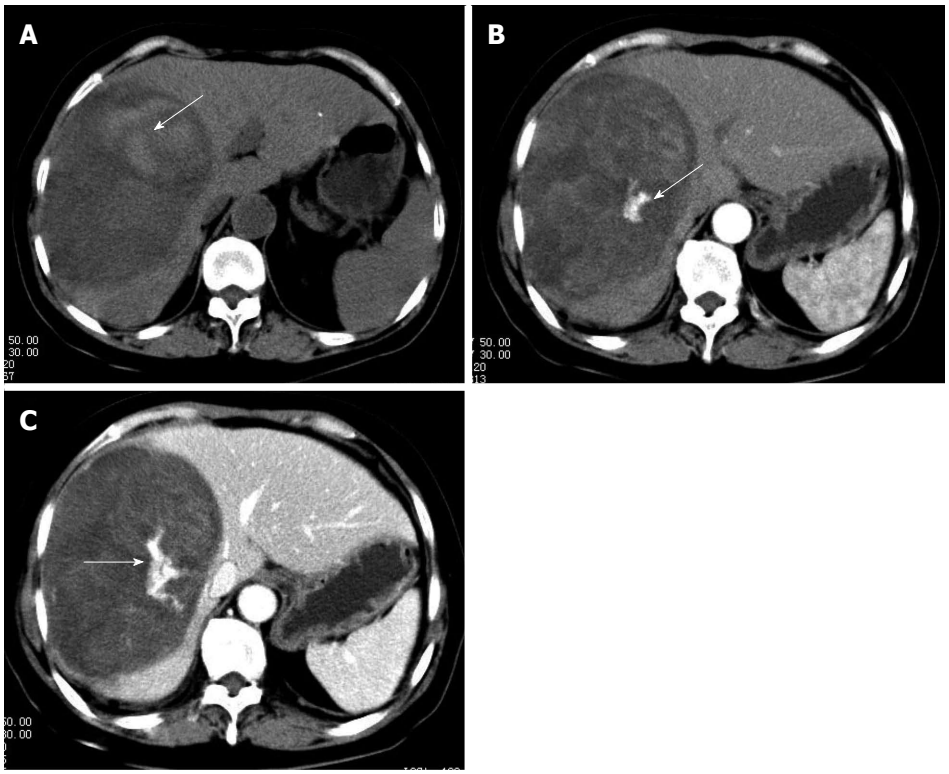

Figure 5 Computed tomography scan. A: Plain computed tomography scan reveals an oval shaped heterogeneous hypoattenuating mass with hyperattenuating foci (arrow) which suggests hemorrhage; B: Dominant mass lesion shows heterogenous enhancement with hyperattenuating enhanced foci (arrow), which indicate vasculature, in arterial phase; C: Dominant mass shows progressive enhancement and hyperattenuating enhanced foci enlarged in portal phase (arrows).

