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EDITORIAL

Acoustic radiation force impulse of the liver

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Abstract

Acoustic radiation force impulse (ARFI) imaging is a new and promising ultrasound-based diagnostic technique that, evaluating the wave propagation speed, allows the assessment of the tissue stiffness. ARFI is implemented in the ultrasound scanner. By short-duration acoustic radiation forces (less than 1 ms), localized displacements are generated in a selected region of interest not requiring any external compression so reducing the operator dependency. The generated wave scan provides qualitative or quantitative (wave velocity values) responses. Several non-invasive methods for assessing the staging of fibrosis are used, in order to avoid liver biopsy. Liver function tests and transient elastography are non-invasive, sensitive and accurate tools for the assessment of liver fibrosis and for the discrimination between cirrhotic and non-cirrhotic liver. Many published studies analyse ARFI performance and feasibility in studying diffuse liver diseases and compare them to other diagnostic imaging modalities such as conventional ultrasonography and transient elastography. Solid focal liver lesions, both benign and malignant, are common findings during abdominal examinations. The accurate characterization and differential diagnosis are important aims of all the imaging modalities available today. Only few papers describe the application of ARFI technology in the study of solid focal liver lesions, with different results. In the present study, the existing literature, to the best of our knowledge, about ARFI application on diffuse and focal liver pathology has been evaluated and results and statistical analyses have been compared, bringing to the conclusion that ARFI can be used in the study of the liver with similar accuracy as transient elastography in diagnosing significant fibrosis or cirrhosis and has got some advantages in respect to transient elastography since it does not require separate equipment, better displays anatomical structures and measurements can be successfully carried out almost in every patient.

Key words: Acoustic radiation force impulse imaging; Sonoelastography; Diffuse liver pathology; Focal liver lesion

Core tip: In the present study, the existing literature, to the best of our knowledge, about acoustic radiation force impulse (ARFI) application on diffuse and focal liver pathology has been evaluated and results and statistical analyses have been compared, bringing to the conclusion that ARFI can be used in the study of the liver with similar accuracy than transient elastography in diagnosing significant fibrosis or cirrhosis and has got some advantages in respect to transient elastography since it does not require separate equipment, better displays anatomical structures and measurements can be successfully carried out almost in every patient.

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INTRODUCTION

Acoustic radiation force impulse (ARFI) imaging is a new and promising ultrasound-based diagnostic technique that, evaluating the wave propagation speed, allows the assessment of the tissue stiffness[1-3]. ARFI is implemented in the ultrasound scanner and by using a conventional probe, without any need for external compression so reducing the operator dependency, it evaluates deep tissues stiffness providing complementary informations potentially useful for the diagnosis[1-6]. By short-duration acoustic radiation forces (less than 1 ms), it generates localized displacements in a selected region of interest (ROI; a box with dimension of 1 cm × 0.5 cm), identified on a conventional B-mode (Figure 1) image[7,8]. Depending on the interactions with the transducer[8,9], the generated wave scan provides qualitative (imaging) or quantitative (wave velocity values, measured in m/s) responses, by Virtual Touch Tissue Imaging and Virtual Touch Tissue Quantification, respectively (Siemens, Erlangen, Germany).

DIFFUSE LIVER DISEASES

Biopsy provides an extremely valuable contribution to the assessment of liver status in the case of chronic disease, offering information both on fibrosis and necro-inflammatory activity. However, not only the risk of complications, which have been reported with a frequency of 5%-20% for minor complications and 0.3%-0.5% for major complications[10] including also exceptional cases of death, but also contraindications, such as coagulopathy, poor patients cooperation or lack of consent, tend to limit its use, especially for repeated use over time. Furthermore, insufficient sampling and inter-observer variability may occur[11].

Considerable efforts have been made to develop non-invasive methods for assessing the staging of fibrosis, in order to avoid liver biopsy. In this setting the ideal method should be simple, inexpensive, easily available, repeatable and accurate.

Liver function tests [alanine aminotransferase (ALT), aspartate aminotransferase (AST), total proteins, serum albumin, gamma-globulins, gamma glutamyl transpeptidase (GGT), total bilirubin, alkaline phosphatase, prothrombin time/international normalized ratio] can be performed prior to the liver biopsy. The 2 main scoring systems used to predict and evaluate liver fibrosis are AST-platelet ratio index (AST level and platelet count) and FIBROMAX (Biopredictive, France) that combines the measurement of 10 indirect parameters adjusted for age, sex, weight and height: α2-macroglobulin, haptoglobin, apolipoprotein A1, total bilirubin, GGT, ALT, AST, fasting glucose, triglycerides and total cholesterol.

Transient elastography (TE) (Fibroscan, Echosense, Paris, France) has proved to be a non-invasive, sensitive and accurate tool for the assessment of liver fibrosis and particularly for the discrimination between cirrhotic and non-cirrhotic liver and its use is rapidly spreading. However, since it requires separate equipment, it means that at least one other examination is necessary in addition to conventional ultrasonography (US) of the liver, requiring additional time and costs after B-mode ultrasonography. Moreover, during TE examination, only A-mode imaging is displayed on the screen in order to select the area of scanning and, consequently, ligaments, vascular structures or even lesions, may inadvertently be included in the ROI, possibly affecting the final results.

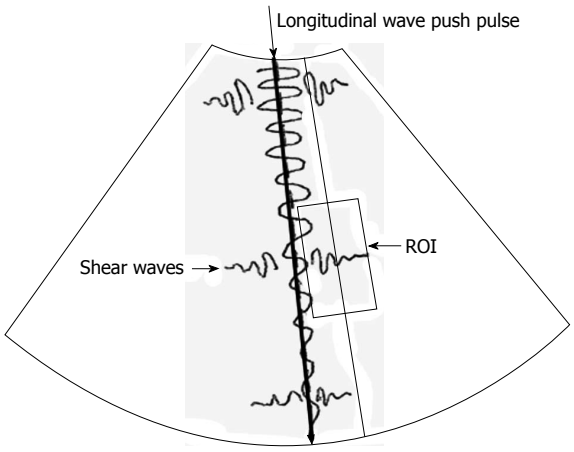

Figure 1 Acoustic radiation force impulse virtual touch tissue quantification technical scheme. ROI: Region of interest.

ARFI imaging offers the possibility of performing a quantitative measurement of the elasticity of the hepatic parenchyma during conventional US evaluations, without requiring additional transducers or other equipment[7].

Many studies analyse ARFI performance in studying diffuse liver diseases. Piscaglia *et al*[12] show that Virtual Touch Tissue Quantification is able to identify the presence of cirrhosis with good accuracy and produces results correlated with those obtained by transient elastography with Fibroscan. They performed measurement in the right lobe, by means of an intercostal scan, a condition which offers high inter-observer reproducibility (r = 0.874 in their series[12]).

The great advantage of fibrosis assessment using Virtual Touch Tissue Quantification is the fact that it can be performed at the same time as conventional US investigation. US is routinely used worldwide in the management of patients with chronic liver disease and is the first imaging technique employed when liver disease is suspected. With conventional US, certain features are highly specific for predicting severe fibrosis or cirrhosis (surface nodularity: specificity 95%; caudate lobe hypertrophy: 91%), but are not very sensitive (sensitivity of 54% and 41% respectively)[13]. Piscaglia *et al*[12] affirm that results in performing ARFI imaging have found to be similar to those of other works, all of which showed an area under the receiver operating characteristic curve (AUROC) above 0.9 for the diagnosis of cirrhosis with a cut-off value of 1.77 m/s (sensitivity 93%, specificity 85.1%). This cut-off value is very similar to those reported by Friedrich-Rust *et al*[7] (1.75 m/s), Sporea *et al*[14] and Takahashi *et al*[15], but differs from the ones obtained

Table 1 Mean wave propagation velocity values

Ref.	Value (m/s)
The right lobe of healthy liver	
Eiler *et al*[33]	1.16
Sporea *et al*[48]	1.19
Karlas *et al*[49]	1.19
Jaffer *et al*[50]	1.12
Marginean *et al*[51]	1.18 ± 0.27
Crespo *et al*[52]	1.06
Kircheis *et al*[53]	1.09 ± 0.13
Yoon *et al*[54]	1.06
Colombo *et al*[18]	1.40
Sporea *et al*[55]	1.28 ± 0.43
Noruegas *et al*[56]	1.11
Rizzo *et al*[57]	0.99
Karlas *et al*[58]	1.15 ± 0.17
Sporea *et al*[59]	0.97 ± 0.19
Son *et al*[60]	1.07 ± 0.11
Rifai *et al*[61]	1.10 ± 0.17
Kuroda *et al*[62]	0.99 ± 0.21
Popescu *et al*[63]	1.15 ± 0.21
Piscaglia *et al*[12]	1.13
Toshima *et al*[64]	1.15
Horster *et al*[65]	1.19
Goertz *et al*[66]	1.16 ± 0.11
Goertz *et al*[35]	1.09
D'Onofrio *et al*[32]	1.56
Fierbinteanu-Braticevici *et al*[17]	< 1.185 (cut-off)
Friedrich-Rust *et al*[7]	1.10
A liver with severe fibrosis (> F3)	
Ye *et al*[67]	1.69
Sporea *et al*[48]	1.43
Karlas *et al*[49]	1.43
Chen *et al*[68]	2.43 ± 0.13
Sporea *et al*[69]	1.60 ± 0.49 HBV; 1.55 ± 0.63 HCV
Crespo *et al*[52]	1.77
Kircheis *et al*[53]	1.44 + 0.26
Yoon *et al*[54]	1.89
Friedrich-Rust *et al*[70]	1.55
Colombo *et al*[18]	1.44
Sporea *et al*[55]	1.64 ± 0.51
Noruegas *et al*[56]	1.48
Rizzo *et al*[57]	1.70
Karlas *et al*[58]	1.70 if non-viral
Sporea *et al*[59]	1.71 ± 0.52
Kuroda *et al*[62]	1.61 ± 0.79 F3; 2.35 ± 1.11 F4
Toshima *et al*[64]	1.88
Sporea *et al*[21]	1.78 ± 0.77
Fierbinteanu-Braticevici *et al*[17]	> 1.54 (cut-off)
Takahashi *et al*[15]	2.57 ± 0.52 mean value for F4 (cut-off > F3 = 1.44)
Lupsor *et al*[16]	1.520 ± 0.575
Friedrich-Rust *et al*[7]	1.64

HBV: Hepatitis B virus; HCV: Hepatitis C virus.

in other studies[16,17] that reported slightly higher thresholds. In their series the good performance of the ARFI technique with the previously reported cut-off value was also confirmed by the testing in a population with cirrhosis proven by biopsy as the reference standard.

Other works compare ARFI imaging to TE by means of Fibroscan, such as the one from Colombo *et al*[18]. They similarly found that TE and ARFI are both highly effective in diagnosing cirrhosis, but they came to the conclusion that TE is probably more accurate in predicting significant fibrosis (AUROC of TE 0.897, AUROC of ARFI 0.815), although they could not demonstrate a statistically significant difference between the two curves. Their results were consistent with Boursier *et al*[19] and Lupsor *et al*[16] who found the same diagnostic accuracy for cirrhosis, but better performance of TE in predicting significant fibrosis (F2 or higher), but were at variance with three studies which instead found similar accuracies of TE and ARFI in diagnosing significant fibrosis[7,20,21].

Another interesting finding was that Virtual Touch Tissue Quantification measurements could be successfully carried out in all patients enrolled[12], while TE was unsuccessful in 7% of cases (*e.g.*, in patients with narrow intercostal spaces and in those with morbid obesity), as reported also in literature[22-26]. A possible explanation for this is that Virtual Touch Tissue Quantification may not be limited by narrow intercostal spaces or even by moderate excess weight, as it only requires the visible liver is not deeper than a fixed distance from the skin surface (in order to put the ROI in the parenchyma), while with TE the liver must not be more than 25 mm from the skin.

Regarding steatosis and inflammatory changes in diffuse liver disease, there is no agreement in the possible use of ARFI in diagnosing these parenchymal changes and in the effects of these changes themselves on ARFI measurements[20,27,28]. It seems unlikely that changes that minimally affect the parenchymal stiffness will be at this moment accurately depicted and diagnosed by using this non invasive technique.

NORMAL AND PATHOLOGICAL VALUES

Mean normal values and mean values indicating severe fibrosis (Table 1) range about 0.8-1.7 m/s (Figure 2A) and about 1-3.4 m/s (Figure 2B) respectively.

Fierbinteanu-Braticevici *et al*[17] demonstrated that ARFI elastography could predict fibrosis of F2 METAVIR stage or higher with a validity of 90.2%. They calculated the optimal cut-off point between stages F1 and F2 to be 1.21 m/s. At this value, ARFI elastography had a sensitivity of 89.4% and a specificity of 100%. In their work, they also demonstrated that ARFI can predict even better F3 and F4 stage fibrosis. The optimal cut-off value to identify fibrosis stage F3 or higher was 1.54 m/s, with sensitivity and specificity of 97% and 100% respectively. The optimal cut-off value in predicting cirrhosis (stage F4) was 1.94 m/s with a sensitivity of 100% and a specificity of 98.1%.

In chronic viral hepatitis, the knowledge of the stage of liver fibrosis is important for prognosis and for decisions about antiviral treatment[29]. Fibrosis staged higher than F2 is an indicator for antiviral treatment, hence the great therapeutic value of a highly accurate diagnostic test. Moreover, early detection of significant fibrosis (F3 or higher) is essential since patients with significant fibrosis are at high risk of developing complications, such as portal hypertension or hepatocellular carcinoma, and consequently need specific follow-up[17].

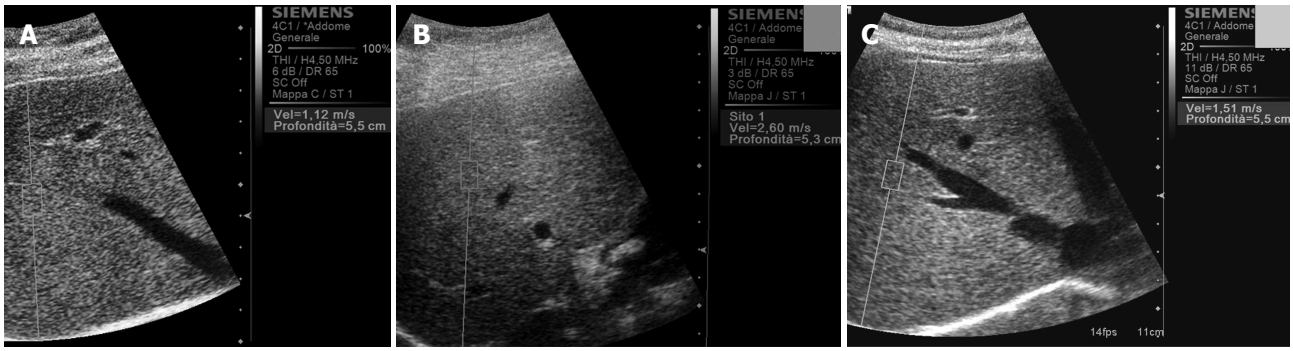

Figure 2 Acoustic radiation force impulse imaging of the liver. A: Normal value in healthy liver. B: Cirrhosis. C: Outlier value in healthy liver.

At present, it's difficult to determine the real impact of ARFI in the early diagnosis of hepatic fibrosis[17,27]. According to Fierbinteanu-Braticevici *et al*[17], in fact, there is a values overlap between F0-F1 and F2 fibrosis stages. The increase in liver propagation velocity has been demonstrated to be more important between stages F2 and F3 than between F1 and F2. This is consistent with the fact that the increase in fibrous tissue is more important between stages F2 and F3 than between F1 and F2. This limit of ARFI was overcome by the fact that fibrosis staged F2 or higher is considered a hallmark of progressive liver disease, therefore these are the patients in which there is a stronger indication for treatment as compared with patients with no or mild fibrosis[30,31].

There is in fact a range of variability of normal and pathological values in the Literature (Table 1). So what is important is to give the correct task to this new technique at present. The correct use of this technique has to be based on the true possibility of this system to detect changes in liver stiffness related to the development of different amount of fibrosis. The risk that absolutely should be avoided is to overestimate pathology and to look for inconsistent diseases. Therefore, in conclusion, the normal cut-off values must not be too strict but perhaps they also have to be adapted from time to time in relation to clinical and technical setting and from measurement to measurement.

In example, variability in the normal value is reported in literature[32] with a mean value of about 1.5 m/s in healthy subjects. This result can be considered an outlier[33] but however possible (Figure 2C). Moreover higher values can be obtained measuring in the left lobe[12,32] and in the superficial part of the right lobe[32]. This last aspect can be contrarily absent in child[33] due to a lower age-related fibrosis in the superficial liver parenchyma. Also in other published series Virtual Touch Tissue Quantification results in the right and left liver lobes did not appear to be strictly similar and, on average, the stiffness values were found to be higher in the left lobe than in the right lobe, at least in patients with chronic hepatitis (68% of patients had higher values in the left lobe than in the right lobe). Furthermore the diagnostic capacity to establish the histological degree of liver fibrosis (with a reference biopsy taken in the right lobe) was lower in Virtual Touch Tissue Quantification measurements from the left lobe than from the right lobe. These data, however, are not to be considered as a limitation of Virtual Touch Tissue Quantification to date, since they may perhaps more correctly reflect real differences and heterogeneity in the disease progression rates between the two lobes. It was in fact demonstrated that when two biopsies were taken in the two lobes during laparoscopy, a difference in one fibrosis stage between the two lobes occurred in up to 33% of cases[34]. However since our reference standard for the assessment of fibrosis in chronic liver disease is biopsy of the right lobe of the liver, it is recommended to measure liver stiffness by Virtual Touch Tissue Quantification in this lobe. Moreover, an approach with multiple measurements in various liver sites is worthy of further investigation as it may lead to interesting and original diagnostic results. In addition, Goertz *et al*[35] suggest to perform Virtual Touch Tissue Quantification *via* intercostal access in order to minimize invalid measurements and standard deviation. In their series, in fact, values taken subcostally were slightly higher than those measured through an intercostal approach.

Also some technical aspects need to be taken into account because they may explain some variability among published data. In example, the new release of the system is based on two acoustic pulses laterally to the ROI one by one at both sides and the maximum depth of the system nowadays achievable is 8 cm. Based on these considerations, the data published in the more recent papers should be more indicative of what can be obtainable with the new systems.

A recent study by Han *et al*[36], compares ARFI performance to Doppler parameters and describes a weak but significant relationship between liver stiffness, measured by ARFI, and the parameters related to the portal pressure, as measured by Doppler US in patients with liver cirrhosis at different Child-Pugh stages, but having no oesophageal varices. The study demonstrates a positive correlation between the median ARFI sonoelastographic velocity, which reveals liver stiffness, and the flow parameters of Doppler US, which reflects portal hypertension. All these features, however, appear in advanced stage of disease.

Regarding the possible role of ARFI, it can be for sure

Table 2 Diagnostic accuracy of non invasive methods for identifying severe liver fibrosis (> F3)

	AUROC	Ref.
Laboratory test (APRI score)	0.80	Lin *et al*[71]
	0.76	Friedrich-Rust *et al*[7]
	0.84	Leroy *et al*[72]
Transient elastography	0.87	Boursier *et al*[73]
	0.96	Ferraioli *et al*[74]
	0.90	Friedrich-Rust *et al*[7]
Acoustic radiation force impulse	0.91	Friedrich-Rust *et al*[7]
	0.90	Lupsor *et al*[16]
	0.99	Fierbinteanu-Braticevici *et al*[17]

AUROC: Area under the receiver operating characteristic curve; APRI: Aspartate aminotransferase platelet ratio index.

employed in the follow up of cirrhotic patients in order to avoid multiple biopsies comparing the result before and after treatment. Liver biopsy is not suitable for repeated evaluations because it is invasive and can cause major complications (0.3%-0.5%)[37]. Moreover liver fibrosis is a sequential and continuous process, and the staging of liver fibrosis should be evaluated frequently (Table 2). In contrast to liver biopsy, ARFI imaging is not invasive and can be repeated many times in the same patient[27].

FOCAL LIVER LESIONS

Solid focal liver lesions are common findings during abdominal examinations. The accurate characterization and differential diagnosis are important aims of all the imaging modalities available today[38-43]. Only three papers describe the application of ARFI technology in the study of solid focal liver lesions, with different results[44-46].

The first human images of hepatic malignancies acquired *in vivo* using the ARFI technique or any other elasticity imaging technique appeared in the work of Fahey *et al*[44]. His group compared B-mode and ARFI images both qualitatively (assessing the lesion margins definition by B-mode ultrasonography and ARFI imaging) and quantitatively (comparing the images contrast for both the techniques). They came to the conclusion that lesions margins definition at ARFI imaging was superior to that seen at B-mode US imaging (qualitative analysis). They also calculated that ARFI imaging can provide improvements in defining the contrast of tissue masses demonstrating, in fact, that the mean contrast for suspected hepatocellular carcinoma (HCC) in B-mode imaging was 2.9 dB (range 1.5-4.2 dB) *vs* 7.5 dB (range 3.1-11.9 dB) in ARFI images, with all HCCs appearing less stiff (brighter) than regional cirrhotic non-tumorous liver parenchyma. Moreover, the mean contrast for hepatic metastases in B-mode images was 3.1 dB (range 1.2-5.2 dB) *vs* 9.3 dB (range 5.7-13.9 dB) in ARFI images. Metastatic lesions in fact are stiffer (darker) than regional non-cirrhotic, non-neoplastic liver parenchyma. Fahey *et al*[44] also stated that combined US/ARFI could find application in tumor screening, lesion characterization and early detection of disease. Since HCC screening is not considered cost-effective in regions with low prevalence, due to the low sensitivity of both sonography and serum AFP sampling[47], ARFI imaging can improve sensitivity and cost-efficiency given its low cost, its capability of improving tumor contrast in comparison to US alone. If ARFI imaging had been proven to be a feasible alternative to contrast-enhanced ultrasound for liver applications, it could hold potential advantages related to the cost and complexity of the imaging protocol used for HCC screening. The authors, however, did not take into account the mechanical response of benign abdominal masses to applied radiation forces, thus they couldn't evaluate the ability of ARFI in differentiating benign from malignant liver masses.

More recent works about ARFI imaging applied to solid focal liver lesions are the ones from Cho *et al*[45] and from Gallotti *et al*[46]. The first one evaluates ARFI values calculated on HCCs, metastases, cholangiocarcinomas and hemangiomas, the second one evaluated in addition adenomas and focal nodular hyperplasia (FNHs), but did not consider cholangiocarcinomas.

Benign lesions

In regard to hemangiomas, Gallotti *et al*[46] agree with Cho *et al*[45] about the great variability of this type of lesions (mean wave velocity value of the lesion 2.30 m/s; mean wave velocity value of the surrounding parenchyma 1.45 m/s), since its stiffness depends on the amount of fibrotic septa which divide the dilated vascular space.

For the first time in Gallotti *et al*[46] paper also FNHs and adenomas were studied. FNH resulted the stiffest lesions after metastases and cholangiocarcinomas, indipendently from their dimensions and from the presence or absence of central scar. In fact, even if present, the ROI has to be located out of the fibrotic central scar. The high stiffness (mean wave velocity value of the lesion 2.75 m/s; mean wave velocity value of the surrounding parenchyma 1.57 m/s[46]) is explained with the well known high fibrotic content of this type of lesion. Thus, if the result will be confirmed by further studies, the cut-off of 2 m/s, suggested by Cho *et al*[45] to distinguish benign from malignant lesions, can no longer be used.

On the other hand, adenomas showed wave velocity values similar to those observed in the surrounding liver[46]: this is a solid focal liver lesion, the softest analysed (mean wave velocity value of the lesion 1.25 m/s; mean wave velocity value of the surrounding parenchyma 1.40 m/s). The presence of cells similar to normal hepatocytes and few stroma explain the low mean wave velocity value calculated in adenomas compared to other focal liver lesions.

Malignant lesions

According to Fahey *et al*[44], but inconsistent with results of Cho *et al*[45] despite the similar diameter of the lesions, in Gallotti *et al*[46] almost all the HCCs evaluated resulted in softer lesions compared to the surrounding cirrhotic liver (mean wave velocity value of the lesion 2.17 m/s;

mean wave velocity value of the surrounding parenchyma 2.99 m/s), and the main elastographic value was significantly lower than that of the surrounding parenchyma. This discrepancy might be explained by the difference in the severity of the cirrhosis of the background liver in each study population. In Cho *et al*[45] the degree of liver cirrhosis of patients with HCCs was likely to be less severe (15 out of 20 patients with HCCs had chronic liver disease of Child-Pugh classification A) compared with that seen in the other studies, assuming that the liver is stiffer with more severe liver cirrhosis.

There is concordance[44-46] about the fact that all metastatic lesions (mean wave velocity value of the lesion 2.87 m/s; mean wave velocity value of the surrounding parenchyma 1.63 m/s[46]) and, when considered, cholangiocarcinomas, are stiffer than the surrounding liver. This is probably due to the presence of fibrous content potentially found in many of these lesions. The presence of necrotic degeneration, mainly in the biggest masses, does not influence the results since the ROI for the stiffness calculation has to be accurately positioned out of the necrotic portion. Summarizing, based on the preliminary results of the study of solid focal liver lesions[44-46], it can be also concluded that ARFI seems to be an useful in the following scenarios: (1) for differential diagnosis between adenomas and FNHs; (2) for the study of metastases; and (3) for the study of HCCs in cirrhotic liver. Future perspective could be the application of ARFI in liver lesion detection by using volumetric automated acquisition.

CONCLUSION

Virtual Touch Tissue Quantification is a new non invasive imaging based technique able to estimate liver stiffness diagnosing cirrhosis with a good accuracy. The first assessment of patients with a suspicion of liver disease can be therefore easily performed with both conventional ultrasonography and Virtual Touch Tissue Quantification for liver stiffness assessment in a single step.

In conclusion, several studies about ARFI application in diffuse liver pathology have been made, and most of them state that ARFI itself can be used in the study of the liver with similar accuracy than transient elastography in diagnosing significant fibrosis[7,20,21] or cirrhosis[12,16,19]. However, ARFI has got some advantages in respect to TE since it does not require separate equipment and consequently it is not necessary an additional examination in addition to conventional US, saving time and costs. Moreover, during TE examination, only A-mode imaging is displayed on the screen in order to select the area of scanning and, consequently, ligaments, vascular structures or even lesions, may inadvertently be included in the ROI, possibly affecting the final results.

Another interesting finding is that Virtual Touch Tissue Quantification measurements can be successfully carried out almost in every patient while TE is unsuccessful in 7% of cases (*e.g.*, in patients with narrow intercostal spaces and in those with morbid obesity), as reported also in literature[22-26]. On the contrary, there are just few indications about ARFI and focal liver lesions, so further studies are needed in order to find ARFI the correct place in the everyday clinical practice.

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REVIEW

New ultrasound techniques for lymph node evaluation

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Abstract

Conventional ultrasound (US) is the recommended imaging method for lymph node (LN) diseases with the advantages of high resolution, real time evaluation and relative low costs. Current indications of transcutaneous ultrasound and endoscopic ultrasound include the detection and characterization of lymph nodes and the guidance for LN biopsy. Recent advances in US technology, such as contrast enhanced ultrasound (CEUS), contrast enhanced endoscopic ultrasound (CE-EUS), and real time elastography show potential to improve the accuracy of US for the differential diagnosis of benign and malignant lymph nodes. In addition, CEUS and CE-EUS have been also used for the guidance of fine needle aspiration and assessment of treatment response. Complementary to size criteria, CEUS could also be used to evaluate response of tumor angiogenesis to anti-angiogenic therapies. In this paper we review current literature regarding evaluation of lymphadenopathy by new and innovative US techniques.

Key words: Lymph nodes; Ultrasound; Endoscopic ultrasound; Lymph node metastasis; Lymphoma

Core tip: The differentiation of malignant from benign lymph nodes by ultrasound, computed tomography and magnetic resonance imaging traditionally relies mainly on size measurements and topographic distribution. However, sensitivity and specificity in the differentiation of benign and malignant lymph nodes are disappointing using only size parameters. The presented paper is intended to discuss, comment and illustrate the clinical important work-up of lymphadenopathy with respect of recently introduced imaging techniques including contrast enhanced ultrasound and elastography.

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INTRODUCTORY CONSIDERATIONS

The differentiation of malignant from benign lymph nodes by ultrasound (US), computed tomography (CT) and magnetic resonance imaging (MRI) traditionally relies mainly on size measurements and topographic distribution[1-3]. However, sensitivity and specificity in the differentiation of benign and malignant lymph nodes are disappointing using only size parameters. Reasons for the low accuracy include that malignant lymph node infiltration occurs in up to 30% in lymph nodes of less than 5 mm which has been shown for lung, esophageal, gastric, pancreatic and rectal carcinoma[4-10]. The evaluation of shape and border often adds no or only little more information

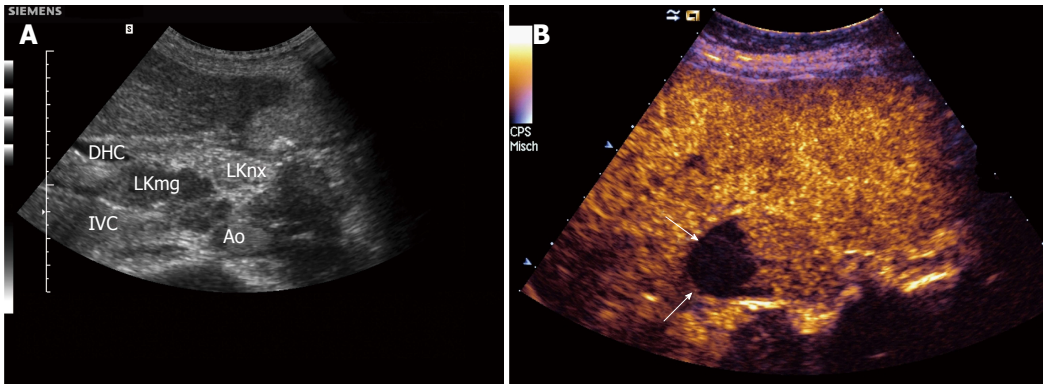

Figure 1 Lymph node infiltration, carcinoma. A: With lymph node (LN) specific contrast agents malignant infiltration can be delineated (LKmg) as focal hypoenhancement in the upper part of this perihepatic LN. The lower part (LKnx) shows normal (physiological) enhancement; B: With SonoVue®. Necrotic (non-enhancing, arrows) areas can be detected within this perihepatic lymph node. Necrotic areas are typically for carcinoma infiltration and tuberculosis. IVC: Inferior vena cava; Ao: Aorta; DHC: Common bile duct.

to exclude malignancy[11,12]. New imaging methods should be able to delineate the early and circumscribed malignant infiltration and to improve ultrasound guided biopsy.

Colour Doppler ultrasound (CDI) adds value for the differentiation of malignant from normal or reactive nodes by displaying the macrovessel architecture. Normal LNs generally show hilar predominant normal vascularity. Inflammatory lymph nodes are typically more vascularised without changes of the predominant hilar vessel architecture. In contrast metastatic lymph nodes present peripheral or mixed vascularity and loss of hilar type of vascularisation[13].

Contrast enhanced CDI has improved the viusalisation of macrovessels (angioarchitecture) but does not allow evaluation of microvessels[14]. Demonstration of malignant neovascularisation, *e.g.*, vessels penetrating the LN capsule, has been used as the characteristic feature of lymph node metastases.

Spectral Doppler ultrasound contributes to differentiation of malignant and benign solid neoplasia[15]. Likewise, normal and inflammatory lymph nodes show lower vascular resistance [resistive index (RI)] as compared to malignant lymph nodes[16] but overall results are disappointing.

Although Doppler ultrasound techniques have extended the opportunities for the differentiation of malignant from benign lymph nodes by displaying changes of macrovascularity and the vascular resistance[13,17,18], they do not improve lymph node detection rate and vascularity is often not detected in small lymph nodes[19]. Therefore, Doppler techniques and contrast enhanced Doppler techniques in general have not significantly improved the diagnostic work up of lymphadenopathy. There is a need for new imaging techniques for better characterisation of lymph nodes with the opportunity to assess also the internal microvessel architecture of lymph nodes and tissue elasticity for detection of early circumscribed malignant infiltration.

In the presented paper we discuss current knowledge about recent advances in ultrasound technology for improved lymph node evaluation.

CONTRAST ENHANCED ULTRASOUND

Contrast enhanced ultrasound (CEUS) is the application of ultrasound contrast agents (UCA) to traditional sonography. The currently used UCA are microbubbles stabilized by a shell which has a high degree echogenicity. Since their physical size is just 1-4 micrometres in diameter (equal to or smaller than red blood cells), UCA allow depiction of both the macrovasculature and the microvasculature[20]. CEUS has been introduced more than ten years ago and guidelines have been published for the liver[20,21] and non-liver indications[22]. Currently 4.8 mL SonoVue® is recommended for imaging superficial LNs with a high frequency probe and for imaging the mediastinal and abdominal LNs with a high frequency endoscopic probe in CE-EUS.

CEUS techniques provide information on vascularisation and perfusion patterns, and exploit the differences in blood flow characteristics between normal and pathological tissue but knowledge about lymph node evaluation is limited[22]. CEUS could be helpful by identifying changes in vascular architecture of macro- and micro-vessels and avascular areas as signs of malignant infiltration.

Carcinoma

Carcinoma infiltration causes the development of pathological vessels (neoangiogenesis) and, therefore, a change of the perfusion pattern with heterogeneous enhancement due to the presence of caliber changes of the neoplastic vessels and arteriovenous shunts[23-27]. Focal hypoenhancement may result from the partial insufficiency of blood-supply due to overpressure in the LN caused by the neoplastic infiltration. Malignant lymph nodes not only have a greater number of peripheral vessels, but also longer contrast enhancement duration than benign lymph nodes[28]. Destructive avascular necroses are an important imaging sign for malignant infiltration (Figures 1-3). Avascular areas are detected by the lack of contrast agent uptake in the necrotic zones and the peripherally located pronounced hyperenhancement (rim enhancement)[29,30]. The contrast enhancement pattern of focal cortical

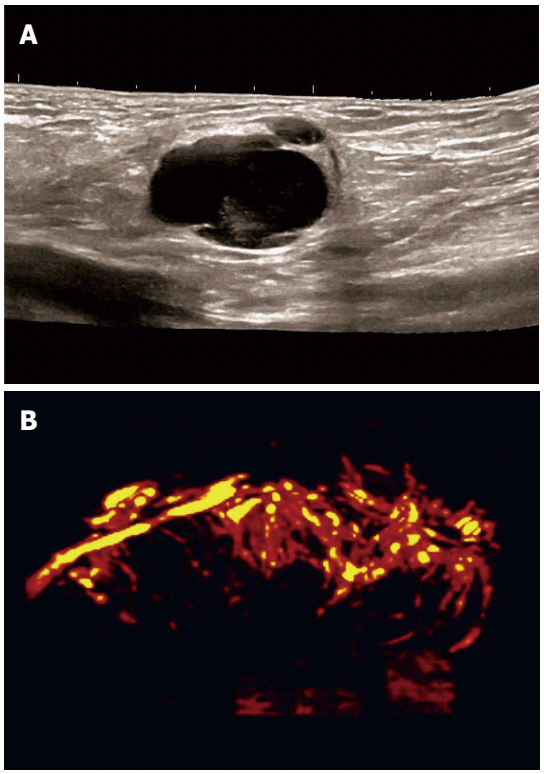

Figure 2 Carcinoma infiltration. Typically vessel destruction with chaotic vessels in the lymph node can be observed. B-mode (A) and 3D angiographic mode (B).

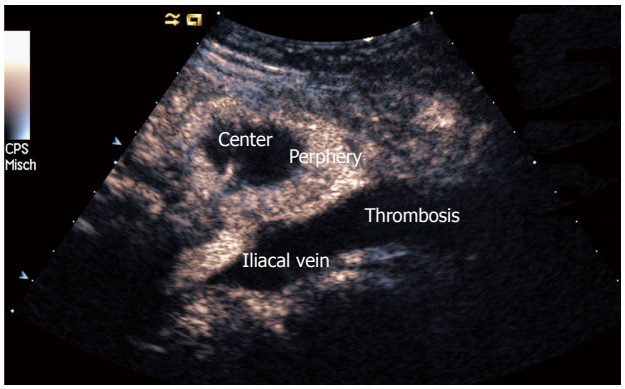

Figure 3 Prostate carcinoma infiltration of the pelvis. Typically vessel destruction with interruption of vessel architecture can be observed in patients with carcinoma. The center of the lymph node is almost non-enhancing except one visible vessel whereas the periphery shows hyperenhancement. Thrombosis of the iliac vein is indicated as well.

thickening has been also identified as an important sign to differentiate benign and malignant lymphadenopathy. In benign lymph nodes contrast enhancement within the cortex is homogeneous, whereas in malignant lymph the cortical thickening is less well vascularized than the adjacent normal lymph node parenchyma[24].

In conclusion, criteria for carcinomatous lymph node infiltration on CEUS are centripetal inhomogeneous enhancement and perfusion defects.

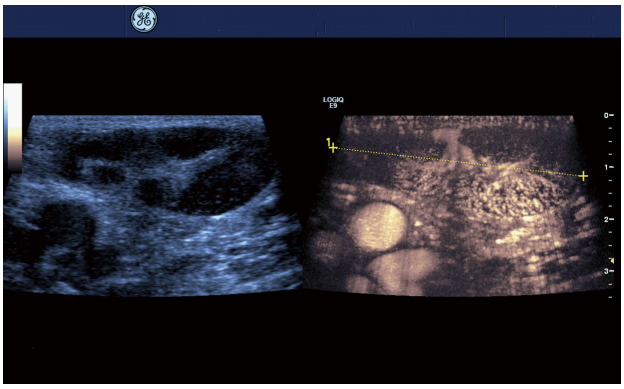

Figure 4 Lymph node infiltration (50 mm), non-Hodgkin's lymphoma. Typically the hilum predominant vessel architecture is preserved (between markers).

Lymphoma

It is essential to consider lymphoma separately because some of its features are different from other LN disease[13,31]. The very few studies published so far showed that in lymphoma contrast enhancement patterns are highly variable. The most often observed pattern is intense homogeneous enhancement, which is not different from reactive inflammatory lymph nodes[25,31] (Figure 4).

In conclusion, there is evidence that the vascular pattern of lymphomatous lymph node infiltration resembles that of non-malignant nodes.

Inflammation

Most inflammatory processes do not change the hilum predominant vessel architecture of lymph nodes. According to the majority of published papers, normal and inflammatory LNs are characterized by a centrifugal and homogeneous enhancement pattern[23-26] (Figure 5). Therefore, inflammation changes the enhancement pattern only by the amount (peak) enhancement but not by changes of distribution. It is worth mentioning that non-destructive necrosis, which is reflected in avascular areas on CEUS, can be also found in granulomatous lymphadenitis, *e.g.*, cat-scratch disease (bartonellosis), tuberculosis and sarcoidosis.

Treatment response

Changes and reduction of intranodal vascularity may be the first sign of response to antineoplastic treatment as shown for gastrointestinal stromal tumors and renal cell carcinoma[22,32]. Since tumour growth depends on neovascularization, CEUS can also help to detect focal nodular tumour recurrence in scars and to guide biopsy[33]. In Hodgkin's disease well demarcated avascular areas have been described as a typical sign of treatment response[34,35].

Dynamic contrast enhanced ultrasound

Quantification software (time intensity curve analysis) has been used for the differential diagnosis of benign and malignant LNs but results so far are conflicting[36]. There

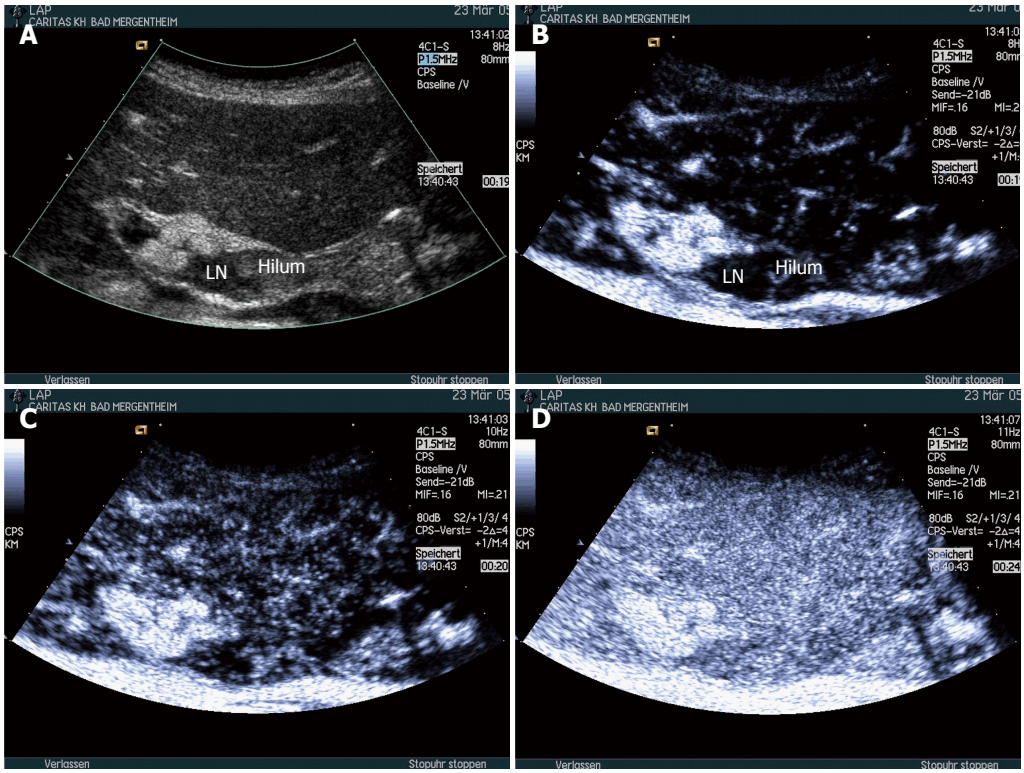

Figure 5 Inflammatory perihepatic lymph node dorsal in the hepatoduodenal ligament. Inflammation most often shows no changes of the symmetric lymph node vascularity and homogenous contrast enhancement (A-D). The hilum is indicated. LN: Lymph node.

are two studies showing that the difference of intensity between the hypervascular and hypovascular regions was significantly higher in metastatic than in non-metastatic LNs[26,37]. Steppan *et al*[38] reported that malignant compared to benign lymph nodes showed higher maximum intensity and duration of enhancement while Yu *et al*[25] reported no significant differences on maximum intensity. Time to peak intensity and area under the curve of malignant lymph nodes and lymphomas were less than that of benign LNs. Ahuja could demonstrate a reduction of vessel density (vascularity) and delay in the time to peak enhancement after treatment. It has to be mentioned that the changes in peak enhancement were operator dependent[33]. Since evidence is inconsistent quantification techniques cannot be generally recommended for clinical use. European Federation for Ultrasound in Medicine and Biology (EFSUMB) has published recommendations on the use of dynamic contrast enhanced ultrasound (DCE-US) discussing the current use and limitations in detail[39].

In conclusion, contrast enhanced techniques compared to conventional ultrasound may improve the differential diagnosis of benign LNs from malignant LNs and provide a more accurate selection of nodes to be submitted to fine-needle aspiration biopsy[25,28].

ELASTOGRAPHY

Elastography is a non-invasive method in which the stiffness of the tissue can be imaged as colour map or shear wave velocity. Two main forms of elastography have been studied for the evaluation of lymph nodes. One form is strain elastography (SE). The ultrasound probe is used to palpate the tissue[40] usually transcutaneously but optionally also intra-operatively or *via* an endoscope[41-45]. The tissue deformation produced (*i.e.*, strain) is assessed by following the way the speckle in the image moves, usually with a tracking algorithm working on the radio-frequency data. The data can then be used to form an image that is coded in colour or grey-scale to show the pattern of strain, which is inversely related to tissue stiffness. Therefore, SE allows assessment and visualization of relative elasticity differences. The area to be evaluated is defined by a ROI in a similar way to CDI[44,46]. New technical developments allow for averaging over several frames to calculate the mean histogram value which corresponds to overall elasticity within a selected area[47]. Comparing two different areas within the ROI allows calculation of the strain ratio. SE is the most commonly used method for the evaluation of lymph nodes. The other forms of elastography are shear wave elastography techniques (SWE) which include transient elastography (TE) (*e.g.*, Fibroscan™, Echosens, France), Acoustic Radiation Force Impulse imaging (*e.g.*, ARFI, Siemens, Germany) and Supersonic Shear Wave Imaging (SSI) (Supersonic, France). In shear wave elastography the "pushing" ultrasound beam causes minute displacements in soft tissue, which depend on the magnitude of tissue stiffness. Using tracking algorithms, the resulting shear waves can be detected sonographically. So far only SSI has been studied for the evaluation of LN. In elastographic images

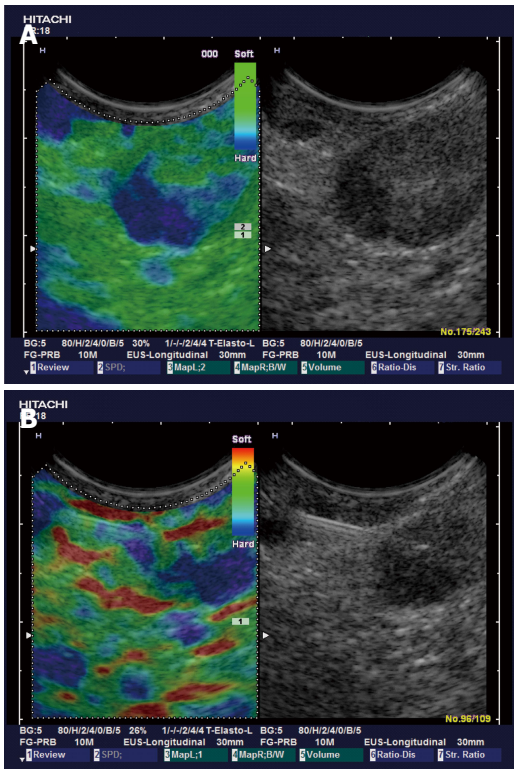

Figure 6 Colorectal carcinoma with presacral circumscribed lymph node metastasis proven by colonic endoscopic ultrasound using Fine Needle Aspiration Cytology. Sonoelastography reliability test evaluation reveals typically harder (blue) area in the lymph node.

of normal lymph nodes the nodal cortex is significantly harder than the medulla and the hilum[48,49].

EFSUMB has prepared recommendations on the use of elastography. In two sets of papers the techniques are explained in more detail[50,51].

Carcinoma

Typically the well differentiated carcinoma at least initially infiltrates lymph nodes in a circumscribed manner (focally stiffer, harder) (Figure 6), whereas the undifferentiated carcinoma leads to a diffuse (stiffer, harder) infiltration.

Suspected cervical LN metastases from hypopharyngeal and thyroid carcinomas have been recently investigated using SE (real time elastography)[39,52]. An elasticity index has been created by comparing the elasticity of the LN with the surrounding head and neck muscle tissue (muscle to LN strain ratio). Using a ratio of > 1.5 as an indicator of malignant infiltration, sensitivity was 82% and specificity 98% which is superior to the best B-mode criteria[52]. These data have been reproduced by Tan *et al*[53]. Moreover, interobserver agreement with SE was very high (kappa 0.828-0.946)[53].

Applying a higher cut-off value for strain ratio (1.78) Teng *et al*[54] at the cost of an only moderate specificity (65%) reported a very high sensitivity (98%) for discriminating malignant from benign suspicious cervical lymph nodes.

Other authors used a scoring system (percentage of blue-coding lymph node area) to differentiate malignant from benign lymph nodes in head and neck cancer patients. A blue coded (hard) area of > 50% of total lymph node area (score 3 and 4) or observation of a central necrosis (score 5) predicted malignant infiltration with high accuracy and added value to traditional ultrasound criteria[55-57].

So far two papers are published for the differential diagnosis of lymph nodes using shear wave elastography on 55 cervical enlarged lymph nodes using SSI. Malignant nodes were homogeneously stiffer than benign lymph nodes. The sensitivity (42%), specificity (100%) and accuracy (62%) were promising defining a cut-off level of 30.2 kPa[58]. The intra- and inter-observer reliability of shear wave elastography using SSI was shown to be fair to excellent for 176 neck lesions according to the intraclass correlation coefficients (0.78-0.85)[59].

In conclusion, elastography seems to be a very promising diagnostic tool for the differentiation between benign and malignant lymph nodes. This is reflected by a recent meta-analysis which reported a pooled sensitivity and specificity for the diagnosis of malignant lymph nodes of 74% and 90% for elasticity scores, and 88% and 81% for strain ratio, respectively[60]. However, to date studies comparing the two techniques of elastography (SE and SWE) are lacking.

Lymphoma

Knowledge of strain imaging in lymphoma is very limited. So far different lymphoma cannot be differentiated. Initial experience suggests that focal lymph node infiltration (Figure 7A) is indicative for low grading of follicular lymphoma whereas diffuse and homogenous lymph node infiltration is typically found in high grade lymphoma (Figure 7B).

Inflammation

Most inflammatory processes do not change the elastographic architecture of lymph nodes. The hilum in normal lymph nodes remains softer than the stiffer cortex also in inflammatory lymph nodes. Circumscribed softer (and stage dependent also stiffer) lymph node areas are found in tuberculosis but this has only been shown in few cases.

ENDOSCOPIC ULTRASOUND

Diagnostic and therapeutic endoscopic ultrasound has been established in the last thirty years[61,62]. The technique can be also applied with colour Doppler imaging as discussed above. Recently CE-EUS and real time endoscopic elastography (RTE-EUS) have been introduced.

Contrast enhanced endoscopic ultrasound

Contrast enhanced endoscopic ultrasound (CE-EUS) is CEUS performed with an endoscopic probe, which can be performed on both Doppler mode with high MI and contrast specific mode with low MI[63] to also guide therapeutic procedures. The dose of the ultrasound contrast agent (UCA) should be 4.8 mL for SonoVue®. CE-EUS

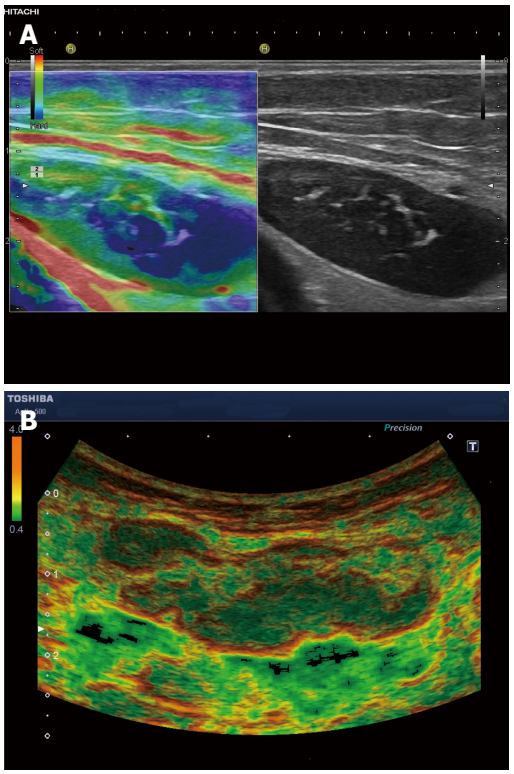

Figure 7 Non-Hodgkin's lymphoma involving the inguinal region. A: Sonoelastography reliability test evaluation reveals typically asymmetric and circumscribed infiltrated harder (blue) lymph node tissue in low grade follicular cell lymphoma; B: Elastography (acoustic structured quantification) reveals mainly homogenous diffuse infiltration in high grade follicular cell lymphoma.

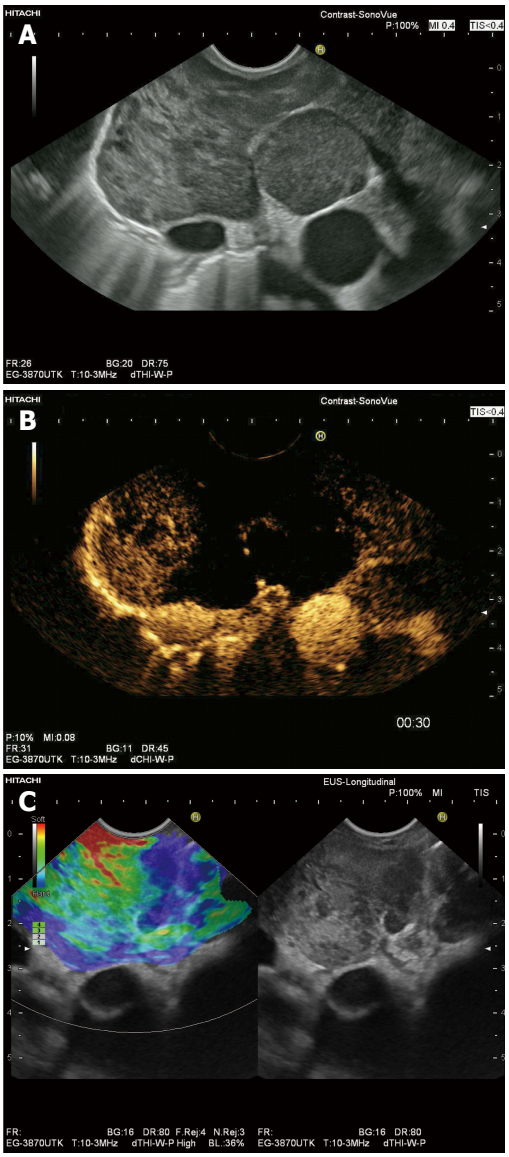

Figure 8 Endosonography of enlarged subcarinal lymph nodes in Non-Hodgkin Lymphoma. A: B-mode reveals two enlarged lymph nodes; B: contrast enhanced endoscopic ultrasound demonstrates extensive avascular (necrotic) areas in the lymph nodes; C: real time endoscopic elastography indicates hard, infiltrated areas (blue color), thus targeting endoscopic ultrasound-guided biopsy.

can improve the detection of small intranodal vessels and thus could be useful in characterization of LNs[3,64] (Figure 8). CE-EUS has improved our understanding of gastrointestinal (subepithelial) tumors[65-67], differential diagnosis of pancreatic neoplasia[15,68-75] and other organ infiltration[74,75] through analysis of perfusion patterns.

There are only a few reports about the usefulness of contrast enhanced endoscopic Doppler ultrasound in the differentiation between malignant and benign lymphadenopathy. Kanamori *et al*[64] performed CE-EUS with high MI on 46 patients in whom EUS revealed LN in the mediastinum or abdominal cavity and suggested that CE-EUS is useful for differentiating benign from malignant LNs by detecting defects of enhancement in malignant nodes. The sensitivity, specificity, and accuracy rate of CE-EUS were 100%, 86.4% and 92.3%, respectively. In another study by Hocke *et al*[3], high MI CE-EUS was performed in 122 patients, and it was found that CE-EUS improved the specificity in diagnosing benign LNs as compared to B-mode EUS by analysing arteries and veins. However, it did not improve the accurate identification of malignant LNs and therefore could not replace EUS-guided fine-needle aspiration[3].

To the best of our knowledge, there is only one report on the application of low MI CE-EUS for the discrimination of benign and malignant abdominal lymph nodes. A Japanese group investigated 43 patients with intra-abdominal lesions of undetermined origin, which were suspected to be malignant lymph nodes, and evaluated the enhancement pattern after injection of the UCA Sonazoid®. Final pathological examination revealed that 35 lesions in fact were lymph nodes. All but one of the malignant lesions showed a heterogeneous enhancement pattern, whereas none of the benign lesion displayed heterogeneous enhancement. Most interestingly the interobserver agreement was very high (kappa 0.953)[78].

Endosonographic elastographic lymph node evaluation (strain imaging)

Endoscopic elastography is real time elastography performed with an endoscopic probe, which has led to further improvement in B mode imaging results for classification of benign and malignant LNs (Figure 8), particularly by

targeting LNs for needle sampling. Janssen *et al*[79] reported on 50 patients, 66 LNs were described elastographically (dominant colour/tissue hardness and guidance for tissue samples) and the elastogram data later compared with the histological findings obtained in the same session from fine needle biopsy. This study revealed that benign LNs exhibited predominantly intermediate homogeneous deformation (yellow/green), while malignant LNs were characterized by a quantitative dominance of hard (blue) units. The accuracy, which could be consistently reproduced by two more reviewers (kappa 0.84), for benign *vs* malignant LNs was about 85%. Intra- and interobserver agreement was also high in one recent study using visual assessment of the elastography image to differentiate between malignant and benign lymph nodes[80]. However, the same group found that EUS elastography did not perform better than EUS morphology in differentiating between malignant and benign lymph nodes in patients with resectable upper gastrointestinal cancer[45]. These findings conflict with the results of two other groups, which showed superior accuracy of EUS elastography strain ratio and histogram analysis, respectively, in comparison with conventional EUS criteria in differentiating malignant and benign lymph nodes in the nodal staging of esophageal cancer[81,82].

Săftoiu *et al*[41] used similar criteria for qualitative analysis in their study. In computer analysis, accuracy for differential diagnosis of malignant *vs* benign LNs increased slightly from 93% to 95%. In a follow-up study[47], they reached an accuracy for differentiation between benign and malignant LNs of 89%, using the computer based histogram analysis of video sequences, while this was significantly superior to the B-Mode image analysis (accuracy 53%). Another recent study with pathological confirmation yielded however lower values for sensitivity, specificity and accuracy, based on strain ratio calculations[45].