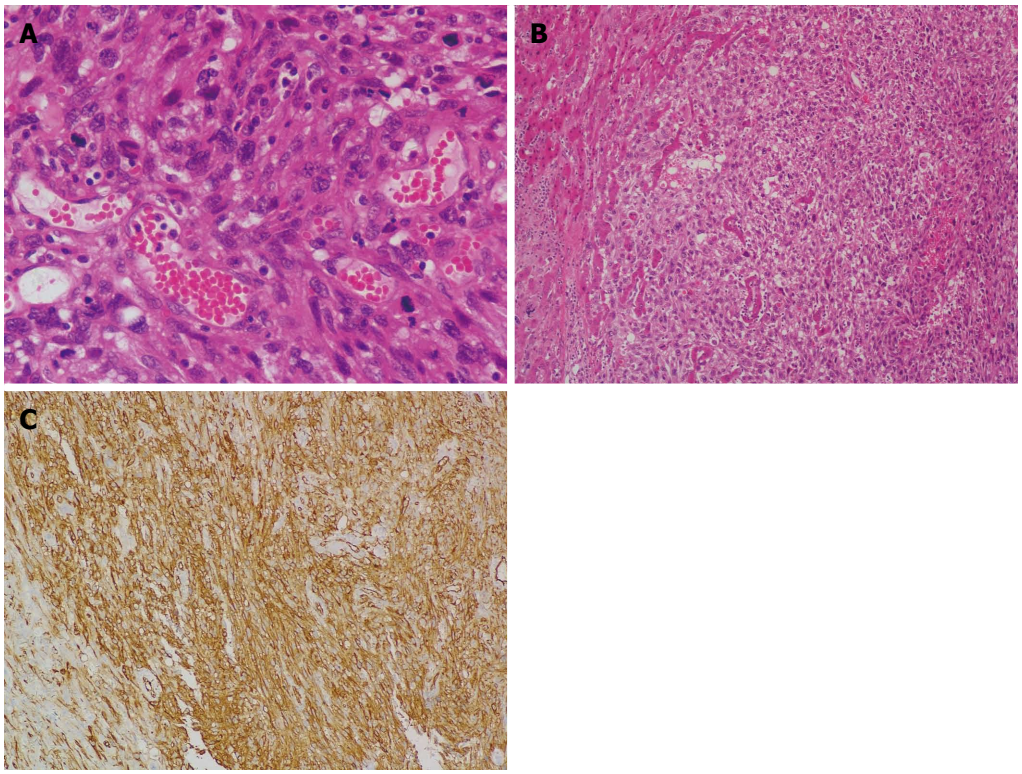

Figure 6 Histological examination. A: Neoplastic cells exhibiting marked nuclear pleomorphism, and vascular channels filled with erythrocytes (HE stain; magnification × 400); B: Clusters of neoplastic cells infiltrating liver parenchyma (HE stain; magnification × 100); C: Immunohistochemical examination reveals positive staining of CD34 (magnification × 200). HE: Hematoxylin and eosin.

contrast-enhanced CT, most nodular lesions appear hypoattenuating and with enhanced foci, and some nodular lesions show irregularity or ring enhancement. Dominant mass lesions show heterogeneous enhancement, suggesting central necrosis and fibrotic changes. A delayed progressive enhancement pattern in all enhanced lesions has been observed[20,21]. MRI reflects the hemorrhagic, heterogeneous, and hypervascular nature of PHA lesions. On T1-weighted MR images, dominant mass lesions often contain irregular areas of high signal intensity, suggesting hemorrhage. On T2-weighted MR images, mass lesions of PHA reveal a markedly heterogeneous architecture, with focal areas of high intensity along with septum-like or rounded areas of low intensity. Areas of high intensity on T2-weighted images suggest hemorrhage or necrosis, while areas of low signal intensity indicate hemosiderin deposition, fibrous solid portions, or occasionally fresh hemorrhage. On dynamic contrast-enhanced MRI, each lesion reveals heterogeneous enhancement on arterial- and portal-phase images. On delayed imaging, however, there is progressive enhancement of the lesion compared with that of early-phase images[14,20]. Pertinent angiographic findings include multiple nodular or mass lesions present with areas of fluffy staining and early pooling of contrast medium that persists and increases over time. Dominant mass lesions reveal poorly defined, irregular tumor with a nonstaining area. The large dominant masses also show early contrast medium pooling and continuously increasing enhancement[12]. Rademaker *et al*[22] have suggested that combined use of common hepatic angiography and dual-phase helical CT provides better identification of PHA.

Differentiating PHA from hepatic hemangioma, hepatocellular carcinoma (HCC), cholangiocarcinoma, and hepatic abscess is difficult. Elevation of AFP, past history of hepatitis B virus infection, and liver cirrhosis favor a diagnosis of HCC. Continuing enhancement on the late-phase of MRI may be helpful in differentiating PHA from HCC[14]. Imaging findings of PHA and hepatic hemangioma are sometimes similar. Rapid growth, tumor rupture or increasing pain may, however, suggest PHA rather than hemangioma[21]. Arterioportal shunting, which is often absent in hemangioma, also favors a diagnosis of PHA[23]. According to experienced radiologists, PHA might be distinguished from hepatic hemangioma *via* biphasic MRI[21,22,24]. It has also been reported that F-18 fluorodeoxyglucose positron emission tomography is helpful in differentiating PHA from giant cavernous hepatic hemangioma[25].

A definitive diagnosis of PHA is always established *via* pathological analysis. PHA has a vascular nature, therefore, liver biopsy is liable to cause bleeding[15,26]. Additionally, due to the high frequency of necrosis and hemorrhage inside the tumor, samples taken from these areas may produce false-negative results[27]. Therefore, nonsurgical liver biopsy is considered to be a dangerous and unreliable method for PHA diagnosis[26]. Nevertheless, Koyama *et al*[20] reported promising results for percutaneous biopsies in their series of PHA patients, with a success rate of 78% and no substantial complications. In our first case, preoperative diagnosis was also determined by CT-guided liver biopsy, implying that while PHA is not an absolute contraindication for liver biopsy, scrutinous observation and surgical evaluation are important prior to the procedure.

Histologically, PHA is composed of spindle-shaped and polyhedral cells. This tumor is characterized by a variety of patterns of vascular channels, ranging from dilated sinusoidal or cavernous spaces to slit-like freely anastomosing vessels, formed by spindle-shaped cells[17,18]. Wang *et al*[28] analyzed 20 cases of PHA immunohistochemically and detected the expression of specific immunohistochemical markers. All cases expressed at least one of the following three markers: CD31, CD34 and FVIIIRag. Ten cases had low expression of PTEN. The Ki-67 proliferative index was > 10% in all cases. Recently, ERG, a transcription factor, was indicated to be a specific and more sensitive diagnostic marker for PHA in comparison to CD31, CD34 and FVIIIRag[29]. Nevertheless, no immunohistochemical marker has been found to be associated with prolonged survival[30].