A recent meta-analysis calculated a sensitivity of 88% and a specificity of 85%, respectively, of EUS elastography for differentiating between benign and malignant lymph nodes[83].

In conclusion, the sensitivity of an imaging procedure critically depends on spatial resolution, which in elastography is as good as in conventional ultrasound since both depend on the same physical rules. The smallest LN metastases may escape both B-mode diagnosis and endosonographic fine needle biopsy. Elastography can detect the smallest metastasis-related changes in tissue hardness and it is considered to be potentially useful for target selection prior to endosonographic guided tissue sampling[10].

RTE can be recommended for discrimination of benign and malignant lymph nodes by identifying malignant regions that should be targeted for EUS-FNA (Figure 8).

SENTINEL LYMPH NODE EVALUATION

The detection or exclusion of sentinel lymph node (SLN) micrometastases is critical in staging cancer, especially breast cancer and melanoma, because it directly affects patient's prognosis and surgical management. It is well known that conventional US is not able to detect SLN in most cases. However, studies showed that low MI CEUS can be used for detecting SLN, which may become a potential application in clinical routine, like lymphoscintigraphy[32,84-89]. The application of CEUS for the investigation of SLN has shown promising results in animal models but the technique has not been sufficiently evaluated in humans. About 1 mL of contrast agent (*e.g.*, SonoVue®) is injected subcutaneously (intralymphatic) near the tumour site and the enhanced lymphatics are traced to the sentinel lymph node. Initial experience indicates that the method is not toxic and performs as well as blue dye or radioisotope methods. The current literature has been recently reviewed[90] and the topic is not the subject of this paper.

PANORAMIC IMAGING, 3D AND 3D-CEUS

Panoramic imaging, 3D[91] and 3D-CEUS[92,93] have been used for improved anatomic and topographic description of lymphadenopathy but have not gained additional information except improved presentation of results to clinicians.

CONCLUSION

The currently possible lymph node detection rate is limited by a minimal required lymph node size which is between 5-10 mm. Since about one third of malignant infiltrations occur in lymph nodes which are not detectable by all imaging methods, reliable exclusion of malignant lymph node infiltration is almost impossible. Therefore, current imaging methods mainly focus on the improved detection of early malignant infiltration in detectable lymph nodes, *e.g.*, to guide neoadjuvant treatment strategies.

Ultrasound techniques (CEUS, CE-EUS and elastography) demonstrate high spatial resolution which is important for early detection of malignant lymph node infiltration (Table 1). CEUS compared with conventional CDI could improve the visualization of vessels in LNs which is essential for the evaluation of vessel distribution. The visualization of avascular necrotic deposits of neoplastic cells is helpful for the differentiation of benign and malignant lymphadenopathy. The identification of hypoenhancing areas in malignant lymph nodes may guide biopsy for improved early detection of malignant infiltration.

In addition, the strictly intravascular distribution of intravenously injected contrast agents (*e.g.*, SonoVue®) allows the assessment of neoangiogenesis which is of importance for treatment evaluation under antiangiogenetic treatment.

CEUS cannot be recommended for the diagnosis of lymphoma so far. However, CEUS may be a tool to assess the treatment response by indentifying the reduction of vascularisation, *e.g.*, in Hodgkin's disease.

Table 1 Criteria on lymph node characterization using different ultrasound modes

Lymphadenopathy more (most) likely	B-mode	(Contrast enhanced) Colour Doppler	Vascular resistance	CEUS (contrast special imaging mode)	Elastography
Inflammatory	Preserved architectur, homogeneous, thin cortex	Preserved vessel architecture, hilar vascularity with or without tree like branching.	Lower, RI < 0.8, PI < 1.6	Homogeneous enhancement from the hilum, centrifugal enhancement	No data, most often normal architecture (except tuberculosis)
Malignant infiltration (metastasis)	Destroyed architecture (capsule), eccentric hypoechoic cortical thickening, inhomogeneity of the internal structure, loss of echogenic hilum, surrounding edema	Peripheral or mixed vascularity, inhomogeneous vessel density, split arteries, torturous course of vessels	Higher, RI > 0.8, PI > 1.6, often variable at different sites	Centripetal enhancement, different intra-nodal enhancement levels, inhomogeneous wash-out, perfusion defects	Initially circumscribed. SR in diffuse infiltration > 1.5 (1.78)
Lymphoma	Focal or global hypoechoic cortical thickening, usually without echogenic hilum, peri-nodular edema, pseudocystic appearance	Often but not always preserved vessel architecture, rich vascularity	Intermediate RI and PI	Intense homogeneous enhancement, starts with diffuse bright spots, peripheral hypo- or non-enhancement	No data; wide range of appearance applying qualitative criteria

CEUS: Contrast enhanced ultrasound; RI: Resistive index; PI: Pulsatility index.

Elastography is mainly helpful in delineating the very early circumscribed malignant infiltration for improved US- and EUS-guided fine needle aspiration (biopsy). Additionally, normal elastographic architecture of enlarged inflammatory lymph nodes can be helpful to prove a benign inflammatory disease, *e.g.*, sarcoidosis.

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REVIEW

Is diabetes mellitus a risk factor for pancreatic cancer?

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Abstract

The relationship between diabetes mellitus and the risk of pancreatic cancer has been a matter of study for a long period of time. The importance of this topic is due to two main causes: the possible use of recent onset diabetes as a marker of the disease and, in particular, as a specific marker of pancreatic cancer, and the selection of a population at risk for pancreatic cancer. Thus, we decided to make an in-depth study of this topic; thus, we carried out an extensive literature search in order to re-assess the current knowledge on this topic. Even if diabetes is found a decade before the appearance of pancreatic cancer as reported in meta-analytic studies, we cannot select those patients already having non detectable pancreatic cancer, at least with the imaging and biological techniques available today. We believe that more studies are necessary in order to definitively identify diabetes mellitus as a risk factor for pancreatic cancer taking into consideration that approximately 10 years are needed to diagnose symptomatic pancreatic cancer. At present, the answer to the as to whether diabetes and pancreatic cancer comes first similar to the adage of the chicken and the egg is that diabetes is the egg.

Key words: Diabetes mellitus; Obesity; Pancreatic neoplasms; Risk factors; Clinical studies

Core tip: Even if diabetes is found a decade before the appearance of pancreatic cancer we cannot select those patients already having non detectable pancreatic cancer, at least with the imaging and biological techniques available today. We believe that more studies are necessary in order to definitively identify diabetes mellitus as a risk factor for pancreatic cancer taking into consideration that approximately 10 years are needed to diagnose symptomatic pancreatic cancer. At present, the answer to the as to whether diabetes and pancreatic cancer comes first similar to the adage of the chicken and the egg is that diabetes is the egg.

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INTRODUCTION

The relationship between diabetes mellitus and the risk of pancreatic cancer has been a matter of study for a long period of time. The importance of this topic is due to two main causes: the possible use of recent onset diabetes as a marker of the disease and, in particular, as a specific marker of pancreatic cancer, and the selection of a population at risk for pancreatic cancer[1].

SEARCH STRATEGY

Taking into consideration diabetes mellitus irrespective of type, there is a lack of agreement regarding the data; thus, we decided to make an in-depth study of this topic. On July 24, 2012, we carried out a PubMed/Medline search using the following strategy: ("Diabetes Mellitus" [Mesh]

Table 1 Diabetes as a risk factor for pancreatic cancer according to diabetes duration

Meta-analysis	Studies evaluated (*n*)		Diabetes duration (yr)	Risk	95%CI
Everhart *et al*[76]	20	All studies evaluated	> 1	RR = 2.1	1.6-2.8
	11	All case-control studies	> 1	RR = 1.8	1.1-2.7
	9	All cohort studies	> 1	RR = 2.6	1.6-4.1
	11	All studies	> 5	RR = 2.0	1.2-3.2
	6	All case-control studies	> 5	RR = 1.8	0.86-3.8
	6	All cohort studies	> 5	RR = 2.4	0.85-7.0
Li *et al*[64]	3		≤ 2	OR = 2.9	2.1-3.9
	3		3-5	OR = 1.9	1.3-2.6
	3		6-10	OR = 1.6	1.2-2.3
			11-15	OR = 1.3	0.9.2.0
			> 15	OR = 1.4	1.0-2.0
Huxley *et al*[80]	9		1-4	RR = 2.05	1.87-2.25
	9		5-9	RR = 1.54	1.31-1.81
	7		≥ 10	RR = 1.51	1.16-1.96
Ben *et al*[84]	3		< 1	RR = 5.38	3.49-8.30
	5		1-4	RR = 1.95	1.65-2.31
	4		5-9	RR = 1.49	1.05-2.12
	4		≥ 10	RR = 1.47	0.94-2.31

or "Diabetes Mellitus, Type 2" [Mesh] or "Diabetes Mellitus, Type 1" [Mesh]) and "Pancreatic Neoplasms" [Mesh] and ("humans" [MeSH Terms] and English [lang]); other papers were manually extracted from the references of the papers selected. From 1966, a total of 787 papers were found and, of these, we selected 74 papers[2-75] and nine meta-analyses[76-84].

ANALYSIS OF LITERATURE AND CLINICAL CONSIDERATIONS

One of the first studies on the relationship between pancreatic cancer and diabetes is that of Maruchi *et al*[2] who found that there was an association between pancreatic carcinoma and diabetes from 1935 through 1974 only in cases of confirmed pancreatic carcinoma in residents of Olmsted County, Minnesota. In their series, 17% of the patients were diabetic (19/113) and nine cases (8%) of diabetes had appeared at least 2 years before the diagnosis of pancreatic cancer. Twenty years later, our group carried out a case-control study matching a large number of patients with and without pancreatic cancer[24]. The main findings were as follows: in the majority of cases, the diabetes was diagnosed at the same time as the cancer or within a few years prior to its identification, suggesting that it was the cancer which caused the diabetes. In fact, diabetes mellitus of long duration (> 7 years) had essentially no association with pancreatic cancer whereas, in a small group of patients who had had diabetes of a 5-7 years duration when the cancer was diagnosed, the association was statistically significant. Finally, all the patients in whom the diagnosis of diabetes had been made prior to that of the tumor had non-insulin-dependent diabetes, and no association was found with the insulin-dependent form.

Taking into account all findings in the literature, all the studies and the meta-analyses found an association between diabetes mellitus and pancreatic cancer at the time of diagnosis. However, little is known about glucose tolerance and insulin secretion in patients with this tumor. Gapstur *et al*[85] prospectively studied the postload plasma glucose concentration in 84 patients with pancreatic cancer in order to determine the presence of an independent association between postload plasma glucose concentration and the risk of pancreatic cancer mortality among people without self-reported diabetes. Compared to a postload plasma glucose level of 119 mg/dL or less and, after adjusting for age, race, cigarette smoking and body mass index, the relative risks (95%CI) of pancreatic cancer mortality were 1.65 (1.05-2.60) for postload plasma glucose levels between 120 mg/dL and 159 mg/dL, 1.60 (0.95-2.70) for levels between 160 mg/dL and 199 mg/dL and 2.15 (1.22-3.80) for levels of 200 mg/dL or more. Such an association appeared to be stronger in men than in women. Estimates were only slightly lower after excluding 11 men and 2 women who died from pancreatic cancer during the first 5 years of follow-up. Elevated body mass index and serum uric acid concentration were also independently associated with an elevated risk of pancreatic cancer mortality in men only. This study provides evidence for a positive, dose-response relationship between postload glycemia and pancreatic cancer mortality. The possible mechanisms underlying the increased pancreatic cancer risk among patients with diabetes mellitus is the involvement of insulin resistance and hyperinsulinemia. In addition, whereas postoperative diabetes was seen in all long-standing diabetic patients, and in some patients with intolerance fasting glucose and normal fasting glucose, the diabetes was resolved in more than 50% of patients with new-onset diabetes despite removal of half of the beta-cell mass[52]. Thus, it seems that diabetes is caused by pancreatic cancer. The answer to whether the diabetes is a specific marker of the disease comes from the study of Aggarwal *et al*[86]; these authors retrospectively reviewed the medical records of 500 consecutive patients with cancer (lung, breast, prostate, colorectal cancers and pancreatic cancer) and 100 non-cancer controls, and found that whereas the prevalence of diabetes mellitus in pancreatic cancer is high, diabetes mellitus prevalence in other common cancers is no different from that in non-cancer controls. Thus, diabetes mellitus is not useful as an early or specific marker of pancreatic cancer.

Controversies also exist between the association of long-standing diabetes mellitus and pancreatic cancer; some, epidemiological studies have excluded the possibility that long-standing diabetes mellitus is a risk factor for pancreatic cancer[2,6,24,32,36,41,44,45,51,52,54,59,60,64,70] whereas others (Table 1) have found a relation-

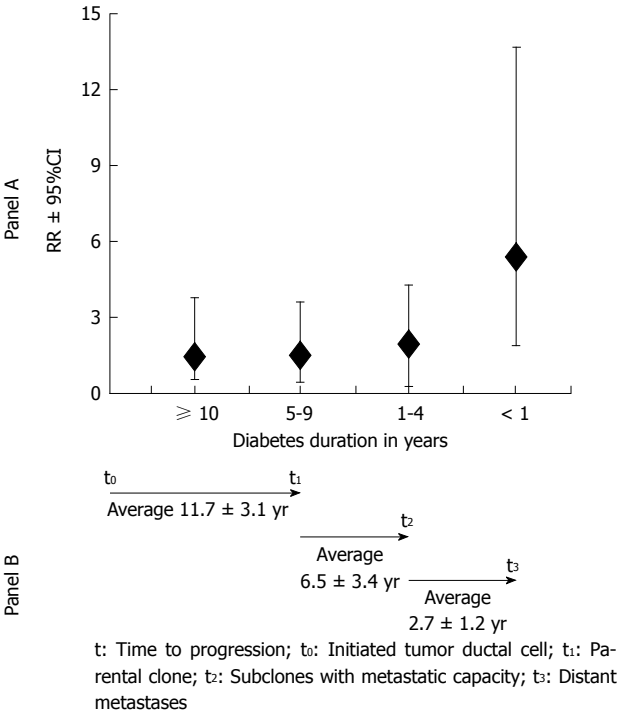

Figure 1 Relative risks and quantitative analysis. Panel A: Relative risks (RR) for the association between diabetes and pancreatic cancer according to the duration of the diabetes (originated from[63]). The risk disappears after 10 years; Panel B: Quantitative analysis of the timing of the genetic evolution of pancreatic cancer indicates that at least a decade is necessary between the occurrence of the initiating mutation and the birth of the parental, non-metastatic founder cell, that at least five more years are required for the acquisition of metastatic ability and patients usually die on an average of 2 years thereafter (originated from[87]).

ship[3-5,7-31,33,35,37-40,42,43,46-49,53,55-58,61,63,65-69,71-75]. It should be pointed out that, in papers showing an association between long standing diabetes and pancreatic cancer there are some biases due to self-reported diabetes which could result in misclassification, heterogeneity among individuals with diabetes in terms of physiologic status, sequelae and treatment which could also confuse this relationship. In addition, Yachida *et al*[87], sequencing the genomes of seven pancreatic cancer metastases to evaluate the clonal relationships among primary and metastatic cancers, found that clonal populations which give rise to distant metastases are represented within the primary carcinoma (but these clones are genetically evolved from the original parental, non-metastatic clone) and they performed a quantitative analysis of the timing of the genetic evolution of pancreatic cancer found at least a decade between the occurrence of the initiating mutation and the birth of the parental, non-metastatic founder cell. At least five more years are required for the acquisition of metastatic ability and patients die an average of 2 years thereafter[87]. Thus, even if diabetes is found a decade before the appearance of pancreatic cancer as reported in meta-analytic studies, we cannot select those patients already having non detectable pancreatic cancer, at least with the imaging and biological techniques available today (Figure 1).

CONCLUSION

We believe that more studies are necessary in order to definitively identify diabetes mellitus as a risk factor for pancreatic cancer taking into consideration that approximately 10 years are needed to diagnose symptomatic pancreatic cancer. At present, the answer to the question posed by Magruder *et al*[83] as to whether diabetes and pancreatic cancer comes first similar to the adage of the chicken and the egg is that diabetes is the egg.

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REVIEW

MicroRNAs may solve the mystery of chronic hepatitis B virus infection

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Abstract

Hepatitis B virus (HBV) infection is a global public health problem that causes persistent liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. A large amount of people die annually from HBV infection. However, the pathogenesises of the HBV-related diseases are ill defined and the therapeutic strategies for the diseases are less than optimum. The recently discovered microRNAs (miRNAs) are tiny noncoding RNAs that regulate gene expression primarily at the post-transcriptional level by binding to mRNAs. miRNAs contribute to a variety of physiological and pathological processes. A number of miRNAs have been found to play a pivotal role in the host-virus interaction including host-HBV interaction. Numerous studies have indicated that HBV infection could change the cellular miRNA expression patterns and different stages of HBV associated disease have displayed distinctive miRNA profiles. Furthermore, the differential expressed miRNAs have been found involved in the progression of HBV-related diseases, for instance some miRNAs are involved in liver tumorigenesis and tumor metastasis. Studies have also shown that the circulating miRNA in serum or plasma might be a very useful biomarker for the diagnosis and prognosis of HBV-related diseases. In addition, miRNA-based therapy strategies have attracted increasing attention, indicating a promising future in the treatment of HBV-related diseases.

Key words: MicroRNA; Hepatitis B virus; Hepatitis B; Host-virus interaction; Biomarker; Therapy

Core tip: The cellular microRNAs (miRNAs) involved in host-hepatitis B virus (HBV) interaction and each stage of HBV-related disease show distinctive miRNA expression profiles at the tissue or serum level indicating that miRNAs have marked potential in detecting or treating of HBV infection.

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INTRODUCTION

The hepatitis B virus (HBV) is a hepadnavirus that causes persistent liver diseases and have a major effect on global public health[1,2]. HBV, discovered in 1966[3], is transmitted among humans by contact with the blood, semen or vaginal fluid of an infected person. Approximately, one third of the world's population have infected HBV, and more than 350 million people have developed chronic HBV infection[4-6]. The severity of HBV-related disease

varies widely, from a self-limited infection to acute hepatitis and from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma[7,8]. The factors affecting the prognosis of HBV infection have not been determined. miRNAs was discovered recently and researchers have determined that it plays a pivotal role in host-virus interactions[9-11]. By using the functions of miRNA, we may explain the mechanism of chronic HBV infection and discover novel biomarkers as well as new therapies for HBV associated diseases.

Since numerous researches discovered that RNA does more than simply serves an intermediary function in "central dogma"[12], the door to a brand new world of RNA had been opened. The genomes of organisms produce two types of RNA, and mRNAs belong to the first type which can be used as translation templates. Besides, genomes manufacture a variety of noncoding RNAs, including the components of the machinery of gene expression and regulatory RNAs[13]. MicroRNAs (miRNAs) are noncoding RNAs, and their mature forms are approximately 22 nucleotides (nt) in length. When these RNAs were initially described in *Caenorhabditis elegans* (*C. elegans*)[14], they were hypothesized to be peculiar to nematodes[12,13]. Subsequent work revealed that miRNAs are common tiny nucleic acid molecules that can be found in plants[15], animals[16] and other organisms[17]. To date, the record of miRNAs has increased significantly. MiRbase 19, released in August of 2012, increased the numbers of recognized hairpin and mature miRNAs to 21264 and 25141, respectively[18-23]. In human, while the expression profiles of some miRNAs in different cells or tissues are similar, other miRNAs may exhibit temporal or tissue-specific patterns[24,25], suggesting that miRNA may be involved in numerous physiological or pathological processes[26].

The biogenesis and action mechanism of these tiny but potential molecules had been detailed described[24,25,27]. Briefly, they are not born so small, in other words, they have some larger progenitors. The processing of the mature miRNA ancestors (primary and precursor miRNAs) is closely related to RNA polymerase Ⅱ (pol Ⅱ), Drosha, the GTP-dependent Ran/Exportin 5 complex, and the Dicer enzyme[24-32]. Generally, by binding to the 3' untraslated regions of their target mRNAs, miRNAs can serve as gene expression regulators, fine-tune the expression primarily at the post-transcriptional level and play critical roles in a variety of physiological and pathological processes, including antiviral defense, developmental timing, cell apoptosis, cell proliferation, tumor generation and so on[24,30,33-39]. One computational prediction indicated that more than 30% of animal genes may be subject to regulation by miRNAs, which emphasizes the importance of miRNA-mediated gene regulation[40,41].

HOST-VIRUS INTERACTION AT THE MIRNA LEVEL

Viruses are generally harmful to human, in order to protect our health, the battle between virus and host break out shortly after infection initiated. In this war, a large amount of reports have indicated that cellular miRNAs serve a key role in protecting the host. However, we may be disappointed at the truth that viruses can use miRNAs as their weapons to fight the host. Remarkably, some features of miRNAs ensure their effectiveness as virally encoded regulators of host and viral gene expression: they are small, lack of immunogenicity and functional flexibility[42]. To facilitate an understanding of the intricacies of host-virus cross-talk mediated by miRNAs, we designed an illustration (Figure 1) base on the review of Scaria *et al*[9] In the interaction between virus and host, miRNAs can be divided into cellular miRNAs and viral miRNAs. To cellular miRNAs, their expression profiles changed at the infected state and the abnormal miRNAs often closely relate to the viral life cycle as well as host disorder. To viral miRNAs, they can evolved to regulate both viral and cellular gene expression[42].

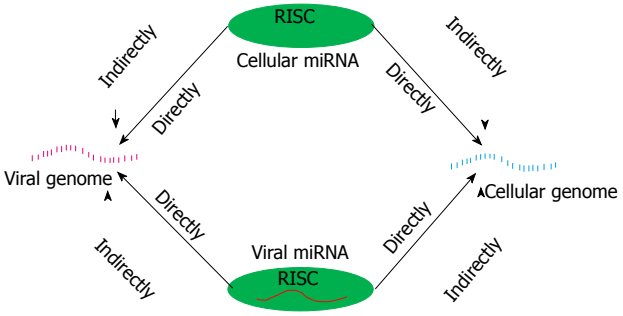

Figure 1 Logical model of host-virus cross talk mediated by microRNAs. Viral microRNAs (miRNAs) and cellular miRNA take part in the host-virus interactions. Moreover, viral and cellular miRNA can influence the expression of viral and cellular genome. RISC: RNA-induced silencing complex.

Cellular miRNAs in host-virus interaction

Studies have noted that miRNA-mediated gene regulation involve in diverse biological processes in the mammalian system, including cellular miRNAs influence viral reproduction and pathogenesis[42,43]. Sometimes, viruses may exploit celluar miRNAs to facilitate certain steps of their life cycle, a living example is hepatitis C virus (HCV) use miR-122, a liver-specific cellular miRNA, to enhance its replication of itself by targeting the viral 5' non-coding region[34,44]. Another study showed that miR-122 knockdown reduced the HCV load in infected chimpanzees[45] and the interferon-mediated down-regulation of miR-122 that contributes to antiviral effects[46]. In contrast, miR-122 serve as an antiviral role in HBV life cycle. For instance, Qiu *et al*[47] found that the miR-122 over-expression inhibited HBV expression, whereas the depletion of endogenous miR-122 resulted in increased production of HBV in transfected cells. Their subsequent study suggested that the miR-122 inhibitor also caused an increase in cellular heme oxygenase-1, which can decrease HBV covalently closed circular DNA (cccDNA) levels both *in vitro* and *in vivo* by reducing the stability of the HBV core protein[48]. A recent study by Wang *et al*[1], indicated that miR-122 expression in the liver was significantly down-

regulated in patients with HBV infection compared with healthy controls. Depletion of endogenous miR-122 and over-expression of miR-122 led to enhanced HBV replication and inhibited viral production, respectively. Cyclin G1 was identified as an miR-122 target that specifically interacted with p53, resulting in the specific binding of p53 to the HBV enhancer elements and simultaneous abrogation of the p53-mediated inhibition of HBV transcription. Ji *et al*[49] found that miR-122 was significantly up-regulated in HBV-infected patients and could inhibit HBV replication in Huh7 and HepG2 cells. Overall, to HCV and HBV, miR-122 can promote and inhibit viral replication respectively. In other words, cellular miRNAs can influence viral lifecycles by accelerative or suppressive mechanisms.

Studies have reported the involvement of cellular miRNAs in numerous host-virus interactions. HIV-1 can use cellular miRNAs to repress the expression of viral proteins and evade the host immune system respose[11,50]. The replication of primate foamy virus can be inhibited by cellular miR-32[43]. miR-24 and miR-93 were responsible for the increased vesicular stomatitis virus replication in variant Dicer1d/d allele mice[51]. The above instances indicate the diversity of miRNA activity and indicate that host-derived miRNAs are essential for the host-virus interactions.

Viral miRNAs in host-virus interaction

A number of the miRNAs that participate in the interaction between host and virus are viral. Pfeffer *et al*[52] initially discovered the existence viral miRNAs in the Epstein-Barr virus (EBV). Analogous to cellular miRNAs, viral miRNAs have multifaceted functions[42], that generally benefit the virus in maintaining its replication, latency and evasion of the host immune system[11]. Barth *et al*[53] showed that miR-BART2 down-regulates the viral DNA polymerase BALF5, inhibiting the transition from latent to lytic viral replication in EBV. Analogously, miR-BART-1p, miR-BART16 and miR-BART17-5p have been found to repress the translation of latency-associated membrane protein LMP-1 mRNA[11,54]. Additional examples of viral miRNAs that regulate viral gene expression are found in HCMV, SV40, MDV, HIV-1 and other viruses[11].

Although numerous miRNA-produced viruses have been identified, the HBV-encoded miRNAs have not been confirmed experimentally but have been suggested by computation[55,56]. This discrepancy may be the result of the limitations of current technology and HBV-derived miRNAs could be found in the future.

EMPHASIZING THE ROLE OF MIRNAS IN HBV INFECTION

A number of cases of host-virus interaction at the miRNA level have been mentioned above. To emphasize the role of miRNAs in HBV infection, we intend to report additional details about the interaction between miRNAs and HBV (Figure 2).

Understanding the mechanisms of miRNAs influence HBV infection requires the knowledge that HBV is a noncytopathic virus that replicates preferentially in the hepatocytes. cccDNA which serves as a template for transcription of all viral RNA is synthetized And after HBV DNA enters the hepatocyte nucleus. The HBV genome is 3.2 kb in length and contains four overlapping open reading frames. It can transcribe viral pregenomic RNA that reverses transcription to synthesize the viral DNA genome and encode the hepatitis B virus surface antigen (HBsAg), hepatitis B virus core protein, viral reverse DNA polymerase (Pol) and X protein. Two enhancers, Ⅰ and Ⅱ, have been shown to function as two master regulators of the four viral promoters[57-59]. Although the viral miRNAs encoded by HBV have not been verified[56], there are cellular miRNAs capable of inhibiting or stimulating HBV viral replication and gene expression. In addition, the products of HBV can alter the miRNA expression profiles.

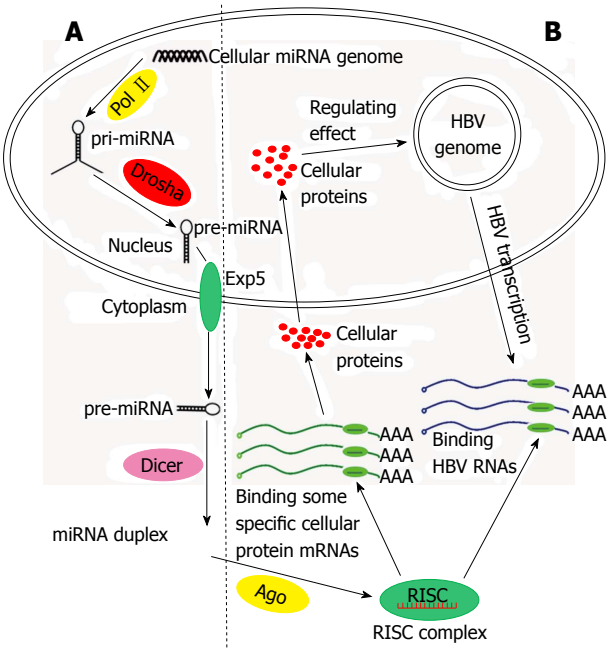

Figure 2 The biogenesis of human cellular microRNAs and the mechanism of the alteration hepatitis B virus gene transcription and replication. For simplicity, not all participators are shown. A: The biogenesis of microRNAs (miRNAs); B: The mechanism of cellular miRNAs regulates hepatitis B virus (*HBV*) gene transcription and replication can be direct and indirect. Cellular miRNAs can target to HBV transcripts (HBV surface antigen mRNA, HBV x mRNA, DNA polymerase mRNA, *etc.*), causing the alteration of HBV expression. Cellular miRNAs can also target to the mRNAs of a number of key regulatory proteins (liver-enriched transcription factors, nuclear receptors, heme-oxygenase-1, DNA methyltransferases, *etc.*) in the process of HBV transcription and replication. Consequently, the amount of these proteins was changed, and the *HBV* gene transcription and replication were altered. RISC: RNA-induced silencing complex. Pol: Polymerase.