PHA is an aggressive malignancy that generally carries a poor prognosis. Complete surgical resection is the only definitive treatment that can improve survival. Groeschl *et al*[31] reported 207 PHA patients with a median overall survival time of only 1 mo. Comparatively, those who had the tumor resected had their survival time prolonged to 6 mo. In our literature review, a total of 32 patients with survival data were analyzed, with a median survival time of 4 mo after initial diagnosis. Nine patients receiving surgical resection with or without adjuvant therapy had longer survival time (median: 10 mo) compared to those who did not have their tumor resected (median: 2 mo). Among them, five patients who underwent tumor excision with negative margins had the best prognoses with a median survival time of 10 mo. Two patients receiving liver transplantation showed rapid recurrence and a subsequent low survival time. Prognosis for four patients presenting with hemoperitoneum due to tumor rupture was dismal (median: 23 d). Curative surgery was difficult to perform in > 80% of PHA patients who were diagnosed at an advanced stage, and presented with a dominant intrahepatic mass and/or extrahepatic metastases[4]. A definite diagnosis of PHA was established only in some cases after surgery, which regretfully did not meet oncological treatment criteria and led to a high risk of tumor recurrence[9]. Spontaneous PHA rupture usually carries a dismal prognosis, even if bleeding is stopped by emergency transcatheter arterial embolization (TAE) or surgery[12]. Due to the high frequency of tumor recurrence (77%) and dismal postoperative survival, PHA is considered to be a contraindication to liver transplantation[32].

PHA is reported to be radioresistant, and radiotherapy is generally not initiated in PHA patients[2,33]. Chemotherapy, including TACE, is considered to be an effective palliative therapy for unresectable PHA[10,15]. However, due to the rarity of PHA and paucity of medical records, articles concerning chemotherapy are few in number. To date, there is no established chemotherapy regimen for advanced PHA. Chemotherapeutic agents such as 5-fluorouracil, carboplatin, doxorubicin and adriamycin have been used in PHA[10,34]. The limited number of studies on this subject have shown that addition of adjuvant chemotherapy may improve survival after surgical resection[35]. Palliative chemotherapy, which is potentially useful, can be an option in advanced hepatic angiosarcoma[10,34]. TACE has shown effectiveness in the treatment of PHA, particularly in patients with dominant masses rather than those with multiple nodules[12]. Although our study reviews the present state of PHA treatment modalities and covers cases viewed in our hospital, a study with a larger sample is required to draw definite conclusions regarding the efficacy of TACE in this setting.

Literature regarding diagnostic methods and treatment regimens for PHA is limited and we hope that our report reminds clinicians of the difficulty in accurately diagnosing this disease, and inspires medical practitioners to study this rare malignancy.

COMMENTS

Case characteristics

Two patients presented with common clinical symptoms including fatigue, weight loss, abdominal pain, chills and fevers.

Clinical diagnosis

Abdominal pain (with or without radiation to the upper extremities), anorexia, fatigue, weight loss, and fever may all be nonspecific symptoms.

Differential diagnosis

Differential diagnoses include hepatic hemangioma, hepatocellular carcinoma (HCC), cholangiocarcinoma and hepatic abscess.

Laboratory diagnosis

α-fetoprotein elevation, along with a past history of hepatitis B virus infection and hepatic cirrhosis favor a diagnosis of HCC over primary hepatic angiosarcoma (PHA). One patient had slightly elevated carbohydrate antigen 19-9, while the other had no suggestive laboratory anomalies apart from elevated alkaline phosphatase.

Imaging diagnosis

Persistent enhancement on late-phase abdominal magnetic resonance imaging seems helpful in differentiating PHA from HCC, while F^{18} fluorodeoxyglucose positron emission tomography has been helpful in differentiating PHA from giant cavernous hepatic hemangioma.

Pathological diagnosis

Pathology reveals spindle-shaped and polyhedral cells, with the specimen possessing a variety of vascular channel patterns, ranging from sinusoidal or cavernous spaces to slit-like, freely anastamosing vessels formed by spindle-shaped cells. Specimens from both patients were stained positive for CD31, a vascular endothelial marker. Both patients were diagnosed with PHA.

Treatment

One patient was initiated on transcatheter arterial chemoembolization postoperatively and survived for 8 mo; the other patient survived for 16 mo after hepatectomy.

Related reports

Most available epidemiological studies link vinyl chloride monomer, angiographic thorotrast, inorganic arsenic and androgenic-anabolic steroid exposure with PHA development in 25% of patients. There are limited studies available and no clearly known etiology has been elucidated. Median survival has been reported to be several months.

Term explanation

PHA is a rare liver tumor, comprising only 0.1%-2% of all primary liver malignancies, with a poor prognosis.

Experiences and lessons

Complete surgical resection remains the definitive treatment and prolongs survival. Curative surgeries are exceedingly difficult to perform and recurrence rates are very high.

Peer-review

Limited studies on PHA have been reported to date. Larger-sized studies are required to draw definite conclusions regarding palliative or adjunctive TACE regimens for PHA treatment. This study underscores the importance of careful imaging study review for timely diagnosis of this aggressive disease.

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