Cellular miRNAs targeting to HBV transcripts

A study of Zhang *et al*[60], in attempt to determine whether host-encoded miRNAs affect HBV replication, antisense oligonucleotides of 328 identified human miRNAs were orderly transfected into HepG22.2.15 cells. The expression level of HBsAg, hepatitis B e antigen and

cell proliferation were detected by enzyme-linked immunosorbent assay and methyltestosterone assay. Compared to the experimental controls, miR-199a-3p and miR-210 efficiently reduced the HBsAg expression without affecting HepG2 2.2.15 cell proliferation. Furthermore, they used the bioinformatics method to analyze six miRNAs, and the outcome suggested a putative binding site for miR-199a-3p in the HBsAg coding region and a binding site for miR-210 in the HBV pre-S1 region, respectively. Potenza *et al*[61] used MiRanda to analyze the HBV genome and found seven sites that were potential targets for human liver miRNAs. Their subsequent validation test found that hsa-miR-125a-5p interferes with the HBV translation and down-regulation of the expression of the surface antigen. These findings indicate that cellular miRNAs can alter HBV gene expression by targeting to HBV transcripts.

Cellular miRNA affects HBV replication

Cellular miRNAs can affect viral translation and change viral replication. In addition to the instance of the miR-122 inhibition of HBV replication, there are other cases about host miRNAs altering HBV replication. A study by Hu *et al*[33] suggested that miR-141 suppressed HBV replication by reducing HBV promoter activities through the down-regulation of peroxisome proliferator-activated receptor alpha. DNA hypermethylation might be closely related to the suppression of HBV cccDNA transcription[56], and miR-152 might be a factor involved in the regulation of the methylation of HBV cccDNA[62,63]. Zhang *et al*[64] revealed that cellular miRNAs do not consistently inhibit HBV replication. Collectively, miRNAs can directly or indirectly alter HBV replication.

HBV infection can change the host miRNA expression pattern

A recently study by Wei *et al*[65] showed that the hepatitis B virus x (HBx) protein expression was found to have a significant inverse correlation with miR-101 expression in HBx-expressing HepG2 cells compared to control HepG2 cells. Ren *et al*[66] found that Drosha (a regulator of the biogenesis of miRNAs) mRNA and protein expression were down-regulated in cells expressing the HBV genome, and that the mechanism was related to a reduction in the activity of the Drosha gene promoter. By using RNA interference to knockdown the HBX gene, the expression of Drosha was significantly restored. Their date showed that HBV could inhibit Drosha expression by inhibiting the promoter activity and in turn, leading to an alteration of the host miRNA profiles[66]. These studies suggested that HBV infection can alter the miRNA expression profiles.

MIRNA PROFILES OF HBV-ASSOCIATED DISEASE

The consequences of HBV infection are diverse and can be ranged from asymptomatic chronic infection to cirrhosis and hepatocellular carcinoma. Numerous studies have detected that cellular miRNAs could influence the lifecycle of HBV and HBV could change the miRNA expression profiles, reversely. Taking these factors into consideration, the miRNA profiles may change along with the severity of HBV associated disease. So, we concentrated on the miRNAs expression patterns and their potential role in HBV associated chronic hepatitis, cirrhosis and HCC in the following contents.

miRNA profiles of chronic hepatitis B

The miRNA profiles of chronic hepatitis B (CHB) from numerous studies are controversial and complicated. On the one hand, a series of study indicated that the miRNA expression patterns of CHB are particular at the tissue or serum level[1,67-69]. For instance, a study of Ura *et al*[68] suggested that the miRNA expression profiles in chronic hepatitis B were different from those in the healthy controls and those in HBV-associated HCC, and hepatitis C. To the contrary, appliying massively parallel signature sequencing to conduct an in-depth analysis of the miRNomes in normal human, hepatitis and HCC liver tissues, Hou *et al*[70] found that, except for in HCC, the known miRNAs exhibited a similar distribution in each library based on classification of the transcripts permillion degrees.

miRNA profiles of liver cirrhosis

A well-known trilogy of hepatitis B is that chronic hepatitis B progresses into liver cirrhosis and HCC. An increasing number of studies have focused on the expression patterns of miRNAs during the cirrhotic stage to uncover their function in the progression of hepatitis B and to seek novel therapies for cirrhosis. Roderburg *et al*[71] investigated the role of miRNAs in liver fibrosis by carbon tetrachloride and bile duct ligation models of liver fibrosis. Fibrosis-inducing injuries cause the abnormal expression of many miRNAs. All three members of the miR-29 family were significantly down-regulated under the disposes of these models. To correlate these findings with HBV in human, they measured the miRNA profiles of human liver samples, and found miR-29 family members were down-regulated in the fibrotic/cirrhotic tissues compared with the non-fibrotic tissues. In conclusion, miR-29 family members were down-regulation both in mouse models and in human fibrotic livers. Hepatic stellate cells (HSCs) play a key role in liver fibrosis[72,73]. Roderburg group's further study revealed that miR-29b was down-regulated in HSCs, upon exposure to fibrotic stress. On a cellular level, miR-29 down-regulation in murine HSC cells was mediated by transforming growth factor (TGF)-β as well as inflammatory signaling and nuclear factor κB (NF-κB). Forced expression of miR-29b in murine HSCs can result in the repression of collagen expression[71].

Additional studies report on miRNA regulation in the progression of liver fibrosis. Compared with quiescent HSCs, Lakner *et al*[73] verified that miR-19b was a regu-

lator of TGF-β signaling in activated HSCs, it play an inhibitory effect in HSC-mediated fibrogenesis. Another study suggested that liver fibrosis could cause the down-regulation of miR-150 and miR-194 in HSC, and that their over expressions could repress HSC activation.

miRNA profiling in HBV-related HCC

Chronic hepatitis B is closely relate to HCC. In recent years, numerous studies that focused on the miRNA profiling in HBV-related HCC identified a number of deregulated miRNAs which are critical for the generation of HCC. Gao *et al*[74] isolated miRNAs from formalin fixed paraffin embedded dysplastic nodules (DNs), small HCCs, and their corresponding nontumorous livers. They investigated the expression changes of seven cancerrelated miRNAs, which have been reported to be frequently deregulated in human cancers and might play a role in liver carcinogenesis. They frequently observed the down-regulation of miR-145 and miR-199b as well as the up-regulation of miR-244 in premalignant DNs, moreover these alterations persisted throughout the HCC development. By restoring miR-145 in both HepG2 and Hep3B HCC cells, they found that cell proliferation, cell migration and cell invasion were significantly inhibited. What's more, an anti-miR-145 inhibitor could impair these inhibitory functions of miR-145. This study suggested that miRNA deregulation was an early event and may accumulate throughout the generation of HBV-associated HCC[74]. A study from Hou *et al*[70] identified miR-199a/b-3p which consistently decreased in HCC, and its decrement significantly correlates with poor survival of HCC patients. Huang *et al*[63] suggested that miR-152 was aberrantly expressed and involved in the regulation of the abnormal DNA methylation status in HBV-related HCC.

Interestingly, one miRNA was found to be up-regulated and contribute to enhancing HBV-related HCC metastasis by repressing the expression of fibronectin[75]. Zhang *et al*[75] reported that the levels of miR-143 were significantly increased in p21-HBx transgenic mice and HCC patients with metastatic HBV-HCC. Furthermore, they found that the over-expression of miR-143 was transcribed by NF-κB and facilitates the invasive and metastatic behavior of liver tumor cell. In an athymic nude mouse model, they found that high levels of miR-143 administered by intratumoral administration could remarkably promote HCC metastasis. And they used p21-HBx transgenic mice to show *in vivo* that local liver metastasis and distant lung metastasis were significantly inhibited by blocking miR-143. What's more, fibronectin type Ⅲ domain containing 3B was identified *in vivo* and *in vitro* as the target of miR-143[75].

A NOVEL DIAGNOSTIC BIOMARKER OR PROGNOSTIC PREDICTOR

The expression profiles of miRNAs in different stages of HBV-associated diseases are always inconsistent. Moreover, a portion of miRNAs are closely related to the stage of this liver disease and often play a crucial role in their progression[68,69,71,73-75]. Therefore miRNAs can serve as the role of biomarker in the diagnosis of HBV-related disease (Figure 3). Studies have reported that miRNAs could be stably detected in plasma and serum[76-78]. Chen *et al*[77] demonstrated that miRNAs could be found in the plasma and serum of humans and that their levels in serum were stable, reproducible, and consistent among individuals of the same species. In their study, Solexa was employed to sequence all of the serum miRNAs of healthy Chinese subjects and to identify specific expression patterns of serum miRNAs for lung cancer, colorectal cancer, and diabetes. They validated two non-small cell lung cancer-specific serum miRNAs in an independent trail using quantitative reverse transcription polymerase chain reaction (qRT-PCR) assays. These results showed the existence of human serum miRNAs and suggested that these miRNAs contain fingerprints for diverse diseases[77]. Hence, assaying miRNA profiles could become a novel approach for detecting HBV-related diseases.

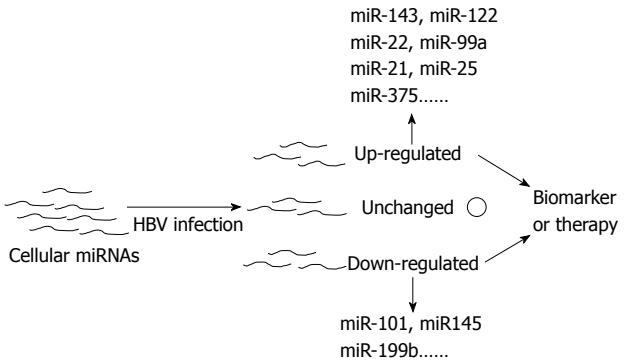

Figure 3 The aberrant expressed microRNAs and their potential use in hepatitis B virus infection. Hepatitis B virus (HBV) infection can alter the expression profiles of cellular microRNAs (miRNAs). Except the unchanged miRNAs, up-regulated (such as miR-143, miR-122, miR-22 *etc.*) and down-regulated miRNAs (miR-101, miR145, miR-199b *etc.*) are promising for detecting and treating HBV-related diseases.

More powerful biomarkers are needed to compensate for the defects of the existing diagnostic means for detecting HBV-related liver injury and HCC[69,79-83]. In blood samples, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are the most widely used enzymatic indicators for liver damage[84]. But enhanced ALT and AST activities have been detected in some other clinical disorders[81,82]. In clinical practice, these two markers are not always consistent with histomorphological alterations[80]. One of the reasons for the high mortality in HBV-related HCC is that the tumors are frequently identified after metastasis at a stage in which curative resection is no longer feasible[69]. We rely on radiology imaging methods such as ultrasonography, computed tomography, and magnetic resonance imaging to find a liver mass to diagnose HCC. These methods can not diagnose small lesions accurately[69]. The most commonly used serum HCC markers, α-fetoprotein (AFP), has insufficient sensitivity and specificity[69,85,86].

Collectively, there is an urgent need for novel strate-

gies in the detection of HBV-related disease, and miRNAs could become a novel and powerful biomarker.

miRNAs can be used to detect liver injury and HBV infection

Several recent reports suggested that miRNAs could be used as an indicator of liver injury and HBV infection[49,67,84,87]. Zhang *et al*[84] selected and validated miRNA biomarkers using an extensive set of plasma samples from patients with HBV infection, patients with skeletal, and healthy controls. Combining these experimental results with their further investigation in liver injury mouse models, these authors reported that the plasma miR-122 concentration presented a disease severity-dependent change in the patients and mouse models that earlier than the alteration in aminotransferase activity. Their findings suggested that miR-122 had potential as a blood marker for liver injury including HBV associated injury[84]. Waidmann *et al*[67] investigated the relationship between miR-122 and HBV infection, suggested that the serum levels of miR-122 can discriminate between HBV infected patients and healthy controls. Ji *et al*[49] found that the numbers of circulating miRNAs increased with the symptom severity of HBV infected patients and that the expression of miR-122 was significantly up-regulated in these patients.

miRNAs other than miR-122 had been reported to get the ability of indicating HBV infection. Hayes *et al*[87] found a number of disease-specific serum miRNAs of HBV infection, including miR-122, miR-22, and miR-99a which were up-regulated at least 1.5-fold in the serum of HBV-infected patients.

miRNAs may become the diagnostic and prognostic marker of HBV-positive HCC or HCC

Early diagnosis of HCC plays a vital role in reducing mortality, but the existing strategies are not effective. A number of miRNAs had been found to have the potential to become the diagnostic and prognostic markers of HCC[69,88-94].

Tomimaru *et al*[69] measured the plasma miR-21 levels of different subjects including HCC patients and chronic hepatitis patients. In their study, plasma miR-21 was significantly reduced after a curative resection in HCC, and the level in the HCC subjects was significantly higher than the levels in the patients with chronic hepatitis and healthy controls. These authors found that miR-21 could differentiate HCC from healthy controls with high sensitivity and specificity. In theory, miR-21 was superior to AFP in the diagnosis of HCC. In a study with several phases, Qi *et al*[93] found that the serum miR-122 level was significantly higher in HCC patients compared to healthy controls and post-operative subjects. Their findings indicate that miR-122 might serve as a novel biomarker for the detection of HCC in healthy subjects but is not useful for the detection of HCC in patients with chronic HBV infections[93].

Li *et al*[92], employed Solexa sequencing to screen and qRT-PCR to validate miRNAs in serum samples. Thirteen miRNAs were found that could accurately distinguish not only HBV cases from healthy and HCV individuals, but also HBV-postive HCC subjects from healthy and HBV subjects. Additionally, in a comparison of miRNA expression in the serum of HCC subjects and healthy controls, six miRNAs were found to be significantly elevated in the samples from HCC. Three miRNAs (miR-25, miR-375, and let-7f) can be used to separate HCC cases from healthy controls. In the prediction of HCC, miR-375 had an ROC of 0.96 (specificity: 96%; sensitivity: 100%).

Although the outcomes of these studies are not uniform, the data have shown that miRNAs are promising for detecting HCC or HBV-positive HCC. A number of reports have indicated that the expression of miRNAs could anticipate the prognosis of HCC[89,91]. Using Kaplan-Meier estimates and the log-rank test, Li *et al*[91] showed that high expression of has-miR-125b was related to good survival and a subsequent transfection assay showed that forced expression of miR-125b in the HCC cell line could perceptibly repress the cell growth and phosphorylation of Akt. Budhu *et al*[89] created a unique 20-miRNA metastasis signature that could significantly predict HCC tissues with venous metastases from metastasis-free solitary tumors with a 10-fold cross-validation. In the corresponding noncancerous liver tissues they could not identify significant miRNAs. A survival risk prediction analysis revealed that the majority of metastasis-related miRNAs were related to survival. Their additional validation experiments revealed that the 20-miRNA tumor signature could serve as a survival and relapse predictor of HCC[89].

Although miRNAs have significant potential, a number of problems remain. Too many miRNAs have been identified to be practically applied for routine clinical use, and the accuracy of the miRNA signatures has not been adequately evaluated[95]. These factors may result in inaccuracy or incorrect diagnosis and prediction outcomes.

EMPLOYING MIRNAS OR ANTAGOMIR IN HBV THERAPEUTIC

The closely relationship between miRNAs and HBV-related diseases offers an opportunity to use miRNAs or antagomir in the treatment of these diseases (Figure 3). The feasibility of this method has been demonstrated[96-99]. Grimm *et al*[100] showed that anti-HBV shRNAs might cause serious toxicity *in vivo*. Although a miRNA-based strategy is promising, its therapeutic application must be dependent on rigorously demonstrated safety, efficient delivery to target tissues and optimization shRNAs dosing and sequencing[100,101]. To obtain an optimal solution for a miRNA-based strategy, Keck *et al*[102] produced improved HBV RNAi triggers, Ely *et al*[103] designed pri-miRNA expression cassettes and linear DNA sequences that expressed antiviral micro-RNA shuttles[104], and Xiangji *et al*[105] developed a lentiviral miRNA-based sys-

tem. Improved miRNA-based therapeutic methods could successfully inhibit HBV replication or expression. A promising miRNA-based HBV therapy method has not been well established but could be designed successfully in the future.

CONCLUSION

In this review, we limited our focus to the role of miRNAs in host-virus interactions, especially in host-HBV interactions. HBV infection is a global issue, but the pathogenesises and therapies of HBV-related diseases are not well defined. In the years since miRNA was discovered in *C. elegans* and subsequent studies revealed that miRNA are involved in many physiological and pathological processes in humans, scientists have observed that miRNAs played a key role in viral diseases and could serve a guardian or aggressor role. Regarding to HBV infection, cellular miRNAs were found to influence HBV translation and replication and HBV was found to change expression profiles of cellular miRNA. This finding led to the possibilities of miRNAs serving as biomarkers and of miRNAs or antagomirs serving as therapeutic tools in HBV-related diseases (Figure 3). Studies have indicated that the blood or tissue samples from the different stages of HBV-related disease presented distinctive miRNA expression patterns and that miRNA-based therapy is feasible.

Although many experimental studies have confirmed the capacity of miRNAs or antagomirs to detect or treat HBV-related diseases, adequate evaluation of their accuracy, efficacy, and cost-effectiveness is required. Further research into the relationship between miRNAs and chronic HBV infection may increase the understanding of hepatitis B virus infection and miRNAs could become accurate biomarkers and powerful therapy tools.

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ORIGINAL ARTICLE

Thiopurines related malignancies in inflammatory bowel disease: Local experience in Granada, Spain

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Abstract

AIM: To investigate the incidence of neoplasms in inflammatory bowel disease (IBD) patients and the potential causative role of thiopurines.

METHODS: We performed an observational descriptive study comparing the incidence of malignancies in IBD patients treated with thiopurines and patients not treated with these drugs. We included 812 patients which were divided in two groups depending on whether they have received thiopurines or not. We have studied basal characteristics of both groups (age when the disease was diagnosed, sex, type of IBD, *etc.*) and treatments received (Azathioprine, mercaptopurine, infliximab, adalimumab or other immunomodulators), as well as neoplasms incidence. Univariate analysis was performed with the student *t* test, χ^2 test or Wilcoxon exact test as appropriate. A logistic regression analysis was performed as multivariate analysis. Statistical significance was establish at *P* values of less than 0.05, and 95%CI were used for the odds ratios.

RESULTS: Among 812 patients included, 429 (52.83%) have received thiopurines: 79.5% azathioprine, 14% mercaptopurine and 6.5% both drugs. 44.76% of patients treated with thiopurines and 46, 48% of patients who did not receive this treatment were women ($P > 0.05$). The proportion of ulcerative colitis patients treated with thiopurines was 30.3% compare to 66. 67% of patients not treated ($P < 0.001$). Mean azathioprine dose was 123.79 ± 36.5 mg/d (range: 50-250 mg/d), mean usage time was 72.16 ± 55.7 mo (range: 1-300 mo) and the accumulated dose along this time was 274.32 ± 233.5 g (1.5-1350 g). With respect to mercaptopurine, mean dose was 74.7 ± 23.9 mg/d (range: 25-150 mg/d), mean usage time of 23.37 ± 27.6 mo (range: 1-118 mo), and the accumulated dose along this time was 52.2 ± 63.5 g (range: 1.5-243 g). Thiopurine *S*-methyltransferase activity was tested in 66% of patients treated with thiopurines, among which 98.2% had an intermediate or high activity. Among the patients treated with thiopurines, 27.27% (112 patients) and 11.66% (50 patients) received treatment with Infliximab and Adalimumab respectively, but only 1.83% (7 patients) and 0.78% (3 patients) received these drugs in the group of patients who did not received thiopurines ($P < 0.001$ and $P < 0.001$ respectively). Finally, 6.8% (29 patients) among those treated with thiopurines have received other immunesupresants (Methotrexate, Tacrolimus, Cyclosporin), compare to 1% (4 patients) of patients not treated with thiopurines ($P < 0.001$). Among patients treated with thiopurines, 3.97% developed a malignancy, and among those not treated neoplasms presented in 8.1% ($P = 0.013$). The most frequent neoplasms were colorectal ones (12

cases in patients not treated with thiopurines but none in treated, $P < 0.001$) followed by non-melanoma skin cancer (8 patients in treated with thiopurines and 6 in not treated, $P > 0.05$).

CONCLUSION: In our experience, thiopurine therapy did not increase malignancies development in IBD patients, and was an efective and safe treatment for these diseases.

Key words: Malignancy; Neoplasm; Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Thiopurines; Azathioprine; Mercaptopurine

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic disorder which is characterised by episodes of inflammatory activity alternated with episodes of remission of this inflammation. Ethiology is mostly unknown, and there is no curative treatment, being the goal of current therapies to maintain patients in remission.

Among the drugs used for the treatment of this disease there are corticoids, immunomodulators (azathioprine, mercaptopurine, cyclosporine or tacrolimus) and biological therapies (infliximab and adalimumab) which interfere in the altered inflammatory and immunological process of these patients in order to induce and maintain clinical remission. Given the usefulness of these drugs for treating this disease, their relative safety with regards to side effects and the tendency to be more strict in symptoms control (top-down *vs* set-up tendency), immunomodulators and biological therapies have become drugs extensively used in this field. However, these treatments imply new challenges, as their hypothetical ability to induce neoplastic diseases, determined by their interference with the immune response which could limit its ability to control dysplastic cells, favouring the development of tumours. Indeed, Azathioprine has been classified as carcinogenic by the International Agency for Research on Cancer. Regarding this relationship between thiopurines and neoplastic diseases, several studies dealing with this adverse effect[1] have been published.

Thiopurines are drugs known to be antiproliferative given that their main effect is that they prevent the proliferation of T lymphocytes, promoting the incorporation of thiopurine analogues into DNA, which prevents the synthesis of purine nucleotides[2], as well as blocking several enzymes involved in the synthesis of DNA, RNA and proteins. All of those mechanisms block the proliferation and functions of the lymphocytes, inhibiting the synthesis of antibodies, and reducing the number of circulating monocytes and granulocytes, which is more evident in tissues and situations with a high cellular turnover.

The presence of thiopurine analogues in the DNA not only alters its structure by preventing cellular proliferation, but also increases the risk of mutagenesis[3-6]. This theoretic possibility becomes more evident in recent studies which describe an increased number of somatic mutations in circulating leukocytes in patients treated with thiopurines in comparison to patients who are not treated[7], more so, the number of mutations is proportional to the dose and duration of the treatment with thiopurines.

Also, this structurally altered DNA becomes more sensitive to radiation, especially ultraviolet radiation (UVA), which creates reactive oxygen species with the potential to modify genetic material and nearby proteins[2,8-11]. Hypothetically, those events determine an increased susceptibility neoplasms. For instance, we find the highest number of non-melanoma skin cancer cases in patients with thiopurines prescribed as immunosuppressants in organ transplant[12]. Other studies have suggested a relation between the development of malignancies and the total thiopurine doses, its metabolite levels and thiopurine *S*-methyltransferase (TPMT) mutations[13-17].

Therefore, with the aim of review the incidence of thiopurine induced neoplasms in our IBD patients and treated with thiopurines at the University Hospital Virgen de las Nieves (Granada, Spain), we designed a retrospective observational study, comparing our results with previously published papers.

MATERIALS AND METHODS

An observational, retrospective study, analysing data from patients with a confirmed IBD diagnosis from 1996 to the present was designed. Data were collected from the electronic clinical charts of each patient, as well as from our local database. We included demographical variables and specific data such as TPMT activity, use of thiopurines (azathioprine and mercaptopurine), as well as other immunosuppressants and biological agents. We also studied the appearance of neoplastic diseases, age of diagnosis and type of disease.

Statistical analysis

Resulting variables were analysed by means of SPSS 18 software (Chicago, IL, United States). Univariate analysis was performed with the χ^2 test, Fisher exact test, Student *t* test or Wilcoxon as appropriate, we used 95%CI for de odds ratios and statistical signification was considered when $P < 0.05$.

Multivariable logistic-regression analyses involving treatment regimen and prespecified baseline characteristics, which were the ones with statistical significance in

Table 1 Patients' characteristics *n* (%)

	Thiopurines (*n* = 429)	No thiopurines (*n* = 383)	*P* value
Age of diagnosis of IBD (yr)	30.85	40.13	< 0.001
Sex			
Female	192	178	> 0.050
Male	237	205	> 0.050
Ulcerative colitis[1]	*n* = 130	*n* = 263	< 0.001
Extension			
E1	5 (3.85)	51 (19.4)	
E2	42 (32.3)	103 (39.2)	
E3	83 (63.85)	109 (41.4)	
Severity			
S1	51 (39.2)	150 (57)	
S2	58 (44.6)	102 (38.8)	
S3	21 (16.2)	11 (4.2)	
Crohn's disease[2]	*n* = 299	*n* = 120	< 0.001
Age of diagnosis (yr)			
A1	33 (11)	6 (5)	
A2	214 (71.6)	68 (56.7)	
A3	52 (17.4)	46 (38.3)	
Location			
L1	137 (45.8)	46 (38.3)	
L2	54 (18)	34 (28.3)	
L3	96 (32.1)	39 (32.5)	
L4	12 (4.1)	1 (0.8)	
Behaviour			
B1	155 (51.8)	105 (87.5)	
B2	55 (18.4)	10 (8.3)	
B3	89 (29.8)	5 (4.2)	
P	73 (24.4)	15 (12.5)	
Neoplasms	*n* = 17	*n* = 31	0.013

[1]Patients with ulcerative colitis treated with thiopurines had more severity and disease extension; [2]Patients with Chron's disease treated with thiopurines more usually had ileal or ileocholic location and an inflammatory or penetrating behaviour; Patients under thiopurine treatment had a lower age at diagnosis. IBD: Inflammatory bowel disease.

Table 2 Montreal classification of inflammatory bowel disease

IBD	Montreal classification
Crohn disease	
Age of diagnosis	A1 below 16 yr
	A2 between 17 and 40 yr
	A3 above 40 yr
Location	L1 ileal
	L2 colonic
	L3 ileocolonic
	L4 isolated upper disease
Behaviour	B1 non-stricturing, non-penetrating
	B2 stricturing
	B3 penetrating
	P perianal disease
Ulcerative colitis	
Extent	E1
	Ulcerative proctitis (distal to the rectosigmoid junction)
	E2
	Left sided UC (distal to the splenic flexure)
	E3
	Extensive UC (proximal to the splenic flexure)
Severity	S0
	Clinical remission (asymptomatic)
	S1
	Mild UC: Passage of four or fewer stools/d with or without blood, absence of any systemic illness, and normal inflammatory markers
	S2
	Moderate UC: Passage of more than four stools per day but with minimal signs of systemic toxicity
	S3
	Severe UC: Passage of at least six bloody stools daily, pulse rate of at least 90 beats/min, temperature of at least 37.5 ℃, haemoglobin of less than 10.5 g/100 mL, and ESR of at least 30 mm/h

IBD: Inflammatory bowel disease; ESR: Erythrocyte sedimentation rate; UC: Ulcerative colitis.

univariate analyses and also the factors with a potential or already recognized influence on neoplasm development, were performed to evaluate the risk of malignancies. A stepwise procedure was used to identify independent risk factors for neoplasm development (with $P = 0.05$ as the threshold level for variables to be entered into the model and retained in the final model).

An online pubmed search was performed with the terms "thiopurines", "azathiopurine", "mercaptopurine", "neoplasm", "malignancy", "inflammatory bowel disease", "ulcerous colitis (UC)" and "Crohn's disease (CD)", in order to review published data on thiopurines, neoplasms and IBD.

RESULTS

Eight hundred and twelve patients with confirmed IBD diagnosis were included; 48.4% had UC and 45.6% were women (Table 1). The average age of diagnosis was 35.23 ± 16.5 years (range: 5-85 years), 34.99 ± 16.7 years for females (range: 5-85 years) and 35.43 ± 16.4 years (range: 5-82 years) for males.

Among CD patients (Table 1), 67.3% had been diagnosed between 16 to 40 years (A2 in Montreal classification), the most frequent location (43.7%) was ileal (L3 of Montreal classification) (Table 2)[18] and the most usual pattern (62.1%) was inflammatory (B1 of Montreal). In UC (Table 1), 48.9% had pancolitis (E3 in Montreal classification) and 51.1% presented as a mild inflammatory bout (S1 of Montreal).

With regards to the treatments used (Table 3), more than a half of the patients (52.8%) have been treated with thiopurines (mostly with azathiopurine) at some point during their illness; other treatments such as biological ones and immunosuppressants (methotrexate, cyclosporine, tacrolimus) have been used less frequently (25.2%). In general, the treatment with thiopurines was more frequently used in CD patients.

The average daily dose of azathiopurine was 123.79 ± 36.5 mg (range: 50-250 mg), with an average usage time 72.16 ± 55.7 mo (range: 1-300 mo), and the accumulated dose for this time was 274.32 ± 233.5 g (range: 1.5-1350 g). With regards to mercaptopurine, the average daily dose was 74.7 ± 23.9 mg (range: 25-150 mg), with an average time under this thiopurine treatment of 23.37 ± 27.6 mo (range: 1-118 mo), and a total accumulated dose of 52.2 ± 63.5 g (range: 1.5-243 g). TPMT activity (tested

Table 3 Treatments *n* (%)

	Thiopurines (*n* = 429)	No thiopurines (*n* = 383)	*P* value
TPMT activity	283 (66)	-	
Low	5 (1.8)	-	
Intermediate	43 (15.2)	-	
High	235 (83)	-	
Azathioprine	341 (79.5)	-	
Mercaptopurine	60 (14)	-	
Azathioprine + Mercaptopurine	28 (6.5)	-	
Infliximab	112 (26.1)	7 (1.8)	< 0.001
Adalimub	50 (11.7)	3 (0.8)	< 0.001
Other immunosuppressants	29 (6.8)	4 (1)	< 0.001

Patients (98.2%) treated with thiopurines have intermediate or high activity. Most of the patients treated with thiopurines take azathioprine. TPMT: Thiopurine S-methyltransferase.

by high-performance liquid chromatography: low < 5 IU/mL, intermediate 5-20 IU/mL, high > 20 IU/mL) has been tested, before using thiopurines, for 66% of the patients treated with these drugs, of which 98.2% had intermediate or high activity. The average usage time for infliximab was 29.09 ± 25.8 mo (range: 1-112 mo) and for adalimumab 25.4 ± 20.4 mo (range: 2-79 mo).

Fifty-one point nine percent of the women received thiopurines, as well as 71.4% of patients with CD and 33.1% of the patients with UC $P < 0.001$ (OR = 5.04, 95%CI: 3.74-6.79).

We found a total of 74 neoplasms in 67 patients (7 patients had 2 neoplasms), 21 of those neoplasms appeared before the diagnosis of IBD, and so they were excluded from the analysis, leaving 53 neoplasms which appeared in 48 patients (5 of which had 2 neoplasms) (Figure 1). Of these neoplasms, 37 (69.8%) appeared after the IBD diagnosis but before the use of thiopurines, while 16 (30.2%) were identified after the treatment with thiopurines had started.

Mean time from IBD diagnosis to malignancy finding was 117 ± 91.8 mo (110.96 ± 99.6 mo in patients with no thiopurine treatment, and 129.21 ± 75.7 mo in treated patients, $P > 0.05$), with a mean time from IBD diagnosis to tiopurine treatment of 49.45 ± 57.05 mo. Mean time from thiopurine initiation to malignancy diagnosis was 67.05 ± 53.7 mo. Mean follow up time was 146.25 ± 91.2 mo, 151.56 ± 98 mo in patients who did not received thiopurines and 141.35 ± 84 mo in patients who did ($P > 0.05$).

The most frequent neoplasms were non-melanoma skin cancer, colorectal cancer and haematological (lymphomas and leukaemia). In general, the neoplasms were less frequent in the group of patients treated with thiopurines except for non-melanoma skin cancer, lymphoma and prostate cancer; however, these differences reached statistical significance only in the cases of breast and colorectal cancer, which had been more frequent in patients not treated with thiopurines (Figure 2).

In the univariate analysis, we identified a higher incidence of neoplasms among patients not treated thiopurines (8.1% *vs* 4%) ($P = 0.013$, OR = 0.469, 95%CI: 0.255-0.861). However, when considering the two types of thiopurines separately [azathioprine (AZA) or 6-mercaptopurine (6-MP)] we did not find these differences: in AZA group, 4.3% of those treated with it developed a neoplasm in comparison to 7.2% of those not treated with AZA ($P > 0.05$); in the 6-MP group, 2.3% of those treated with it developed a neoplasm, in comparison to 6.4% of those not treated with 6-MP ($P > 0.05$). Moreover, we did not observe differences in the incidence of neoplasms in relation to other treatments (infliximab, adalimumab or other non-thiopurine immunosuppressants).

With regard to sex, 4.3% of the women have developed a neoplasm in comparison to 7.2% of the men ($P > 0.05$); however, we observed a higher incidence of neoplasms among women not taking thiopurines compared to the ones treated with this drugs; $P = 0.039$ (OR = 0.294, 95%CI: 0.093-0.930), these differences are not seen among men ($P > 0.05$).

With respect to the type of IBD, we have identified more neoplasms among patients with UC (7.9%) than

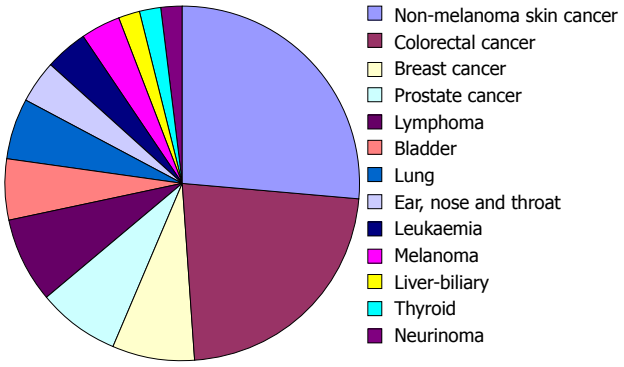

Figure 1 Types of neoplasms (*n* = 53). The most frequent neoplasms were non-melanoma skin cancer, colorectal cancer and haematological (lymphomas and leukaemia).

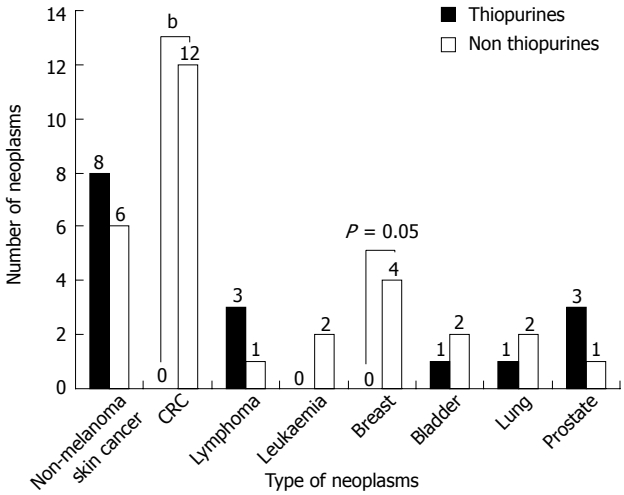

Figure 2 All the neoplasms, except for non-melanoma skin cancer, lymphomas and prostate, have been more common in patients not treated with thiopurines, however, significant differences have only been identified in colorectal and breast cancer. Non thiopurines *vs* thiopurines, [b]*P* value < 0.01 (OR = 0.96, 95%CI: 0.94-0.98); *P* value = 0.05 (OR = 0.99, 95%CI: 0.98-1).

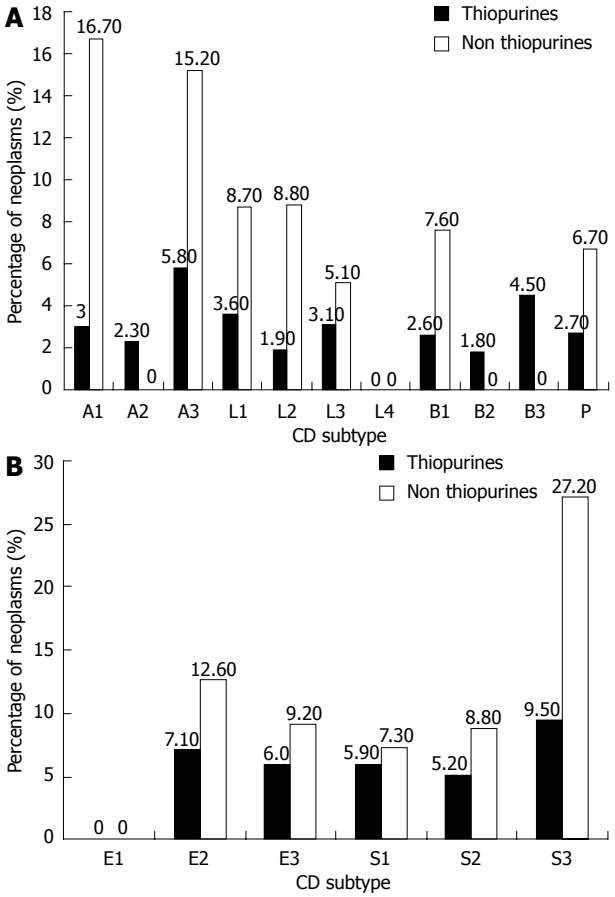

Figure 3 There seem to be no statistically significant differences in the appearance of neoplasms in any of the locations, behaviours or ages of diagnosis of the patients with Crohn's disease and ulcerative colitis in relation to thiopurines intake. A: Percentage of neoplasms according to Crohn's disease (CD) subtype; B: Percentage of neoplasms according to ulcerative colitis (UC) subtype.

among patients with CD (4.1%), $P = 0.021$ (OR = 0.494, 95%CI: 0.269-0.907). However, when we studied the emergence of neoplasms in relation to the treatment with thiopurines in each type of IBD (UC and CD), we found no differences in any of the groups ($P > 0.05$).

In patients with CD, after stratifying patients by age of diagnosis, location and disease pattern, we noted a higher number of neoplasms among patients not treated with thiopurines in all groups, without reaching statistical significance (Figure 3A). This also occurred in patients with ulcerative colitis when stratifying them regarding the extension of the disease and its severity (Figure 3B).

In the logistic regression analysis, we only identified the age of diagnosis of IBD as a risk factor ($P = 0.03$, OR = 1.34, 95%CI: 1.003-1.066).

DISCUSSION

For years, the aim of treating IBD has been to control the patient's symptoms, but as we have access to new treatments for this disease, the goal is becoming more ambitious, even trying to obtain mucosal healing. This is the reason for international guidelines to propose the use of stronger treatments in earlier stages and, although with this form of treatment we improve our control of the disease and its complications, we also increase the number of patients with treatments with potentially significant side effects (immunosuppressants, biological therapies, *etc.*). One of these undesirable effects is the development of a neoplastic disease which could be increased by the use of immunosuppressants and immunomodulators, given that these drugs block the natural containment mechanisms of neoplastic diseases as has been previously described in solid organ transplantation receptors.

On the other hand, in IBD as a chronic inflammatory disorder, there is a higher risk of neoplasms (it is known that there is a higher risk of colorectal cancer especially in more severe a long-lasting forms of the condition) and although the use of immunosuppressants such as thiopurines may reduce this risk by controlling the underlying inflammatory process, it is hard to establish whether the increased risk of malignancies in patients with IBD treated with thiopurines is due to the disease itself or to the treatment. Aiming to clarify this matter, there is an increasing number of publications suggesting a causative relationship between neoplasms affecting IBD patients and the treatment provided[1].

In our study, the incidence of neoplasms has been double among patients not treated with thiopurines despite having used them for a prolonged period of time (an average of 72.16 mo for AZA and 23.37 mo for MP) and with high doses (average accumulated dose of 274.3 g of AZA and 52.2 g of MP), which could be due to an improved control of the inflammatory process preventing the appearance of dysplasia and its subsequent progression to neoplasm.

The most frequent neoplasms have been skin ones, excluding melanoma, of which we have identified 14 cases (8 in the thiopurines group and 6 in the non-thiopurines group, $P > 0.05$). Although there are few studies published, the risk of developing this type of neoplasm is estimated to be higher in patients who are treated with thiopurines, especially in relation to the duration of the treatment and the accumulated dose of thiopurines. In the Long *et al*[19] study published in 2010 a relation was established between the risk of developing skin cancer in 50000 patients with IBD, suggesting that there is an increased risk of non-melanoma skin cancer in patients with IBD (RR = 1.64, 95%CI: 1.51-1.78) and the use of thiopurines increases this risk in direct relation to the duration of the treatment (OR = 3.56, 95%CI: 2.81-4.5 at 90 d; OR = 4.27, 95%CI: 3.08-5.92 at 1 year). The risk with other biological treatments was also increased (OR = 2.18, 95%CI: 1.07-4.46). In another study[20] in which 9618 patients with IBD were enrolled, the risk of developing non-melanoma skin cancer was also higher among those who were treated with thiopurines. Furthermore, in a recent paper[21] including 1084 patients, the risk of developing non-melanoma skin cancer was estimated to be 5 times higher among those who were treated with thiopurines, especially in Caucasian patients (12 times

higher). On the other hand, nearly all the studies refer to exposure to sunlight and UVA as a risk factor for this type of neoplasm, recommending to patients treated with thiopurines avoiding this exposure. Indeed, in our study, even though the absolute number of patients affected with this type of cancer is higher in the group of patients treated with thiopurines, there were no significant differences, which could be due to the low number of cases. Despite these data the risk/benefit balance is in favor of using thiopurines, given the low aggressiveness of these neoplasms. However, these patients must be advised to use sunscreen and avoid prolonged exposure to sunlight.

In our study, the second most common neoplasm was colorectal cancer, which was only identified in the group of patients which were not treated with thiopurines (and had not been treated with biological drugs either), of which most are men with UC. It is a well-known fact that patients with uncontrolled IBD have a higher risk of developing this type of neoplasm, especially in extensive UC with a long-term course. It is known that the use of treatments such as 5-aminosalicylates which help to control the inflammatory activity of the IBD reduce the incidence of colorectal cancer[22-26]. The effect of the thiopurines is hard to establish given that many studies refer to a neutral risk[27-31], however, in the 2009 study by Beaugerie *et al*[32] with 19438 patients included, they observed a seemingly protective effect derived of the use of thiopurines, an effect which has also been seen in another study published in the same year by Andrews *et al*[33]. Our results show a lower risk of colorectal cancer in patients with IBD treated with thiopurines ($P < 0.001$, OR = 0.961, 95%CI: 0.942-0.981), probably due to an improved control of the disease and a reduction of the sustained colonic inflammation. The fact that in our study colorectal cancer is more frequent in patients with UC, may be due to the fact that in these patients the inflammation of the colon increases its risk as well as the fact that CD patients received thiopurines more frequently, which would have played a protective role in relation to colorectal cancer by improving control over the inflammatory process.

Among the 812 patients, 5 lymphomas have been identified, of which 1 was diagnosed prior to the CD diagnosis, thus, it has been excluded from the analysis. Of the 4 remaining, there has been 1 case identified among the patients not treated with thiopurines and 3 among those treated with thiopurines, without significant differences. There are contradictory data regarding the appearance of lymphomas in patients with IBD among different studies[34-38]. In the paper by Beaugerie *et al*[39] published in Lancet in 2009, with 19486 patients, an increased risk of developing intestinal lymphoma was identified in patients with IBD, which suggests that this disease *per se* entails a higher risk of developing lymphoma. The authors observed an increased risk of lymphoma among the patients on thiopurines therapy (HR = 5.28, 95%CI: 2.01-13.9, $P = 0.0007$) which decreased to a similar risk in patients never treated with these drugs when they were suspended; however, in a subgroup of patients who stopped taking thiopurines, inflammatory activity was greater and the risk of lymphoma was lower, concluding that the risk was associated to thiopurines and not to inflammatory activity. In other studies[40], there are even cases describing lymphoma regression in CD after suspending treatment with Azathioprine. In a 2005 meta-analysis[41] there was a RR of 4 in the patients with IBD who received thiopurines in relation to those who did not take them. However, the absolute risk of lymphoma continues to be low and is estimated in 300-1400 cases-year of treatment, although the subgroup of elderly patients with IBD who would comprise a higher risk of this malignancy.

Moreover, there is a clear relationship, already known from other studies[42-46], between the Epstein-Barr virus (EBV) infection and the development of lymphoma. In our series only one patient had a positive serology to EBV.

T cells hepatosplenic lymphoma is a highly aggressive variant of lymphoma, described in patients who were co-treated with thiopurines and infliximab[47-50], although there are other published cases of patients treated with TNF inhibitor monotherapy[51-53] or azathioprine alone[54-57], from which we deduce that the increased risk of developing this special form of lymphoma may be due to the IBD itself, its treatment (monotherapy or combined treatment) or a combination of both. For thiopurines, the cases published occur after 1-2 years of treatment. In our study we have not observed any case of this nature due to its low incidence.

Another malignant haematological disorder frequently associated with IBD and thiopurines is acute myeloid leukaemia (AML). We have identified two cases of AML, both of which have been in patients not treated with thiopurines (neither have they been treated with Infliximab or Adalimumab) which entails a 0.5%. In the study by Caspi *et al*[58], the risk of presenting this entity for patients with IBD has been established at 5.3 times higher than in the general population, and it has been mainly related to the dose and the duration of the treatment[59,60], increasing when this exceeds 5 years or 600 g of accumulated AZA doses. On the other hand, in other studies there is a subgroup of patients in which the accumulated dose is low and the risk of AML continues to be high, this leads us to think of an increased susceptibility to this drug within a subgroup of patients, which could be explained by the TPMT activity, as was clarified in the paper by Relling *et al*[61] and Bo *et al*[62] in which the low TPMT activity was related to the AML risk and secondary myelodysplastic syndromes.

Therefore, despite the existence of contradictory data, there is a higher risk of malignant haematological disorders in patients with IBD and this risk would be higher, although only slightly, in patients treated under thiopurines therapy (depending on the total accumulated doses, duration of the treatment, TPMT activity and the aggressiveness of the treatment measured through metabolites or toxicity such as leucopenia); however, this

risk is overcome by the benefit of its use. In our study we observed no significant differences in these neoplasms incidence. However, we observed all the cases of leukemia neither in patients treated with thiopurines nor with TNF inhibitors, and most of the lymphomas appeared in patients treated with thiopurines. These differences with regard to the published data could be explained by to the low number of cases observed and the high TPMT activity found. Although some studies establish a relationship between the risk of developing these neoplasms with the levels of active thiopurines metabolites, this measurement is unavailable in the University Hospital Virgen de las Nieves, (Granada-Spain), thus, we are unable to provide more data.

Another important neoplasm observed in our series is breast cancer, which appeared in 4 cases, all of which occurred in women not treated with thiopurines ($P = 0.05$, OR = 0.99, 95%CI: 0.98-1). There are little data regarding the occurrence of this neoplasm in patients treated with thiopurines or with IBD and they don't seem to have a higher risk compare to the general population[63,64]. However, there are more data regarding the development of other gynecological neoplasms such as cervical cancer. As most of them are related to the infection by human papillomavirus types 16 and 18, we could hypothesize that the use of immunosuppressants would increase the risk of this type of neoplasms; however, this has not been proved in the initial studies[37,65]. Indeed, other studies showed an increase incidence in cervical dysplasia in patients with IBD[66,67] and subsequent papers showed a higher risk of cervical cancer in relation to the duration of the treatment with azathioprine, combined use of corticoids or the use of tobacco[68-71]. In conclussion, the risk of breast or cervical cancer in patients with IBD is similar to the general population, and it seems not affected by thiopurines.

Among the risk factors identified in other studies, the accumulated doses of thiopurines, the usage time, the TPMT activity and the use of tobacco have been recognized. None of those factors has been found as a risk factor for any of the neoplasms described in our series. Regarding tobacco, we are unable to provide data as this information is lacking in our database. However, it is an obvious risk factor for the development of neoplastic pathology and it might have played a role.

In conclusion, in our study neoplasms were more frequent among the patients who have not been treated with thiopurines, as well as in patients diagnosed with UC (the treatment with thiopurines was more usual among CD patients), regardless of the sex. Considering the type of neoplasm, we observed significant differences in colorectal and breast cancer, for which the use of thiopurines has a protective role. In the rest of neoplasms we did not observe any difference, but the lymphomas and non-melanoma skin cancer had a higher incidence among patients treated with thiopurines, as occurs in the previously published studies. Due to this, we can conclude that the benefit provided by thiopurines use surpasses the risk.

COMMENTS

Background

Inflammatory bowel disease (IBD) is a chronic condition with bouts of bowel inflammation, resulting in abdominal pain, diarrhea, weight loss, and bleeding among other symptoms. Pathogenesis is mostly unknown, but autoimmunity is involved, and immunosupressants like thiopurines (azathioprine and mercaptopurine) are one of the available therapeutic options. However, these drugs have the theoretical potential to facilitate the development of other diseases controlled by the immune system, as infections or neoplasms. Therefore, it is essential to determine whether thiopurines therapy has an influence on neoplasms development, and if this effect is more intense that the benefits for IBD.

Research frontiers

A variety of drugs are used for IBD therapy, and changes have to be done depending on disease control or adverse events. Because of this, it is very difficult to determine whether the risk of neoplasm is attributable to a given drug, its combination with other, the time under treatment, doses or the disease itself. Moreover, intervening diseases, therapies, smoking and the severity of the disease itself could also have a role in neoplasms development.

Innovations and breakthroughs

Early thiopurines therapy provides a better control of IBD symptoms and prevents its complications. Nevertheless, a higher risk of neoplasms has been suggested in some papers after an early immunosuppressants introduction, although in other studies addressing this same issue this effect is not clear. They present a group of 812 IBD patients, 52.8% under thiopurines treatment, in which they have studied malignancies development.

Applications

Their results suggest that thiopurine treatment does not increase the risk of neoplasms in IBD patients. They have shown to be safe and effective drugs for disease control.

Terminology

IBD is a chronic sistemic condition that affects chiefly the gastrointestinal tract. The disease consists in inflammatory changes that involve the target organs. Thiopurines (azathioprine and mercaptopurine) are drugs known as immunosupresants, with an effect on the immune system and the inflammatory changes triggered by it.

Peer review

In this paper, the authors describe a retrospective analysis of 812 patients with inflammatory bowel diseases who were treated in a single center in Spain since 1996. The authors mainly compare malignancy incidences between patients treated with thiopurines and those who never received thiopurines.

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ORIGINAL ARTICLE

Histopathology of type C liver disease for determining hepatocellular carcinoma risk factors

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Abstract

AIM: To evaluate the histopathological findings of type C liver disease to determine risk factors for development of hepatocellular carcinoma (HCC).

METHODS: We studied 232 patients, who underwent liver biopsy for type C chronic liver disease between 1992 and 2009, with sustained virological response (SVR) after interferon therapy. The patients were divided into two groups according to the F stage 0 + 1 + 2 group (n = 182) and F3 + 4 group (n = 50). We prospectively observed and compared the incidence of HCC of the patients with SVR in the F0 + 1 + 2 and F3 + 4 groups. Then, the background factors and liver histopathological findings, including the degree of fibrosis, F stage, inflammation, necrosis, bile duct obstruction, fat deposition, and degree of irregular regeneration (IR) of hepatocytes, were correlated with the risk of developing HCC.

RESULTS: HCC developed in three of 182 (1.6%) patients in the F0 + 1 + 2 group, and four of 50 (8.0%) in the F3 + 4 group. The cumulative incidence of HCC in the former group was found to be significantly lower than in the F3 + 4 group (log rank test P = 0.0224). The presence of atypical hepatocytes among IR of hepatocytes in the F3 + 4 group resulted in a higher cumulative incidence of HCC, and was significantly correlated with risk of HCC development (RR = 20.748, 95%CI: 1.335-322.5, P = 0.0303).

CONCLUSION: Atypical hepatocytes among the histopathological findings of type C liver disease may be an important risk factor for HCC development along with progression of liver fibrosis.

Key words: Hepatocellular carcinoma; Irregular regeneration of hepatocytes; Liver fibrosis; Type C chronic liver disease; Histopathology of liver biopsy

Core tip: To evaluate the histopathological findings of type C liver disease to determine risk factors for the development of hepatocellular carcinoma (HCC), we studied 232 patients, who underwent liver biopsy, with sustained virological response after interferon therapy. We investigated in detail the histopathological findings, and analyzed the findings to determine the risk factors. Consequently, atypical hepatocytes among irregular regeneration of hepatocytes may be an important risk factor for HCC development, along with progression of liver fibrosis. Clarification of atypical hepatocytes as a risk factor of carcinogenesis may aid in the early diagnosis of HCC.

Matsumura H, Nirei K, Nakamura H, Higuchi T, Arakawa Y,

Ogawa M, Tanaka N, Moriyama M. Histopathology of type C liver disease for determining hepatocellular carcinoma risk factors. *World J Gastroenterol* 2013; 19(30): 4887-4896 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4887.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4887

INTRODUCTION

At our facility, we maintain a database of hepatitis patients who were recruited with informed consent from the 1990s. We previously reported the natural progression of patients with type C chronic hepatitis (CH) and liver cirrhosis (LC) - both attributable to hepatitis C virus (HCV) infection - over the course of about 30 years. The incidence of hepatocellular carcinoma (HCC) among those with LC was 7% per annum[1,2]. Many other studies have reported the progression of liver disease and the risk of development of HCC with HCV infection[3-8].

In this study, we collected liver biopsy samples from type C chronic liver disease patients before interferon (IFN) therapy and examined in detail the histopathological findings of the liver tissue. According to the progression of liver fibrosis (F stage), we examined the factors that may contribute to the development of HCC. We further studied the association of the condition of the hepatocytes, present during regeneration of the liver parenchyma destroyed by infection, with the incidence of HCC. The irregular regeneration (IR) of hepatocytes, which occurs as a result of repeated cycles of necrosis and regeneration of the liver parenchyma in CH, was found to be important for the prognosis of HCC[9-15]. A few studies have sought to determine the association between the histopathological findings from liver biopsies and the risk of developing HCC[4]. Therefore, patients with type C chronic liver disease who underwent liver biopsy were investigated in detail for findings on the sites and degree of fibrosis and inflammation, necrosis, IR of hepatocytes, and fat deposition in the liver. These findings were analyzed to determine the risk factors for development of HCC.

MATERIALS AND METHODS

Study population

In this study, samples from 482 patients with type C liver disease who underwent liver biopsy between 1992 and 2009 were collected prior to the start of IFN therapy. Two hundred and thirty-two patients achieved a sustained virological response (SVR) to IFN therapy. We determined the F stage in the liver (stages 0-4, as described for histological scoring) by liver biopsy according to the method of Desmet *et al*[16], Knodell *et al*[17] and Ishak *et al*[18]. Then, 232 patients were divided into two groups according to the F stage: stages 0, 1 and 2 (F0 + 1 + 2 group, n = 182) and stages 3 and 4 (F3 + 4 group, n = 50). We prospectively observed and compared the incidence of HCC in the patients with SVR in the F0 + 1 + 2 and F3 + 4 groups. The observation period was from the time of diagnosis of SVR to the time of the final examination or of diagnosis of HCC. Then, the clinical background factors and the histopathological findings at the liver biopsy were correlated with the risk of developing HCC together with progressive liver fibrosis.

All of the patients were positive for serum HCV antibody (2nd generation ELISA; Dinabot, Tokyo, Japan) and HCV RNA in serum, and negative for serum hepatitis B surface antigen (HBsAg ELISA; Dinabot), anti-nuclear antibody (indirect immunofluorescence assay: IF, Special Reference Laboratory, Tokyo, Japan), anti-smooth muscle antibody (IF), and anti-mitochondrial antibody (IF). No heavy drinkers (more than 30 g of ethanol intake daily) were included.

We confirmed the positivity and measured the concentration of HCV RNA in the blood samples using the competitive reverse transcriptase-polymerase chain reaction and DNA probe methods (Special Reference Laboratory, Tokyo, Japan) or Amplicor monitoring method (Amplicor HCV Monitor, Roche Diagnostic K.K., Tokyo, Japan). Subjects without HCC were confirmed by abdominal computed tomography (CT) or ultrasonography within the 6 mo prior to initial liver biopsy. At the time of liver biopsy, we determined the patient's body mass index, serum aspartate aminotransferase (AST) level (U/L), serum alanine aminotransferase (ALT) level (U/L), serum γ-glutamyltransferase (GT) level (U/L), serum platelet count ($\times 10^4/\mu L$), and HCV genotype, and carried out a histopathological examination of the biopsy specimens. We recorded the patient's sex and age, period of observation (years), and incidence of HCC. All patients gave their consent to be included in this study according to the Declaration of Helsinki.

Administration of IFN

Initial IFN therapy varied according to the time period when the patients were treated. From 1992 to 2001, IFN monotherapy was administered using natural (n) IFN (n-IFNα: Sumiferon, Sumitomo Pharma Co., Osaka, Japan) in 155 cases, recombinant (r)-α2a (r-IFNα2a: Canferon, Takeda Pharmaceutical Co., Osaka, Japan) in 44 cases, and r-α2b (r-IFNα2b: Intron, Schering-Plough Co., Osaka, Japan) in 121 cases. From 2001 to 2007, we treated 68 patients with combination therapy of IFN-α2b and ribavirin (Rebetol; Schering-Plough Co.). From 2007 to 2009, we administered combination therapy of Peg-IFN-α2b (Peg Intron; Schering-Plough Co.) and ribavirin in 93 cases. In accordance with the protocol based on the medical insurance system in Japan, considering the HCV genotype, we administered IFN therapy for 6-12 mo. The following classification of outcomes was determined according to the effects of IFN therapy: SVR was achieved in patients who became negative for serum HCV RNA for > 6 mo after termination of IFN therapy.

Liver biopsy

Liver tissues were taken from patients by percutaneous

needle biopsy (MONOPTY, 14 or 16 gauge; C. R. Bard Inc., Tempe, AZ, United States) with the aid of an ultrasonic echo guide within the 6 mo preceding the start of IFN therapy. These tissue specimens were fixed in 2.5% formalin, embedded in paraffin, sectioned at 3-4 μm, and stained with hematoxylin and eosin (HE).

Histological scoring

The tissues obtained by liver biopsy were evaluated for the sites and degree of fibrosis, inflammatory reaction, and necrosis, as well as the degree of IR of hepatocytes, bile duct obstruction, and fat deposition in the liver.

The histopathological findings were scored using 5 grades: score 0, none of the above features; score 1, minimal detection (observed in less than one-third of the field); score 2, mild (observed in one-third to less than two-thirds of the field); score 3, moderate (observed in two-thirds or more of the field), and score 4, severe (diffusely in all fields). The samples were scored blindly by two pathologists (Moriyama and the author), and the final value was calculated as the average of their scores (mean ± SD).

Only minimal fibrosis was observed in the portal areas of normal livers. Pericellular fibrosis was defined as fibrosis around the hepatocytes, perivenular fibrosis as fibrosis around the central vein, and portal sclerosis as fibrosing expansion of the portal area. The degree of pericellular fibrosis, perivenular fibrosis, and portal sclerosis were evaluated and assigned a score from 0 to 4 as described above for histopathological scoring. According to the method of Desmet *et al*[16] and Knodell *et al*[17], we determined the F stage in the liver (stages 0-4, as described above for histopathological scoring), including bridging fibrosis.

In evaluating hepatocellular necrosis, focal necrosis was defined as necrosis of several hepatocytes with infiltration of inflammatory cells such as macrophages and lymphocytes. Bridging necrosis refers to necrosis connecting areas, such as two portal tracts, leading to extensive hepatocellular necrosis and a state of acute hepatic failure. The patients were further defined as with (Y) or without bridging necrosis (N).

Infiltration of lymphocytes is a characteristic of viral hepatitis. The degree of inflammatory cell infiltration into the liver parenchyma (parenchymal infiltration) and portal area (periportal infiltration) were assessed, and assigned a score from 0 to 4 as described above for histopathological scoring. The degree of lymphocytic infiltration into the entire hepatic lobule (lymphoid reaction) and portal area (portal lymphocyte infiltration) was also evaluated and assigned a score from 0 to 4 as described above for histopathological scoring.

We also investigated the histopathological abnormalities characteristic of IR of hepatocytes (Figure 1), which were evaluated in each sample according to the criteria by Uchida[9], Shibata *et al*[10], Ueno *et al*[11] and Moriyama *et al*[12]. IR comprises the following six elements: dysplastic change, map-like distribution, bulging, oncocytes, nodularity, and atypical hepatocytes. Based on these, we examined the liver biopsy samples for: presence and degree of dysplastic change defined as anisocytosis characterized by variability of the cells; presence and degree of map-like distribution, which is defined as distinct populations of hepatocytes with a homogeneous appearance within each population and separated from each other by a sharp outline; presence and degree of bulging, which is defined as expansive proliferation of hepatocytes compressing the surrounding parenchyma; presence of oncocytes with nonuniformity of the cytoplasm; presence and degree of nodality, defined as nodular appearance of hepatocytes; and presence of atypical hepatocytes characterized by cellular degeneration. Each finding of IR was evaluated and assigned a score from 0 to 4 as described above for histopathological scoring.

Bile duct obstruction was investigated according to the degree of cholangitis by lymphocytes (bile duct damage) and assigned a score from 0 to 4 as described above for histopathological scoring. Finally, the degree of fat deposition in liver (steatosis) was assessed according to the method by Brunt *et al*[19] and Matteoni *et al*[20], and assigned a score from 0 to 4 as described above for histopathological scoring.

Evaluation of the long-term outcome of patients

Patients with IFN therapy underwent abdominal ultrasonography every 3 or 6 mo and abdominal CT examination every 6-12 mo to check for the occurrence of HCC. When space occupying lesions (SOLs) were detected in the livers of the patients by dynamic CT, enhancement of SOLs was observed in the early phase of dynamic CT and the disappearance of SOL staining was observed in the late phase. A precise diagnosis was made by abdominal angiography. When SOLs in the liver were not enhanced in the early phase of dynamic CT, or if a precise diagnosis could not be made by abdominal angiography, tumor biopsy was carried out and a precise diagnosis was made on the basis of the pathological findings.

Statistical analysis

Sex, genotype, and bridging necrosis (Y/N) were compared using the χ^2 test for independence. The remaining parameters including the clinical characteristics at the time of liver biopsy and the liver histopathological findings are shown as mean ± SD and were compared using the Mann-Whitney *U* test. Cumulative incidence curves were determined with the Kaplan-Meier method and the differences between groups were assessed using the log-rank test. Analysis of risk factors for HCC occurrence was made using the Cox proportional hazard model and these were compared by multivariate analysis. These were performed using SPSS 11.0 software (SPSS Inc., Chicago, IL, United States). $P < 0.05$ was considered significant.

RESULTS

Clinical background factors at time of liver biopsy and liver histopathological findings in SVR patients according to F stage

Comparison of clinical background factors at the time of liver biopsy and liver histopathological findings in

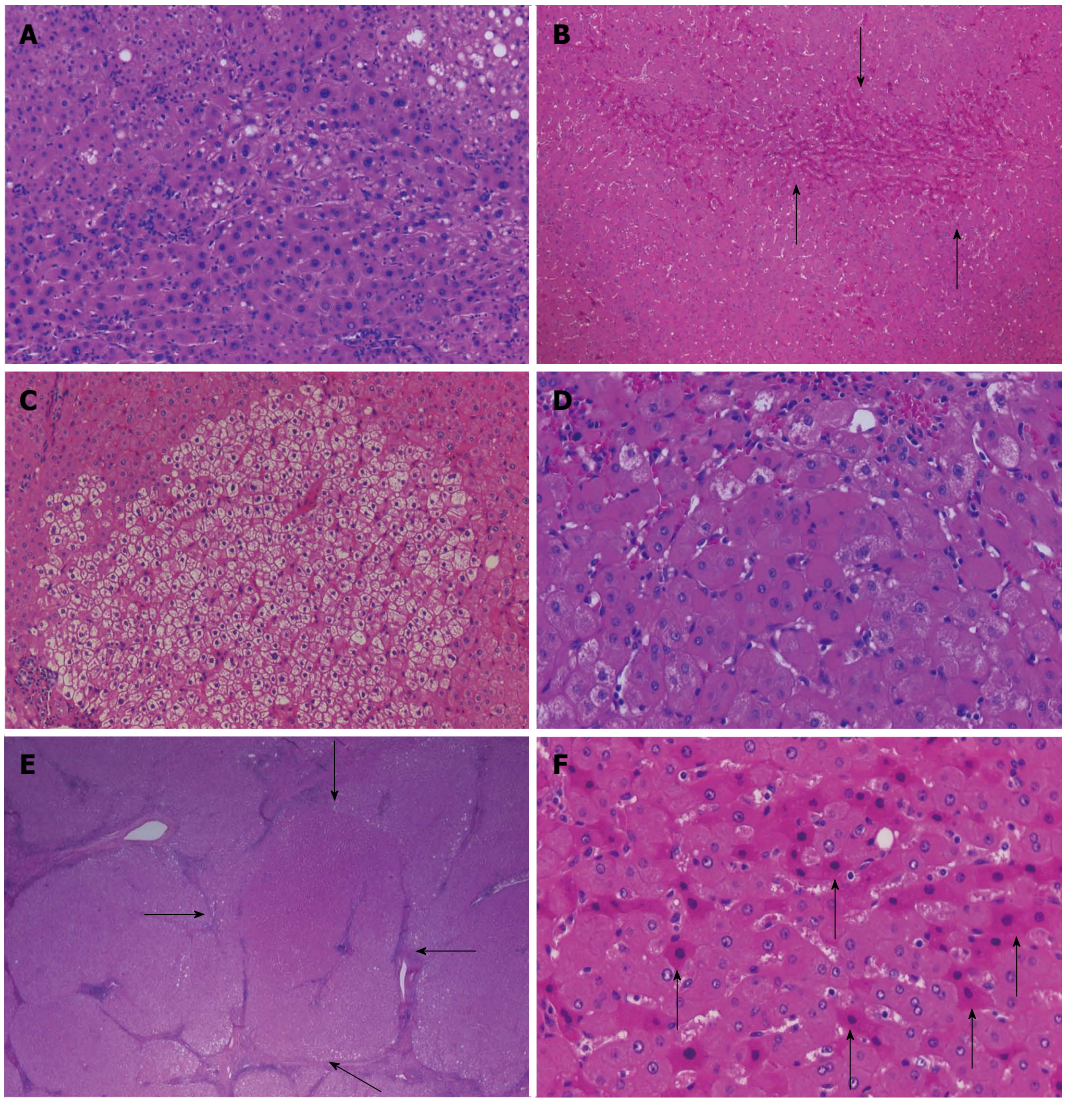

Figure 1 Features of irregular regeneration of hepatocytes. Microscopic views are shown of the biopsied liver tissues (F1 and F3 stage) of representative patients with hepatitis C virus infection. A: Dysplastic change; anisocytosis characterized by variability of cell size with focal dysplastic change [hematoxylin and eosin (HE); × 200]; B: Map-like distribution; distinct populations of hepatocytes with a homogeneous appearance within each population are separated from each other by a sharp outline (arrows) (HE; × 100); C: Bulging; expansive proliferation of hepatocytes compressing the surrounding parenchyma (HE; × 100); D: Oncocytes; oncocytic change of hepatocytes (HE; × 400); E: Nodularlity; nodular arrangement of the parenchyma (arrows) (HE; × 40); F: Atypical hepatocytes; degeneration of hepatocytes (arrows) (HE; × 400). These histopathological findings were scored using 5 grades: score 0, none; score 1, minimal (observed in less than one-third of the field); score 2, mild (observed in one-third to less than two-thirds of the field); score 3, moderate (observed in two-thirds or more of the field) and score 4, severe (diffusely in all fields). The findings were scored as the average (mean ± SD).

SVR patients according to the F stage (F0 + 1 + 2, n = 182 and F3 + 4, n = 50) was performed (Tables 1 and 2). As demonstrated in Table 2, the number of patients for each score was compared, then the presence of bridging necrosis (Y/N) was compared, and the remaining parameters are presented as mean ± SD. The findings demonstrated that there was a higher level of deterioration of the liver in the F3 + 4 group as compared to the F0 + 1 + 2 group; liver fibrosis was more progressive, age, AST, ALT, and α-fetoprotein (AFP) were higher, platelet count was lower, and inflammatory reaction was stronger.

Cumulative incidence of HCC between patients with SVR in the F0 + 1 + 2 and F3 + 4 groups

The cumulative incidence of HCC was compared between patients with SVR in the F0 + 1 + 2 and F3 + 4 groups by the Kaplan-Meier method (Figure 2A). HCC developed in seven of 232 (3.0%) patients, which included three of 182 (1.6%) patients in the F0 + 1 + 2 group, and four of 50 (8.0%) patients in the F3 + 4 group. The cumulative incidence of HCC in the former group was found to be significantly lower than in the F3 + 4 group (log rank test P = 0.0224).

Cumulative incidence of HCC in the F0 + 1 + 2 and F3 + 4 patients with or without atypical hepatocytes

Among the histopathological findings in the liver, the occurrence of the IR of hepatocytes is analyzed to determine the risk factors for development of HCC together with progressive liver fibrosis. In order to correct for the

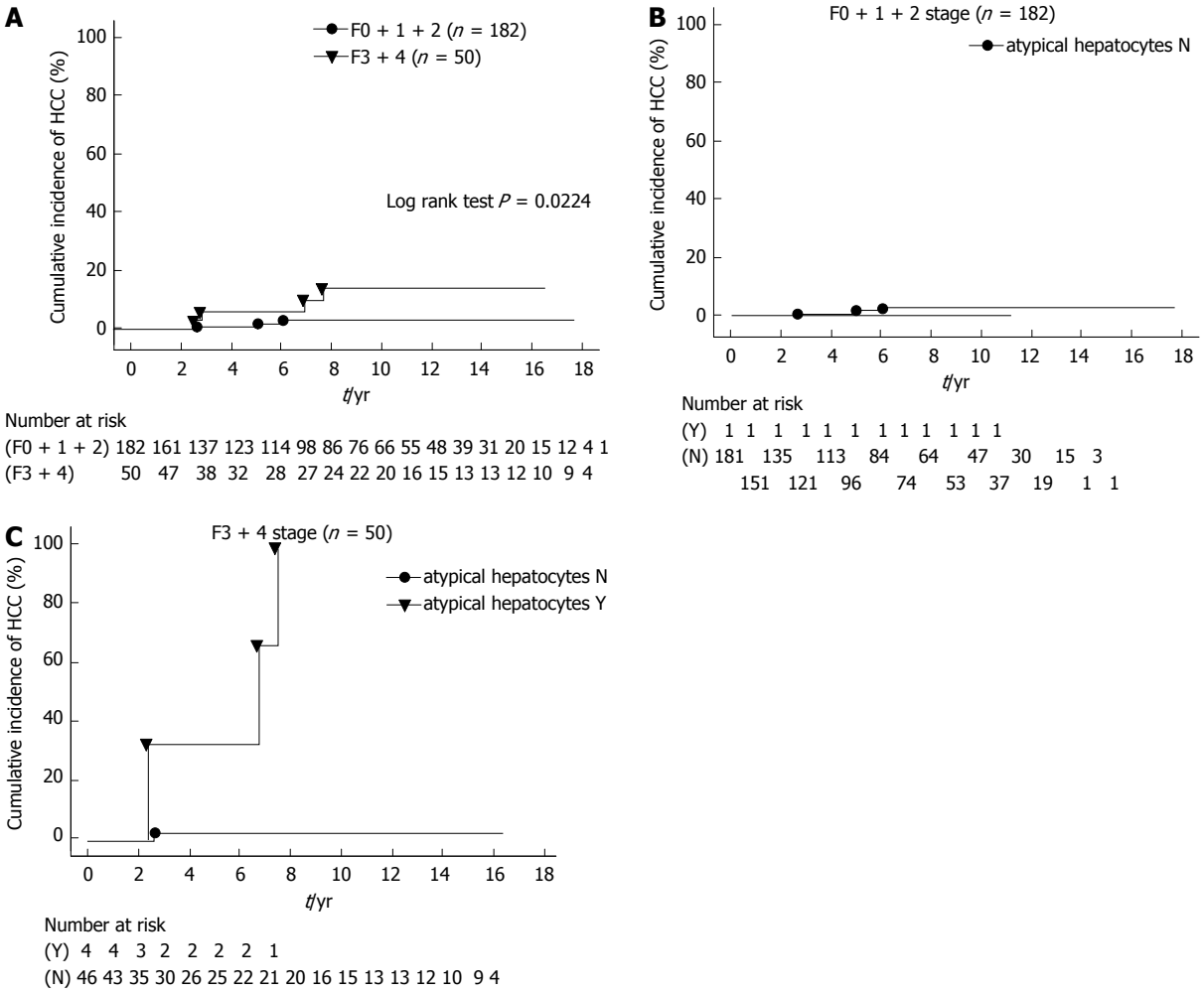

Figure 2 Comparison of the cumulative incidence of hepatocellular carcinoma. A: Between patients with sustained virological response in the F0 + 1 + 2 and F3 + 4 groups. The cumulative incidence of hepatocellular carcinoma (HCC) was compared between patients with sustained virological response in the F0 + 1 + 2 and F3 + 4 groups by the Kaplan-Meier method. HCC developed in seven of 232 (3.0%) patients, which included three of 182 (1.6%) patients in the F0 + 1 + 2 group, and four of 50 (8.0%) patients in the F3 + 4 group. The cumulative incidence of hepatocellular carcinoma in the former group was found to be significantly lower than in the F3 + 4 group (log rank test $P = 0.0224$). Values below the graphs show the numbers at risk; B and C: Between patients with sustained virological response in the atypical hepatocytes (N) and (Y) groups, according to the F0 + 1 + 2 and F3 + 4 groups. HCC developed in seven of 232 (3.0%) patients, of which three belonged to the F0 + 1 + 2 group and four to the F3 + 4 group. To correct for the atypical hepatocytes, the 232 patients were divided into two groups as follows: those with an absence (score 0) of atypical hepatocytes (N group, $n = 227$) and those with presence (score 1) of atypical hepatocytes (Y group, $n = 5$). The Y group included all cases with scores 1-4, but actually there were only cases with score 1. The cumulative incidence of HCC was compared in the F0 + 1 + 2 and F3 + 4 patients according to the atypical hepatocyte status by the Kaplan-Meier method. For the F3 + 4 patients, we found that there was a significantly lower cumulative incidence of HCC in the N group as compared to the Y group (log rank test, $P = 0.0001$). Values below the graphs show the numbers at risk.

IR of hepatocytes, dysplastic change, map-like distribution, bulging, oncocytes, nodularity, and atypical hepatocytes, the 232 patients were divided into two groups as follows: those with an absence (score 0) of IR of hepatocytes (N group) and those with presence (score 1-4) of IR of hepatocytes (Y group) according to the F0 + 1 + 2 and F3 + 4 groups. The cumulative incidence of HCC was compared with the IR of hepatocytes according to the F0 + 1 + 2 and F3 + 4 patients by the Kaplan-Meier method.

For example, to correct for the atypical hepatocytes, the 232 patients were divided into two groups as follows: those with an absence (score 0) of atypical hepatocytes (N group, $n = 227$) and those with presence (score 1-4) of atypical hepatocytes (Y group, $n = 5$). The Y group included all cases with score 1, but there were only cases with score 1. For the F3 + 4 patients, we found that there was a significantly lower cumulative incidence of HCC in the N group as compared to the Y group (log rank test, $P = 0.0001$, Figure 2B and C). However, there was no significant difference in the remaining IR of hepatocytes (data not shown). Thus, our findings demonstrate that with the progression of liver fibrosis, those patients with atypical hepatocytes among the IR of hepatocytes have a higher cumulative incidence of HCC.

Comparison of clinical background factors to determine association with development of HCC

In the F0 + 1 + 2 and F3 + 4 groups, the findings were analyzed to determine the risk factors for development of HCC using the Cox proportional hazard regression method. We compared the clinical background factors at

Table 1 Comparison of clinical background factors at the time of liver biopsy in sustained virological response patients, between F0 + 1 + 2 and F3 + 4 groups *n* (%)

Parameter		F0 + 1 + 2	F3 + 4	*P* value
Number	232	182	50	
Gender (male)	136 (58.6)	104 (57.1)	32 (64.0)	0.3832
Age (yr)	47.9 ± 12.2	45.9 ± 12.4	55.2 ± 8.3	0.0001
BMI	22.6 ± 3.3	22.7 ± 3.3	22.2 ± 3.6	0.5466
AST (U/L)	55.2 ± 45.9	49.8 ± 46.6	74.5 ± 37.6	0.0007
ALT (U/L)	75.5 ± 62.7	70.5 ± 63.0	93.5 ± 58.9	0.0216
γ-GT (U/L)	62.3 ± 71.8	61.4 ± 76.7	65.8 ± 50.4	0.7048
Platelet count ($\times 10^4/\mu L$)	18.8 ± 6.2	20.2 ± 6.0	13.9 ± 3.9	0.0001
Genotype type 1/2	108 (46.5)/ 124 (53.5)	86 (47.2)/ 96 (52.8)	22 (44.0)/ 28 (56.0)	0.6830
AFP (ng/mL)	7.7 ± 13.3	5.4 ± 8.6	15.5 ± 21.4	0.0008
Observation period (yr)	7.5 ± 4.9	7.4 ± 4.7	8.0 ± 5.5	0.4350

These 232 patients were divided into two groups according to the F stage: stages 0, 1, and 2 (F0 + 1 + 2 group, *n* = 182) and stages 3-4 (F3 + 4 group, *n* = 50). Comparison of clinical background factors at the time of liver biopsy in SVR patients according to the F stage (F0 + 1 + 2 and F3 + 4) was performed, and the data are shown. The clinical background factors demonstrated that there was a higher level of deterioration of the liver in the F3+4 group as compared to the F0 + 1 + 2 group; Age, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and α-fetoprotein (AFP) was higher, and platelet count was lower. The parameters are presented as mean ± SD. AST: Upper limit of the normal range is 38 U/L; ALT: Upper limit of the normal range is 44 U/L; γ-glutamyltransferase (GT): Upper limit of normal is 73 U/L; AFP: Upper limit of normal is 20.0 ng/mL; Platelet count: reference value is 15.0 × 10^4-35.0 × 10^4/μL. BMI: Body mass index.

liver biopsy of the patients with SVR in the F0 + 1 + 2 and F3 + 4 groups (Table 3). Univariate analysis using the Cox proportional hazard regression method was applied to these parameters to determine the clinical background factors that were associated with the development of HCC. None of the clinical background factors showed any significant associations with HCC. Blank values in the table signify that the bias was applied, but could not be statistically processed.

Comparison of liver histopathology to determine factors associated with development of HCC

We compared the histopathological findings of liver biopsy in patients with SVR in the F0 + 1 + 2 and F3 + 4 groups (Table 3). Univariate analysis using the Cox proportional hazard regression method was applied to these parameters to determine the liver histopathological findings that were associated with the development of HCC. Significant correlations were found for map-like distribution (RR = 3.082, 95%CI: 1.066-8.913, *P* = 0.0378) and atypical hepatocytes (RR = 42.055, 95%CI: 4.303-411.0, *P* = 0.0013) in the F3 + 4 patients. In the F0 + 1 + 2 patients, none of the liver histopathological findings showed any significant associations with HCC. Blank values in the table signify that the bias was applied, but could not be statistically processed.

Multivariate analysis using the Cox proportional hazard regression method was applied to the parameters that could be of statistical significance in Table 3 in each of the F stage groups, and the findings are shown in Table 4. In the F0 + 1 + 2 group, none of the factors showed any significant correlation with the development of HCC. In the F3 + 4 group, map-like distribution (RR = 1.687, 95%CI: 0.404-7.053, *P* = 0.4734) was not found to have any correlation while the presence of atypical hepatocytes (RR = 20.748, 95%CI: 1.335-322.5, *P* = 0.0303) was shown to be significantly associated with HCC development.

DISCUSSION

In Japan, ⩾ 90% of cases of LC associated with HCC are attributable to hepatitis B or C virus infection. Many studies of hepatitis C have reported risk factors for HCC other than the virus, such as the progression of fibrosis, male sex, increasing age, consumption of a large amount of alcohol, and excess iron in the liver[7,21-23]. It has been reported that the occurrence of HCC may be suppressed by reducing the degree of inflammation, regardless of the presence or absence of HCV[24-26]. Furthermore, among the liver histopathological findings, it has been reported that the progression of liver fibrosis (F stage) constitutes a risk factor for carcinogenesis[4]. Therefore, we sought to identify the liver histopathological findings, other than the F stage, that are correlated with the risk of carcinogenesis. Previously, we have reported that the degree of IR is related to factors other than liver fibrosis that contribute to liver carcinogenesis. It has been reported that the cumulative incidence of HCC was significantly lower in cases in which the degree of IR had improved after IFN therapy than in cases without IFN therapy[11,12]. If the degree of inflammation and necrosis in the liver were also improved by IFN therapy, this would be reflected by an improvement in the histopathological findings, particularly the degree of IR and liver fibrosis, and by a reduction in the incidence of HCC. Moreover, it is generally accepted that HCV may be a direct cause of HCC[27]. Thus, by considering the differences in liver histopathological findings in patients with SVR following viral clearance, it was possible to eliminate the direct effects of the virus on HCC progression. If the degree of inflammation and necrosis in the liver was improved following elimination of the virus by IFN therapy, the development of HCC would be attributable, at least in part, to nonviral factors. Therefore, we investigated the risk factors in detail, including the degree of liver IR and the histopathological findings of liver biopsy specimens, and prospectively examined the findings separately according to the degree of liver fibrosis in SVR patients.

In type C chronic disease, it is said that active inflammation persists in the liver, with the formation of lymphoid follicles, varying degrees of liver parenchyma failure, and IR of hepatocytes, ultimately leading to the formation of small regenerative nodules. In other words, IR of hepatocytes, which occurs in the liver as a result of repeated cycles of necrosis and regeneration, is recognized as a population of hepatocytes that differ from normal ones in size, appearance, and arrangement of

Table 2 Comparison of liver histological findings at the time of liver biopsy in sustained virological response patients, between F0 + 1 + 2 and F3 + 4 groups

Parameter	Score					F0 + 1 + 2	F3 + 4	*P* value
	0	1	2	3	4			
Irregular regeneration								
Dysplastic change	109	50	49	21	3	0.769 ± 0.998	1.660 ± 1.081	0.0001
Oncocytes	211	16	4	1	0	0.066 ± 0.290	0.300 ± 0.647	0.0003
Map-like distribution	160	37	22	13	0	0.396 ± 0.756	0.960 ± 1.142	0.0001
Nodular arrangement	216	13	1	2	0	0.027 ± 0.164	0.320 ± 0.713	0.0001
Bulging	206	18	6	2	0	0.104 ± 0.400	0.340 ± 0.688	0.0022
Atypical hepatocytes	227	5	0	0	0	0.005 ± 0.074	0.080 ± 0.274	0.0012
Inflammatory cell infiltration								
Peri-portal	0	65	127	35	5	1.819 ± 0.652	2.260 ± 0.828	0.0001
Parenchymal	0	37	149	44	2	1.978 ± 0.585	2.300 ± 0.678	0.0010
Portal lymphocyte	0	5	103	121	3	2.456 ± 0.562	2.780 ± 0.507	0.0003
Portal lymphoid reaction	0	6	47	78	101	3.132 ± 0.850	3.360 ± 0.802	0.0904
Fibrosis								
F stage	5	128	49	34	16			
Portal sclerosis	113	81	32	6	0	0.615 ± 0.783	1.020 ± 0.795	0.0014
Pericellular fibrosis	89	90	41	11	1	0.802 ± 0.870	1.260 ± 0.853	0.0011
Perivenular fibrosis	63	66	65	25	0	1.236 ± 1.016	1.243 ± 0.895	0.9691
Bridging necrosis Y (presence)						0/228	4/228 (1.7%)	0.0020
Bile duct damage	123	71	26	9	2	0.597 ± 0.861	1.000 ± 0.904	0.0041
Steatosis	96	46	68	18	1	0.944 ± 0.990	1.429 ± 1.118	0.0035

Comparison of liver histological findings in sustained virological response patients according to the F stage (F0 + 1 + 2 and F3 + 4) was performed, and the data are shown. The histological findings demonstrated that there was a higher level of deterioration of the liver in the F3 + 4 group as compared to the F0 + 1 + 2 group; liver fibrosis was more progressive and inflammatory reaction was stronger. The number of patients for each score was compared, then the presence of bridging necrosis (Y/N) was compared and the remaining parameters are presented as mean ± SD. Scoring: score 0, none; score 1, minimal (observed in less than one third of the field); score 2, mild (observed in one third to less than two thirds of the field); score 3, moderate (observed in two thirds or more of the field) and score 4, severe (diffusely in all fields).

the nucleus. As a result, nodular lesions often are detectable in the livers of patients with type C chronic disease. These nodular lesions may be classified into two types: dysplastic nodules and early HCC. In addition, dysplastic nodules are divided into low-grade and high-grade dysplastic nodules or borderline lesions[28]. Fatty changes may be seen in 40% of high-grade dysplastic nodules, but are not observed in the low-grade ones, and regenerative large nodules are observed at high frequency in early HCC[29,30].

We compared the cumulative incidence of HCC between patients with low (F0 + 1 + 2) and high (F3 + 4) degree of liver fibrosis, and it was found that the former group had a significantly lower rate than the F3 + 4 group, suggesting that as fibrosis progresses in the liver, HCC is more likely to occur. It is clear that liver fibrosis progresses and also contributes to an increased risk of the development of HCC. In this study, factors associated with the development of HCC were identified by comparing patients with SVR by multivariate analysis using the Cox proportional hazard regression method, which was applied to the clinical and histopathological parameters. We found that among all the investigated factors, the presence or absence of atypical hepatocytes among IR of hepatocytes may be an important risk factor for HCC development along with progression of liver fibrosis.

Moreover, patients with progressive liver fibrosis and atypical hepatocytes among the IR of hepatocytes also had a higher cumulative incidence of HCC. Therefore, our results clearly demonstrate the occurrence of atypical hepatocytes in progressive liver fibrosis as a risk factor of HCC. This finding is also considered important for determination of a patient's therapeutic options.

HCC, stemming from LC induced by HCV infection, and other nonviral liver diseases, such as nonalcoholic steatohepatitis (NASH)[31-33] and autoimmune hepatitis[34], may develop through carcinogenic mechanisms based on inflammation[35,36]. Accordingly, we consider that the atypical hepatocytes may also contribute to the development of HCC in these nonviral diseases. Thus, during the assessment of the histopathological findings from liver biopsies, attention should be paid to the liver fibrosis, and additionally the presence or absence of atypical hepatocytes according to the type of liver disease.

In conclusion, among the histopathological findings in the liver of type C chronic disease, the occurrence of atypical hepatocytes in the IR of hepatocytes is significantly correlated with the risk of developing HCC together with progressive liver fibrosis. We believe that clarification of this finding as a risk factor of carcinogenesis may aid in the early diagnosis of HCC, and it would be meaningful to perform liver biopsy in patients with progression of liver fibrosis. Thus, by treating patients for both hepatitis C infection and atypical hepatocytes, we may attain an increase in the survival rate and a lower incidence of HCC.

Table 3 Factors associated with the development of hepatocellular carcinoma, identified by comparing patients with sustained virological response by univariate analysis using Cox proportional hazard regression method (n = 232)

Parameter	F0 + 1 + 2			F3 + 4		
	RR	95%CI	*P* value	RR	95%CI	*P* value
Clinical background factors[1]						
Gender (male)	1.200	0.109-13.252	0.8815	-	-	-
Age (yr)	1.308	0.985-1.737	0.0635	1.123	0.959-1.314	0.1494
BMI	1.121	0.693-1.814	0.6415	0.987	0.549-1.772	0.9644
AST (U/L)	0.996	0.964-1.028	0.7903	1.003	0.971-1.037	0.8540
ALT (U/L)	0.992	0.965-1.020	0.5678	0.996	0.972-1.020	0.7494
γ-GT (U/L)	0.996	0.975-1.018	0.7169	1.001	0.977-1.025	0.9436
Platelet count (× 10^4/μL)	1.004	0.837-1.204	0.9634	1.088	0.855-1.384	0.4936
Genotype type 1	-	-	-	0.285	0.030-2.752	0.2781
AFP (ng/mL)	0.989	0.867-1.129	0.8698	0.995	0.919-1.077	0.9046
Histological findings						
Irregular regeneration[2]						
Dysplastic change	1.920	0.720-5.124	0.1926	1.330	0.543-3.256	0.5322
Oncocytes	-	-	-	1.788	0.510-6.265	0.3639
Map-like distribution	-	-	-	3.082	1.066-8.913	0.0378
Nodularity	-	-	-	-	-	-
Bulging	-	-	-	-	-	-
Atypical hepatocytes	-	-	-	42.055	4.303-411.029	0.0013
Infiltration						
Peri-portal	3.248	0.537-19.655	0.1995	0.477	0.110-2.067	0.3225
Parenchymal	3.028	0.389-23.604	0.2902	0.420	0.092-1.921	0.2634
Portal lymphocyte	2.085	0.217-20.014	0.5241	1.213	0.177-8.333	0.8443
Portal lymphoid reaction	2.362	0.345-16.168	0.3812	3.364	0.484-23.407	0.2202
Fibrosis						
F stage						
Portal sclerosis	0.551	0.078-3.884	0.5500	1.293	0.444-3769	0.6373
Pericellular fibrosis	0.963	0.243-3.822	0.9576	0.534	0.117-2.440	0.4184
Perivenular fibrosis	0.627	0.168-2.344	0.4878	0.429	0.076-2.412	0.3370
Bridging necrosis Y	-	-	-	-	-	-
Bile duct damage	0.678	0.113-4.061	0.6707	1.305	0.374-4.555	0.6766
Steatosis	1.064	0.343-3.306	0.9142	1.660	0.623-4.421	0.3106

We compared the clinical background factors and histological findings at liver biopsy of the patients with sustained virological response in the F0 + 1 + 2 and F3 + 4 groups, data of which are shown. Univariate analysis using the Cox proportional hazard regression method was applied to these parameters to determine the clinical background factors that were associated with the development of hepatocellular carcinoma (HCC). [1]None of the clinical background factors showed any significant associations with HCC. Blank in the table signifies that the bias was applied, but could not be statistically processed; [2]Significant correlations were found for map-like distribution (RR = 3.082, 95%CI: 1.066-8.913, P = 0.0378) and atypical hepatocytes (RR = 42.055, 95%CI: 4.303-411.0, P = 0.0013) in the F3 + 4 patients. None of the liver histopathological findings showed any significant associations with HCC in the F0 + 1 + 2 patients. Blank values signify that the bias was applied, but could not be statistically processed. AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; AFP: α-fetoprotein; BMI: Body mass index; GT: glutamyltransferase.

Table 4 Factors associated with the development of hepatocellular carcinoma, identified by comparing patients with sustained virological response by multivariate analysis using Cox proportional hazard regression method

F3 + 4 parameter	RR	95%CI	*P* value
Map-like distribution	1.687	0.404-7.053	0.4734
Atypical hepatocytes	20.748	1.335-322.530	0.0303

Multivariate analysis using the Cox proportional hazard regression method was applied to the parameters in each of the F stage groups. In the F0 + 1 + 2 group, none of the factors showed any significant correlation with the development of hepatocellular carcinoma (HCC). In the F3 + 4 group, map-like distribution (RR = 1.687, 95%CI: 0.404-7.053, P = 0.4734) was not found to have any correlation while the presence of atypical hepatocytes (RR = 20.748, 95%CI: 1.335-322.5, P = 0.0303) was shown to be significantly associated with HCC development.

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COMMENTS

Background

A few studies have sought to determine the association between thehistopathological findings from liver biopsies and the risk of developing hepatocellular carcinoma (HCC). The authors studied 232 patients who underwent liver biopsy, with a sustained virological response (SVR) after interferon (IFN) therapy. They investigated in detail the histopathological findings and analyzed the findings to determine the risk factors.

Research frontiers

It has been reported that progression of liver fibrosis (F stage) constitutes a

risk factor for carcinogenesis. Therefore, the authors sought to identify the liver histopathological findings, other than F stage, that are correlated with the risk of carcinogenesis. If the degree of inflammation and necrosis in the liver were improved following elimination of the virus by IFN therapy, the development of HCC would be attributable, at least in part, to nonviral factors. Then, they investigated the risk factors in detail, including the degree of liver irregular regeneration (IR) and the histopathological findings of liver biopsy specimens, and prospectively examined the findings separately according to the degree of liver fibrosis in SVR patients.

Innovations and breakthroughs

Atypical hepatocytes among IR of hepatocytes may be an important risk factor for HCC development along with progression of liver fibrosis.

Applications

The authors believe that clarification of this finding as a risk factor of carcinogenesis may aid in the early diagnosis of HCC, and it would be meaningful to perform liver biopsy for patients with progression of liver fibrosis. Then, by treating patients for both hepatitis C infection and atypical hepatocytes, they may attain an increase in the survival rate and a lower incidence of HCC.

Terminology

IR of hepatocytes, which occurs as a result of repeated cycles of necrosis and regeneration of the liver parenchyma in chronic hepatitis, is divided into six elements: dysplastic change; anisocytosis characterized by variability of cell size with focal dysplastic change; map-like distribution; distinct populations of hepatocytes with a homogeneous appearance within each population, which are separated from each other by a sharp outline; bulging; expansive proliferation of hepatocytes compressing the surrounding parenchyma. Oncocytes: oncocytic change of hepatocytes. Nodularlity: nodular arrangement of the parenchyma. Atypical hepatocytes: degeneration of hepatocytes.

Peer review

In this study, the authors analyzed the risk factors for HCC associated with type C chronic liver disease. They found that the presence of atypical hepatocytes was significantly correlated with risk of HCC development, and therefore concluded that atypical hepatocytes among the histopathological findings of type C liver disease may be an important risk factor for HCC development along with progression of liver fibrosis.

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ORIGINAL ARTICLE

Infective severe acute pancreatitis: A comparison of ^{99m}Tc-ciprofloxacin scintigraphy and computed tomography

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Abstract

AIM: To evaluate ^{99m}Tc-ciprofloxacin scintigraphy compared with computed tomography (CT) for detecting secondary infections associated with severe acute pancreatitis (SAP) in swine.

METHODS: Six healthy swine were assigned to a normal control group (group A, n = 6). SAP was induced in group B (n = 9) and C (n = 18), followed by inoculation of the resulting pancreatic necroses with inactive *Escherichia coli* (*E. coli*) (group B) and active *E. coli* (group C), respectively. At 7 d after inoculation, a CT scan and a series of analyses using infecton imaging (at 0.5, 1, 2, 3, 4 and 6 h after the administration of 370 MBq of intravenous infecton) were performed. The scintigrams were visually evaluated and semi-quantitatively analyzed using region of interest assignments. The differences in infecton uptake and changes in the lesion-background radioactive count ratios (L/B) in the 3 groups were recorded and compared. After imaging detection, histopathology and bacterial examinations were performed, and infected SAP was regarded as positive. The imaging findings were compared with histopathological and bacteriological results.

RESULTS: In group A, 6 animals survived without infection in the pancreas. In group B, 7/9 swine survived and one suffered from infection. In group C, 15/18 animals survived with infection. Hence, the number of normal, non-infected and infected SAP swine was 6, 6 and 16, respectively. The sensitivity, specificity, accuracy, positive predictive value and negative predictive value of the infecton method were 93.8% (15/16), 91.7% (11/12), 92.9% (26/28), 93.8% (15/16) and 91.7% (11/12), whereas these values for CT were 12.5% (2/16), 100.0% (12/12), 50.0% (14/28), 100.0% (2/2) and 46.2% (12/26), respectively. The changes in L/B for the infected SAP were significantly different from those of the non-infected and normal swine (P < 0.001). The mean L/B of the infectious foci at 0.5, 1, 2, 3, 4 and 6 h was 1.17 ± 0.10, 1.71 ± 0.30, 2.46 ± 0.45, 3.36 ± 0.33, 2.04 ± 0.37 and 1.1988 ± 0.09, respectively. At

3 h, the radioactive counts (2350.25 ± 602.35 k) and the mean L/B of the infectious foci were significantly higher than that at 0.5 h (P = 0.000), 1 h (P = 0.000), 2 h (P = 0.04), 4 h (P = 0.000) and 6 h (P = 0.000).

CONCLUSION: ^{99m}Tc-ciprofloxacin scintigraphy may be an effective procedure for detecting SAP secondary infections with higher sensitivity and accuracy than CT.

Key words: Pancreatitis; Infection; Radionuclide imaging; Ciprofloxacin; X-ray computed tomography

Core tip: We successfully used a specific inflammatory agent, ^{99m}Tc-ciprofloxacin, which non-invasively detected secondary infections in an infective severe acute pancreatitis (SAP) model with higher sensitivity and accuracy than computed tomography. This method may be an effective tool for accurately diagnosing and assessing the severity of secondary infections in human SAP patients in the future. To our knowledge, there have been no previous studies that have compared the differential diagnosis of non-infectious and infectious SAP using ^{99m}Tc-ciprofloxacin imaging and histopathological and biological methods.

Wang JH, Sun GF, Zhang J, Shao CW, Zuo CJ, Hao J, Zheng JM, Feng XY. Infective severe acute pancreatitis: A comparison of ^{99m}Tc-ciprofloxacin scintigraphy and computed tomography. *World J Gastroenterol* 2013; 19(30): 4897-4906 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4897.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4897

INTRODUCTION

Infection of pancreatic or peripancreatic necroses occurs in 30%-70% of patients with severe acute pancreatitis (SAP)[1,2]. This disease is often accompanied by a late deterioration of organ function or generalized systemic illness[3], which is the leading cause of SAP-related deaths, with mortality rates of more than 30%[4-6]. Infected pancreatic necrosis in patients with clinical signs and symptoms of sepsis is an important indication for interventional therapy including surgery and drainage[7,8], whereas patients with sterile necrosis should be managed conservatively and undergo intervention only under certain circumstances[9].

However, until recently, the differential diagnosis of sterile and infectious SAP has remained a challenging issue. Effective biomarkers that will enable the localized diagnosis of infected pancreatic necrotic tissue are still under development and need to be confirmed in studies of larger patient cohorts[10,11]. Ultrasonography, computed tomography (CT) and magnetic resonance imaging have been used widely in the evaluation of pancreatitis, and CT is usually the first choice, however, these techniques have limitations in detecting secondary infections in cases of SAP *i.e.*, unusual gas bubbles and typical manifestations of an abscess on the images. White blood cell (WBC) imaging is regarded as the principal nuclear medicine method for the imaging of infection and inflammation[12]. However, it is also difficult to distinguish between infective and sterile inflammatory conditions using this method[13]. Fine-needle aspiration has contributed to making a definitive diagnosis of infected pancreatic necrosis, but is an invasive procedure and there are difficulties in applying this technique to critically ill patients[10].

It is therefore essential to develop a sensitive and specific imaging methodology that will non-invasively detect secondary infections in SAP patients. Over the past few decades, a number of radiopharmaceuticals have been developed to investigate infective and non-infective inflammatory disorders[13-16]. In this regard, ^{99m}Tc-ciprofloxacin may be one of the most promising agents in the field of nuclear medicine[17,18]. This radiochemical combines the advantages of a ^{99m}Tc label and the broad-spectrum bacteria-localizing capability of ciprofloxacin, which has a higher sensitivity and specificity for bacterial infections than WBC scans[14,17-22].

We speculate that ^{99m}Tc-ciprofloxacin may have efficacy in the diagnosis of SAP secondary infections. In our study, a SAP secondary infection model was developed in swine as previously reported[23]. The features and effectiveness of ^{99m}Tc-ciprofloxacin scintigraphy in the diagnosis of secondary bacterial infection in this infective SAP animal model were then evaluated and compared with contrast-enhanced CT, and with histopathological and bacteriological testing.

MATERIALS AND METHODS

This study was approved by the Animal Care Committee of Changhai Hospital. Healthy female Taihu swine (Experimental Animal Center of the Second Military Medical University, Shanghai, China), weighing 20-25 kg were acclimatized for one week before the start of the experiments. The animals had no access to food for 1 d and to water for 4 h prior to the start of the experiment.

Preparation of the SAP animal model

Six healthy swine were assigned to group A as normal controls. SAP was induced in 27 animals as previously reported[23], and these animals were randomly assigned to group B (n = 9) and group C (n = 18). Two days after the onset of SAP, 4 mL of inactive *Escherichia coli* (*E. coli*) and active (10^8/mL) *E. coli* were inoculated into necrotic foci of the pancreas in group B and C swine by CT-guided puncture, respectively (Table 1). Imaging examinations were performed 7 d after inoculation. The swine received ketamine hydrochloride (0.1 mL/kg) before imaging examinations and received 2 mL pentobarbital (Bioszune Life Sciences, Beijing, China) solution (3% w/v) at 20-min intervals during the examinations.

Radiopharmaceuticals

^{99m}Tc-ciprofloxacin was prepared by mixing 2 mg

Table 1 Results of secondary bacterial infection in a severe acute pancreatitis animal model

	n	Inoculation	Survival number	Pathologic diagnosis and biological results	
				Infection (bacteria)	Non-infection
Group A	6	No	6	0	6
Group B	9	Inactive *E. coli*	7	1 (*S. aureus*/*E.coli*)	6
Group C	18	Active *E. coli* (10^8/mL)	15	15 (14 *E. coli* and 1 *S. aureus* /*E.coli*)	0

E. coli: *Escherichia coli*; *S. aureus*: *Staphylococcus aureus*.

ciprofloxacin (Radiopharmaceuticals Laboratory of Beijing Normal University, China), 500 μg stannous tartrate, and 370 MBq freshly eluted sodium pertechnetate; then placed for 15 min at room temperature. Radiochemical purity was determined with a simple thin-layer chromatography technique, using 1-mm filters (Xinhua Group Co., Ltd., Hangzhou, China) in methyl ethyl ketone. ^{99m}Tc-ciprofloxacin remained at the base, and free pertechnetate moved with the solvent front. The Rf values of ^{99m}Tc-ciprofloxacin and $^{99m}TcO^{4-}$ were 1.0 and 0.0, respectively. The radiochemical purity and labeling rate of the radiopharmaceutical preparations were found to be greater than 90% at 6 h.

^{99m}Tc-ciprofloxacin scintigraphy and data analysis

^{99m}Tc-ciprofloxacin was administered into the ear vein of the swine. Abdominal imaging was then performed using a dual-head single photon emission CT (SPECT) scanner (Philips, Forte, Netherlands). The energy peak was controlled at 140 KeV with a 15% window. Each animal underwent a ^{99m}Tc-ciprofloxacin scan at 0.5, 1, 2, 3, 4 and 6 h after the injection of radiolabeled ciprofloxacin. Multi-position graphic information with a total of 64 tomographic images was acquired continuously. The radioactivity counts for each frame were 300 k and the matrix size was 64 pixel × 64 pixel. Following acquisition, filter back projection reconstructions were performed.

The ^{99m}Tc-ciprofloxacin scintigrams were visually evaluated by three experienced nuclear medicine physicians in a blind fashion based on CT anatomic images. Sequential images captured from 0.5-6.0 h were mandatory for inclusion and interpretation. The scans were read independently, and any disagreements in interpretation were discussed and a consensus was reached on a majority basis. They were considered positive for infection when the pancreatic necrosis and peripancreatic tissue had a higher radionuclide uptake with a clear edge than the surrounding tissue, and negative for infection when the pancreatic necrosis and peripancreatic tissue had no significant radionuclide uptake. Diagnostic results were compared to bacterial culture and smear results. Semi-quantitative analysis was performed by determining the radioactivity counts of the pancreas, liver, spleen, renal, intestinal track, muscle and infectious foci in the pancreas using region of interest techniques, and the radioactivity of the muscle at the level of the pancreatic body was considered to be background. The measured values were averaged by three physicians. The lesion-to-background (L/B) ratios were then scored. The L/B curves changed with time in the groups and the optimal imaging time for diagnosis of infective SAP was thereby investigated.

CT scan and imaging analysis

CT was performed using a Sensation 64 scanner (Siemens Medical Solutions, Forchheim, Germany) 15 min after the SPECT scan. CT scanning (plain plus enhanced) was performed using the following parameters: a 3 mm slice thickness, 120 kV, 110 mAs, a 512 × 512 matrix, and 1.5 mL/kg of contrast material (Ultravist 300 mg I/mL; Schering AG, Germany) at a rate of 2 mL/s. The images were read by the same three nuclear medicine physicians and a consensus was reached on a majority basis according to the following criteria: visible gas bubbles scattered within the pancreatic necrosis or peripancreatic fluid on the CT images were considered positive[24].

Pathologic study, bacterial culture and smear testing

After image examination, the animals were euthanized to remove the pancreas, and fluid was aspirated from the injection area or necrotic focus for bacterial culture or smear testing. Tissue samples were stained with hematoxylin and eosin (HE), and observed for evidence of pathologic changes to the pancreas. The criteria for diagnosing SAP secondary infection were as follows: (1) the appearance of the isolated pancreatic specimen was consistent with the pathologic diagnosis of SAP, whereby acute purulent inflammatory foci were present; and (2) the result of bacterial cultures from the necrotic area were positive or the presence of infection was confirmed using a smear. Diagnoses were made independently by a senior pathologist with no prior knowledge of the specimens.

Statistical analysis

Quantitative data were expressed as the mean ± SD. The sensitivity, specificity, accuracy, positive predictive value (PV^+), and negative predictive value (PV) of each imaging diagnosis were calculated. The effect of group and time on L/B was analyzed using two factor-repeated measure analysis of variance, the comparisons for the changes of L/B over time among groups were analyzed using one factor-repeated measure analysis of variance, the comparisons at the different time points in the same group and the comparisons among groups at the same time point were subjected to the Bonferroni test. Comparison of two rates was subjected to the χ^2 test. SPSS 10.0 software (SPSS, Chicago, IL, United States) was used for analysis and $P < 0.05$ was considered statistically significant.

RESULTS

In group A, all six swine survived. In group B, 1 animal was excluded due to a main pancreatic duct intubation

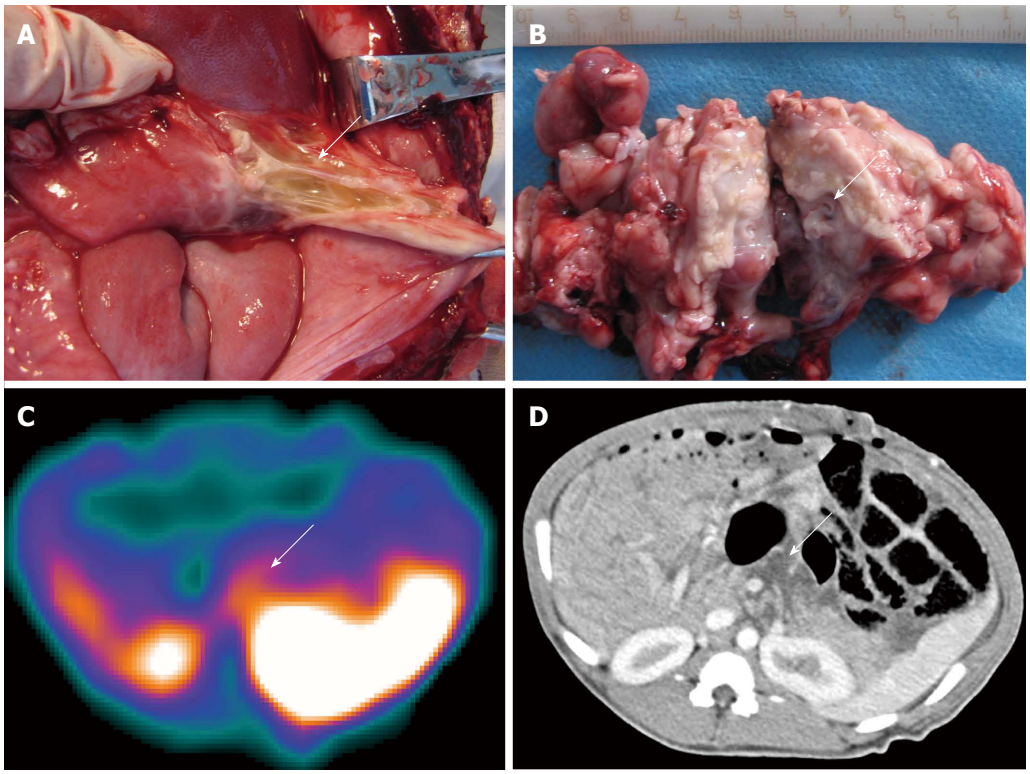

Figure 1 ^{99m}Tc-ciprofloxacin scintigraphy has higher sensitivity and accuracy in the detection of bacterial infections than computed tomography (the swine came from group C). A: The evidence of secondary infections in the peripancreatic fluid were confirmed by pathological examination and by bacterial smear or culture testing; B: Pancreatic necrosis was confirmed by pathological examination and by bacterial smear or culture testing; C: ^{99m}Tc-ciprofloxacin scintigraphy at 3 h demonstrated accumulation of radioactivity in the peripancreatic fluid and pancreatic necrosis (L/B = 3.37), indicating a true positive result; D: Enhanced computed tomography analysis showed low-density necrosis and peripancreatic fluid collection (arrow) without any indication of infection.

failure and another died of asphyxiation during anesthesia. In group C, one animal was also excluded from further analysis due to a main pancreatic duct intubation failure and two animals died of disease progression after the onset of SAP. Thus, in groups B and C, 7/9 and 15/18 were subjected to imaging analysis, respectively (Table 1).

Pathology findings

In group A, none of the swine showed infectious foci in the pancreas (Table 1). In group B, one of the seven animals (1/7) showed a focus with a *Staphylococcus aureus*/*E. coli* mixed infection. Light microscopy analysis of HE-stained sections revealed liquefactive necrosis in the center of this infectious focus. The remaining 6 animals in group B showed no bacterial infection (Table 1). In group C, 15 swine showed successful induction of a secondary infection. Bacterial culture and smear analysis of the necrotic foci in the pancreas showed that 14 SAP swine were infected with *E. coli* alone and 1 with a mixture of *E. coli* and Streptococcus, and showed intestinal perforation caused by this SAP secondary infection after paunching (Table 1). A total of 16 foci were found and yellow liquid flowed out of the cross-sections (Figure 1A and B). One animal had two cystic lesions in the pancreatic body and tail, with diameters of 19 and 5 mm, respectively. HE staining of infectious foci in group C showed liquefactive necrosis in most parts of the focal center, structureless substances in the fat cytoplasm, and coagulative necrosis in part of the foci.

Visual analysis

In group A, ^{99m}Tc-ciprofloxacin scintigraphy revealed high radionuclide uptake in the kidneys, liver and spleen with excretion to the urinary bladder. No activity was observed in the area of the pancreas, normal bone marrow, muscle or gastrointestinal tract at any time point (Figure 2A). The CT images showed that the pancreatic parenchyma of all 6 animals were homogeneous and uniformly enhanced after contrast administration (Figure 2A).

In group B, no radionuclide uptake in the pancreatic areas was detected by SPECT in 5 of 7 animals at any time point (Figure 2B) and these swine were therefore diagnosed as negative for secondary infection (Table 2). Mild uptake in the pancreatic area was evident in one animal and the L/B was 2.15 at 3 h after administration, indicating a positive diagnosis of secondary infection (Table 2). However, pathology only displayed significant proliferation of granulated tissue at the edge of the necrotic area, no infectious focus was found in this animal by either pathologic or bacterial examinations (Figure 3A-C). Radionuclide uptake in the pancreatic area was detected in one animal, which was subsequently found to be infected with a mixture of *S. aureus* and *E. coli* in the pancreatic necrosis (Table 2). CT images revealed the pancreas had enlarged markedly and that the gastroin-

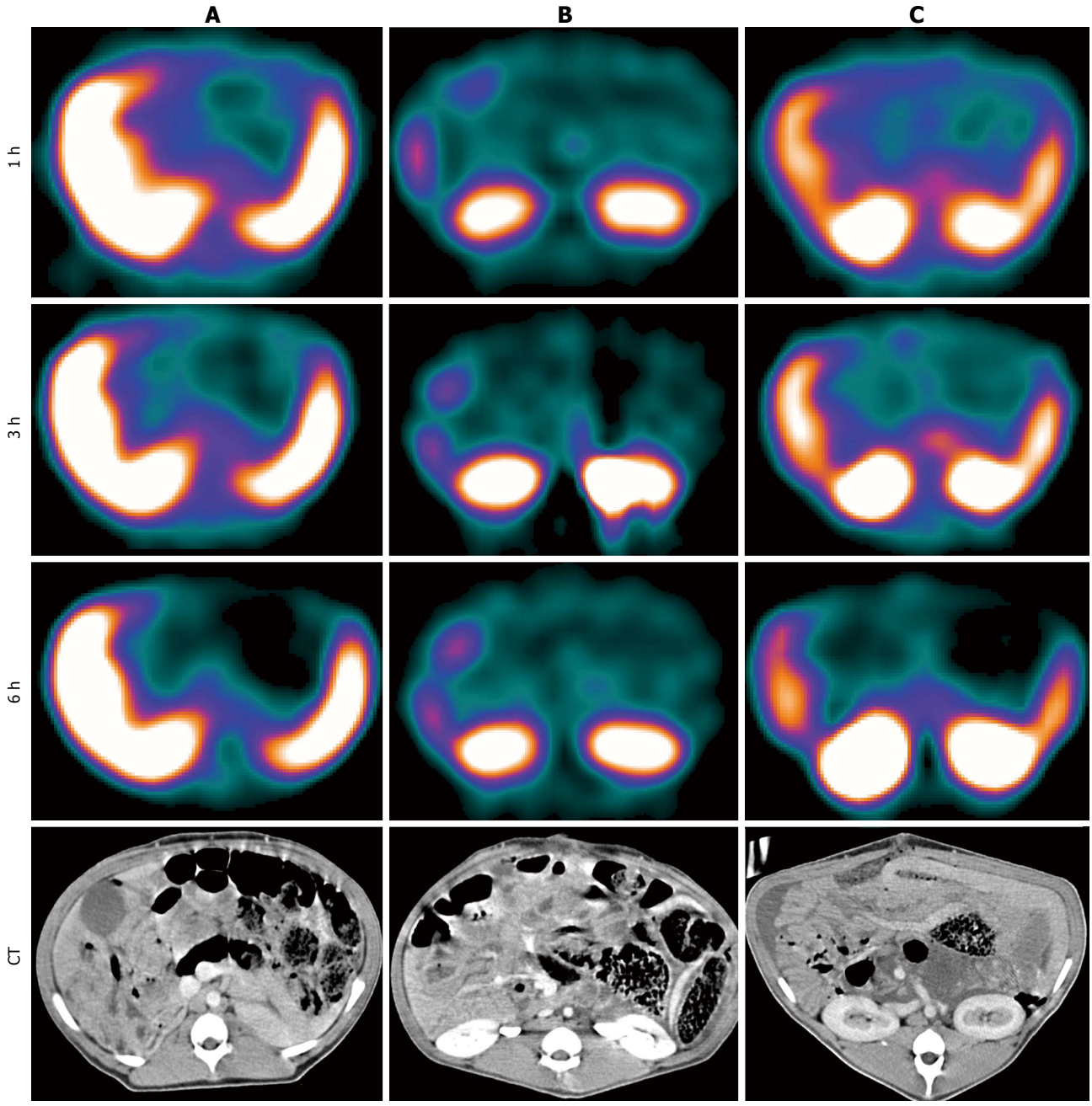

Figure 2 ^{99m}Tc-ciprofloxacin and computed tomography imaging of a normal swine pancreas and both non-infected severe acute pancreatitis swine and an animal with secondary infection associated with severe acute pancreatitis. A: No uptake indicating no infection, and a homogenous pancreatic parenchyma is evident in the normal pancreas by computed tomography imaging; B: A lower accumulation of ^{99m}Tc-ciprofloxacin in the pancreatic necroses of non-infected severe acute pancreatitis (SAP) swine; C: A higher accumulation of this agent in SAP swine with a secondary infection focus is evident.

testinal tract was expanded and associated with effusion. Focal or pathy hypoattenuated areas within the pancreatic parenchyma were observed in all animals in group B and there were no signs of gas bubbles (Figures 2B and 3D).

In group C, one animal was interpreted as negative by scintigraphy, which was proven to be a misdiagnosis by pathological examination, and by bacterial culture and smear testing. The remaining 14 animals in this group showed radioactive accumulations in the pancreatic area and were diagnosed as positive for secondary infection (Figures 1C and 2C), which was confirmed in each case by pathologic examination and bacterial culture (Table 2). One animal showed irregular patches of radioactivity accumulation around the pancreatic area due to intestinal perforations (Figure 4A). The animal with two lesions in the pancreatic body and tail was found to have a bigger focus in the pancreatic body, while smaller lesions were unclear.

CT images revealed that the pancreas was enlarged with irregular patchy and round-like cystic low-density necrotic areas, and effusion around the pancreas (Figures 1D and 2C). One animal had two round-like cystic lesions in the pancreatic body and tail, with diameters of 19 and 5 mm, respectively, without significant enhancement after

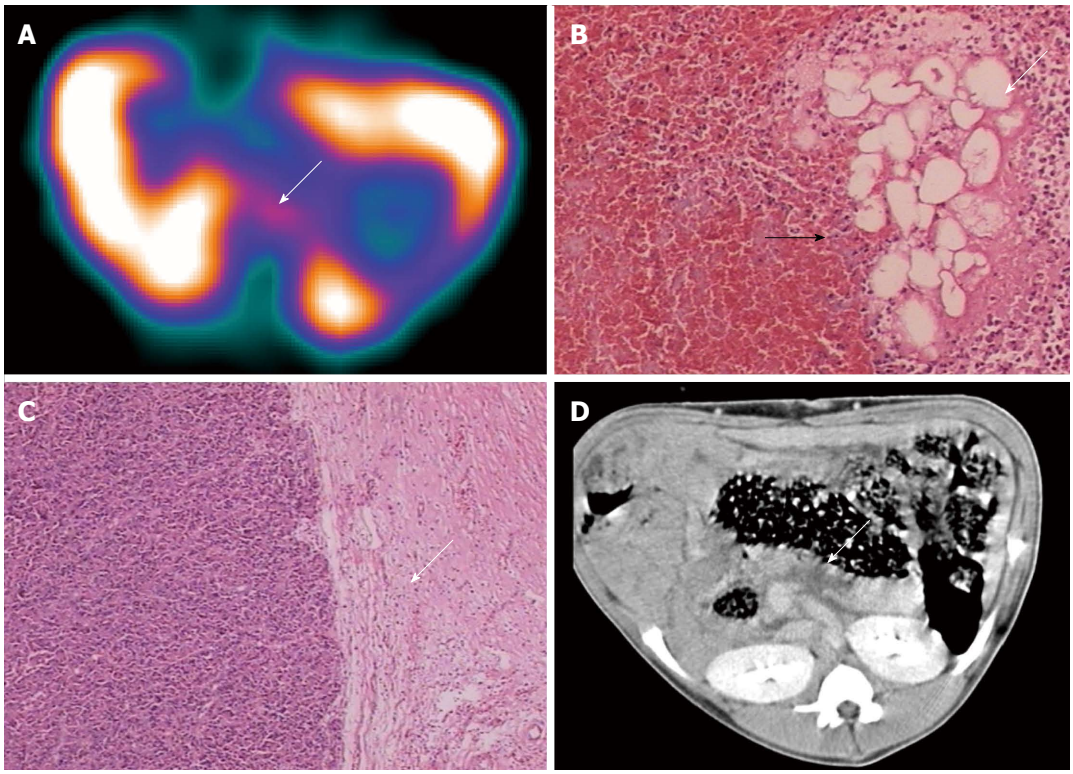

Figure 3 False-positive case of ^{99m}Tc-ciprofloxacin scintigraphy (the swine came from group B). A: ^{99m}Tc-ciprofloxacin scintigraphy (arrow) is indicative of secondary infection (L/B = 2.15); B: However, light microscopy analysis showed pancreatic tissue and fat tissue necrosis (arrow), surrounding a marked hyperplastic area in granulated tissue (black arrow); C: Fibrous tissue (arrow), but no associated bacterial infection; D: Computed tomography image showed focal hypoattenuated areas within the pancreatic parenchyma (arrow) without gas bubbles.

Table 2 Pathologic diagnosis, ^{99m}Tc-ciprofloxacin and computed tomography imaging analysis of secondary bacterial infection in a severe acute pancreatitis animal model

	Pathologic diagnosis	SPECT		CT	
		Infection	Non-infection	Infection	Non-infection
Group A	Infection	0	0	0	0
	Non-infection	0	6	0	6
Group B	Infection	1	0	0	1
	Non-infection	1	5	0	6
Group C	Infection	14	1	2	13
	Non-infection	0	0	0	0

SPECT: Single photon emission computed tomography; CT: Computed tomography.

contrast administration and were diagnosed as pseudocysts. Gas bubble signs were found in 2 swine, and one showed intestinal perforations and gas bubbles scattered throughout the necrotic area and in the peripancreatic fluid (Figure 4B), and the other showed gas bubbles in the pancreatic necrosis.

Quantitative analysis

Based on our histopathological and biological results, the number of normal, non-infected and infected SAP swine was 6, 6 and 16, respectively (Table 1). It was calculated that ^{99m}Tc-ciprofloxacin scintigraphy had a sensitivity of 93.8% (15/16), a specificity of 91.7% (11/12), an accuracy of 92.9% (26/28), a PV+ of 93.8% (15/16), and a PV of 91.7% (11/12) for detecting secondary bacterial infection associated with SAP (Table 3), and these values for CT were 12.5% (2/16), 100.0% (12/12), 50.0% (14/28), 100.0% and 46.2% (12/26), respectively. Of these parameters, sensitivity, accuracy and PV were significantly lower than those of ^{99m}Tc-ciprofloxacin scintigraphy ($P < 0.01$) (Table 3).

^{99m}Tc-ciprofloxacin scintigraphy results at different time points

In infected SAP swine, the infectious foci in the pancreatic tissues showed no radionuclide uptake at 0.5 h, mild uptake at 1 and 2 h, and peak radioactivity counts at 3 h (2350.25 ± 602.35 k), and then gradually decayed from 4-6 h. The change was different in the kidney, liver, spleen, gastrointestinal tract and muscle (Figure 5A). The L/B in 6 normal swine, 6 non-infected SAP and 16 infected SAP animals at 0.5, 1, 2, 3, 4, 6 h after the administration of ^{99m}Tc-ciprofloxacin are presented in Figure 5B. There were significant differences in the L/B changes over time among the three study groups ($F = 95.66$, $P < 0.001$). These changes in the infected SAP animals differed significantly from those in the non-infected SAP ($F = 88.63$, $P = 3.1e^{-16}$) and normal swine ($F = 63.61$, $P = 8.2e^{-13}$). In contrast, no significant differences were found between the non-infected SAP and normal groups ($t = 1.17$, $P = 0.251$). The L/B ratio at 3 h after the administration of

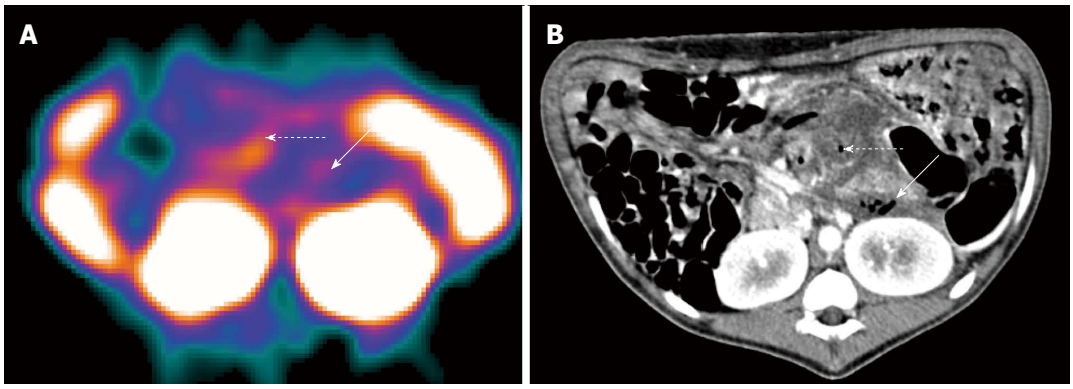

Figure 4 Swine with severe acute pancreatitis and secondary bacterial infection (from group C). A: The highest level of radioactivity accumulation was found by ^{99m}Tc-ciprofloxacin scintigraphy at 3 h (L/B = 3.42); B: Multiple bubbles scattered in the necrotic area and peripancreatic fluid were demonstrated on computed tomography images. Intestinal perforation caused by severe acute pancreatitis was found at autopsy.

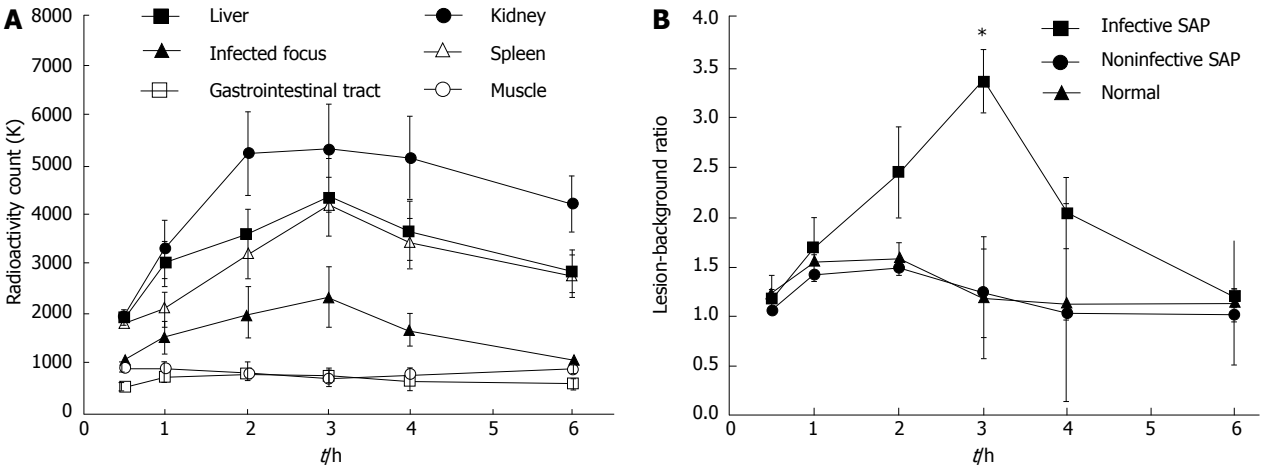

Figure 5 ^{99m}Tc-ciprofloxacin scintigraphy results at different time points. A: Curves showing radioactivity count changes over time in the abdominal tissues of swine with infected severe acute pancreatitis (SAP) are shown. These curves show high uptake in the kidneys and moderate uptake in the liver and spleen. No activity was observed in the areas of the muscle or gastrointestinal tract. The radioactive uptake by infectious foci increased gradually over time, reached a peak at 3 h, and then gradually decayed; B: Lesion-background ratio change curves for the normal pancreas, and non-infected and infected SAP pancreas in the swine model are shown. The curves show that the optimal lesion-background ratio occurred at 3 h after administration of Infecton in positive SAP animals.

Table 3 Results of imaging diagnosis for secondary infection of animals with severe acute pancreatitis *n* (%)

	Imaging results	Pathologic results		Sensitivity	Specificity	Accuracy	PV+	PV-
		+	-					
Infection	+	15	1	15 (93.8)[b]	11 (91.7)	26 (92.90)[b]	15 (93.8)	11 (91.7)[b]
	-	1	11					
CT	+	2	0	2 (12.5)	12 (100.0)	14 (50.0)	2 (100.0)	12 (46.2)
	-	14	12					

[b]$P < 0.01$ *vs* computed tomography (CT). +: Infection; -: Non-infection; PV+: Positive predictive value; PV-: Negative predictive value.

^{99m}Tc-ciprofloxacin in the infected SAP swine reached 3.36 ± 0.33, which was significantly higher than at all other time points (*P* values were 1.1e^{-35}, 3.5e^{-27}, 3.9e^{-13}, 4.2e^{-21} and 2.5e^{-35}, respectively).

DISCUSSION

Secondary infection of pancreatic necrotic tissue is accepted as one of the most important prognostic indicators of disease severity and outcomes in SAP cases[4-6]. Early diagnosis is the key to improved treatment outcomes and reduced mortality. Although CT plays an important role in the diagnosis of SAP, it can not detect the sites of secondary infection with sufficient sensitivity as it can only do so if gas bubbles appear within the lesion, which occurs infrequently. Indeed, Bhansali *et al*[25]

reported previously that bubbles were detectable in only 16% of SAP patients with secondary infections using CT imaging (21/131 cases). Our present experimental results indicate that the sensitivity of CT is too low (12.5%, 2/16) to accurately diagnose a SAP secondary infection.

The novel radiopharmaceutical used in our scintigraphy analysis is based on the 4-fluoroquinolone broad spectrum antibiotic ciprofloxacin. Following intravenous injection, ciprofloxacin is widely distributed in the body and is excreted *via* the kidneys. The mode of action of ciprofloxacin is mediated *via* inactivation of the bacterial DNA gyrase, which results in the retention of this agent at the sites of active bacterial infection[18]. Sierra *et al*[26] analyzed the mechanism of intracellular accumulation of ^{99m}Tc-ciprofloxacin in *Staphylococcus aureus* and *Pseudomonas aeruginosa* strains, and found that ^{99m}Tc-ciprofloxacin was equally accumulated intracellularly in all tested strains, while $^{99m}TcO^{4-}$ did not show accumulation in any of the strains. The absence of intracellular $^{99m}TcO^{4-}$ indicated that the entire radioactivity detected in the ^{99m}Tc-ciprofloxacin assay was due to the accumulation of the radiopharmaceutical compound, rather than free $^{99m}TcO^{4-}$.

^{99m}Tc-ciprofloxacin has been widely tested in clinical studies and shown to be taken up by a wide range of live (but not dead) bacteria *in vitro* and *in vivo* [12,13,17,18]. It has also been reported that the specificity of ^{99m}Tc-ciprofloxacin scintigraphy reached 85%-96% in detecting both bone and joint bacterial infections[27-29] and showed a sensitivity and specificity of 91.7% and 75%, respectively, in the diagnosis of acute bacterial cholecystitis[19]. In our present study, the sensitivity, specificity and accuracy of ^{99m}Tc-ciprofloxacin were found to be 93.8%, 91.7% and 92.9%, respectively. The accuracy was higher than that of CT. We found that ^{99m}Tc-ciprofloxacin scintigraphy had some important advantages over WBC imaging which have also been reported by other studies. Firstly, the technique used for preparation is easy, and can be carried out without withdrawing blood, purifying leucocytes, labeling and reinjecting the radiolabeled cells, compared to WBC imaging. Secondly, no adverse effects were reported in response to intravenous administration of ^{99m}Tc-ciprofloxacin. Thirdly, both the radiochemical purity and labeling rate were found to be over 90% within 6 h at room temperature. Hence, ^{99m}Tc-ciprofloxacin is an ideal specific targeting agent for the detection of bacterial infection[17,27-29].

In infected SAP cases, the radioactivity levels in the bacterial foci were higher than in the surrounding SAP, muscle or soft tissues. Pronounced focal accumulation was evident at 3 h after administration, when both the radioactivity counts and lesion-background ratios were at peak levels. This suggests that ^{99m}Tc-ciprofloxacin scintigraphy is a suitable diagnostic test for SAP patients with a suspected secondary infection. However, both false-positive and false-negative results still arise in ^{99m}Tc-ciprofloxacin scintigraphy. In our present analyses, one false-negative result was found in a group C animal for which the pathology showed a pancreatic necrosis with a mild infection. This may suggest that the detectable uptake of ^{99m}Tc-ciprofloxacin has a severity of infection threshold. One false-positive result was also found in an animal showing marked hyperplasia of granulated and fibrous tissue at the edge of the pancreatic necrosis, which suggested that radioactive uptake may be related to granulated tissue repair at the edge of necrotic foci, as well as an increase in blood perfusion or capillary permeability[30].

We also found that SPECT visualization has disadvantages, including low resolution and poor display of anatomic structure. It can not display the shape and area of pancreatic necrotic foci, nor display the non-infected foci with peripancreatic fluid or pseudocysts. However, while the clinical use of SPECT-CT is widely accepted, the resolution and capability for displaying anatomical details of the SPECT-CT scanner are much improved[31]. Incorporating SPECT with CT in one scanner, which has the advantages of the two imaging techniques, makes it possible to evaluate and diagnose the infected foci with indefinite anatomical localization and low radioactive uptake.

In summary, ^{99m}Tc-ciprofloxacin scintigraphy has a higher sensitivity and accuracy in the detection of bacterial infections than CT. Moreover, this agent is not taken up in a normal pancreas and non-infected SAP, which could be highly useful in the detection of infectious SAP. This method may therefore become an effective tool in the future for accurately diagnosing and assessing the severity of secondary infections in human SAP patients. Undoubtedly, it is very important for clinicians to develop treatment programs and improve the efficacy of SAP. ^{99m}Tc-ciprofloxacin scintigraphy, ^{18}F-FDG PET and diffusion-weighted imaging (DWI) have been applied widely for the diagnosis of infection, but they have advantages and limitations[32-36]. In the future, we will evaluate the efficacy of these commonly used techniques in the diagnosis of secondary infection of SAP.

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COMMENTS

Background

Secondary infection is one of the most challenging problems in the treatment of severe acute pancreatitis (SAP). Infected pancreatic necrosis in patients with the clinical signs and symptoms of sepsis is an important indication for interventional therapy including surgery and drainage, whereas patients with sterile necrosis should be managed conservatively and undergo intervention only under certain circumstances. Conventional scintigraphic and radiologic methods have limitations in the detection of secondary infection of SAP. It is therefore essential to develop a sensitive and specific imaging methodology that will non-invasively detect secondary infections in SAP patients. However, to their knowledge, only a few authors have applied traditional radionuclide imaging agents for the diagnosis of pancreatitis associated with infection. The utility of infecton in the detection of SAP secondary infection is still unclear.

Research frontiers

In the area of SAP, one of the research hotspots is the detection of second-

ary infection of pancreatic necrosis. Over the past few decades, a number of radiopharmaceuticals have been developed to investigate infective and non-infective inflammatory disorders. In this regard, ^{99m}Tc-ciprofloxacin may be one of the most promising agents in the field of nuclear medicine. This radiochemical combines the advantages of a ^{99m}Tc label and the broad-spectrum bacteria-localizing capability of ciprofloxacin, which has a higher sensitivity and specificity for bacterial infections than white blood cell scans.

Innovations and breakthroughs

The authors successfully used a specific inflammatory agent, ^{99m}Tc-ciprofloxacin, which non-invasively detected secondary infections in an infective SAP model with higher sensitivity and accuracy than computed tomography (CT). To our knowledge, there have been no previous studies that have compared the differential diagnosis of non-infectious and infectious SAP using ^{99m}Tc-ciprofloxacin imaging and histopathological and biological methods.

Applications

This method may be an effective tool in the future for accurately diagnosing and assessing the severity of secondary infections in human SAP patients. Undoubtedly, it is very important for clinicians to develop treatment programs and improve the efficacy of SAP.

Terminology

SAP is defined as necrosis involving at least 30% of the pancreas as visualized by contrast-enhanced CT, with greater involvement indicating greater severity of necrosis. It is a special type of acute pancreatitis with more complications and high mortality. The 4-fluoroquinolone broad spectrum antibiotic, ciprofloxacin, was labeled with $^{99m}TcO^{4-}$. The mode of action of ciprofloxacin is mediated *via* the inactivation of bacterial DNA gyrase, which results in the retention of ^{99m}Tc-ciprofloxacin at the sites of active bacterial infection.

Peer review

This is a well-presented manuscript describing a good research protocol on the use of ^{99m}Tc-ciprofloxacin in the detection of infection in severe acute pancreatitis in an animal model. The single photon emission CT (SPECT) images, with high background activity in the nearby organs, are indistinct, as is to be expected at this resolution. As the authors themselves have pointed out, SPECT-CT hybrid imaging would provide better images and localize the abnormal tracer activity more precisely to the focus of infection. The results are interesting and suggest that ^{99m}Tc-ciprofloxacin scintigraphy may therefore become an effective tool in the future for accurately diagnosing and assessing the severity instances of secondary infections in human SAP patients.

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ORIGINAL ARTICLE

Pancreatitis in patients with pancreas divisum: Imaging features at MRI and MRCP

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Abstract

AIM: To determine the magnetic resonance cholangiopancreatography (MRCP) and magnetic resonance imaging (MRI) features of pancreatitis with pancreas divisum (PD) and the differences *vs* pancreatitis without divisum.

METHODS: Institutional review board approval was obtained and the informed consent requirement was waived for this HIPAA-compliant study. During one year period, 1439 consecutive patients underwent successful MRCP without injection of secretin and abdominal MRI studies for a variety of clinical indications using a 1.5 T magnetic resonance scanner. Two experienced radiologists retrospectively reviewed all the studies in consensus. Disputes were resolved *via* consultation with a third experienced radiologist. The assessment included presence and the imaging findings of PD, pancreatitis, and distribution of abnormalities. The pancreatitis with divisum constituted the study group while the pancreatitis without divisum served as the control group. MRCP and MRI findings were correlated with final diagnosis. Fisher exact tests and Pearson × 2 tests were performed.

RESULTS: Pancreatitis was demonstrated at MRCP and MRI in 173 cases (38 cases with and 135 cases without divisum) among the 1439 consecutive cases. The recurrent acute pancreatitis accounted for 55.26% (21 of 38) in pancreatitis patients associated with PD, which was higher than 6.67% (9 of 135) in the control group, whereas the chronic pancreatitis was a dominant type in the control group (85.19%, 115 of 135) when compared to the study group (42.11%, 16 of 38) (χ^2 = 40.494, P < 0.0001). In cases of pancreatitis with PD, the dorsal pancreatitis accounted for a much higher percentage than that in pancreatitis without PD (17 of 38, 44.74% *vs* 30 of 135, 22.22%) (χ^2 = 7.257, P < 0.05).

CONCLUSION: MRCP and MRI can depict the features of pancreatitis associated with divisum. Recurrent acute pancreatitis and isolated dorsal involvement are more common in patients with divisum.

Key words: Pancreas divisum; Pancreatitis; Diagnosis; Magnetic resonance imaging; Magnetic resonance cholangiopancreatography

Core tip: We reviewed 1439 cases of abdominal magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP). There were 122 cases of pancreas divisum (PD) and 38 of them were diagnosed as pancreatitis. The pancreatitis associated with PD was usually distributed in dorsal pancreas and presented as recurrent acute type. MRCP in combination with MRI can accurately detect ductal and paren-

chymal abnormalities of pancreas. Therefore, MRCP and MRI should be referred to as primary diagnostic tools for pancreatitis with PD whereas endoscopic retrograde cholangiopancreatography can be reserved for those who require therapeutic interventions.

Wang DB, Yu J, Fulcher AS, Turner MA. Pancreatitis in patients with pancreas divisum: Imaging features at MRI and MRCP. *World J Gastroenterol* 2013; 19(30): 4907-4916 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4907.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4907

INTRODUCTION

Pancreas divisum (PD) is the most common developmental anatomic variant of pancreatic duct with a reported incidence of 4%-14% in the population at autopsy series, 3%-8% at endoscopic retrograde cholangiopancreatography (ERCP), and 9% at magnetic resonance cholangiopancreatography (MRCP)[1-5]. This abnormality occurs when the dorsal and ventral pancreas anlage fails to fuse during the 6th-8th week of gestation. PD is characterized not only by the anatomical morphology but also by the physiology in which the majority of pancreatic juice drains through the duct of Santorini into the duodenum at orifice of minor papilla while the minority (about 10%) drains through the (ventral) duct of Wirsung into the duodenum at major papilla[1]. Although the clinical significance still remains controversial, there seems to be an association between PD and chronic abdominal pain and recurrent acute pancreatitis. Moreover, the timely and appropriate therapeutic interventions such as minor papillotomy or stent placement in the dorsal pancreatic duct or surgical procedures can benefit the patients with symptomatic PD remarkably from reducing the pressure in the main pancreatic duct[6].

The manifestations of acute and chronic pancreatitis at magnetic resonance imaging (MRI) and magnetic resonance (MR) cholangiopancreatography (MRCP) have been well described in previous studies[7-10], however, to our knowledge, there is no published literature on imaging features of pancreatitis in patients with PD using MRI together with MRCP without secretin injection. Although ERCP is considered as a gold standard of diagnosis, prior studies have shown that there is a great correlation between MRCP and ERCP in detecting PD[11,12]. Currently, the multidetector computed tomography (MDCT) has been reported to be valuable in the detection of PD[13]. As a noninvasive approach, MRCP can be used much more extensively than ERCP when radiation is in consideration and can always be performed together with MRI, which can depict the morphologic changes in detail[9,14]. Therefore, since MRI and MRCP can be employed to establish a diagnosis non-invasively, including for patients who are unable to undergo diagnostic ERCP, the ERCP can be reserved for those who require therapeutic intervention.

Therefore, the purpose of this study was to retrospectively evaluate the imaging features of pancreatitis in patients with PD at MRI and MRCP without injection of secretin and to describe the differences of MR imaging between pancreatitis with and without divisum.

MATERIALS AND METHODS

Patient population and proof of diagnosis

During one year period, a total of 1439 consecutive patients (age range, 16-95 years; 698 men and 741 women) consecutively underwent successful abdominal MRI and MRCP without injection of secretin in our institution for a variety of clinical indications. Among them, 173 cases were finally diagnosed as pancreatitis based on clinical presentations, laboratory values, and imaging findings. Of the 173 cases, 38 cases associated with PD constituted the study group in this study. A total of 42 times of ERCP examination and interventional therapy were performed in 21 cases in the study group. Among them, minor papillotomy and temporary transpapillary stent placement in main pancreatic duct (n = 15) through minor papilla, stent placement in common bile duct (CBD) (n = 3), and surgery of Puestow procedure (n = 1) were performed. Eighteen patients were male and 20 were female, with a mean age of 43.6 years (range, 20-79 years). The remaining 135 cases of pancreatitis without PD (66 male and 69 female) served as control group, aged from 19 to 85 years with a mean age of 53.4 years. We obtained the institutional review board approval and waiver of informed consent for this retrospective HIPPA-compliant study.

The recurrent acute pancreatitis was defined in this study as a clinical setting in which the clinical or/and serologic features were characteristic of acute pancreatitis with a history of recurrence at least 2 times. The clinical data, which included symptoms at presentation, history of previous episodes of pancreatitis, associated symptoms involving other systems, and laboratory findings, were reviewed for all of the 173 cases of pancreatitis in this study.

Imaging techniques

All the MR studies including coronal MRCP and axial MRI were performed with a 1.5-T MR imager (Magneton Vision; Siemens, Erlangen, Germany) using a phase array body coil. The MRCP images were initially obtained and axial MR imaging followed. The pancreaticobiliary tract was localized with a thick-slab (40 mm) half-Fourier RARE image in coronal-oblique (25 degrees) and axial planes, which necessitated an acquisition time of 7 s. The thin-slab MRCP acquisitions were obtained at various angles that allowed optimal visualization of the bile and pancreatic ducts; the number of thin-slab acquisitions per patient ranged from 3 to 15 (mean, 7 acquisitions). Both the thick-slab and thin slab images were obtained during breath hold. The half-Fourier RARE parameters included repetition time ms/echo time ms (effective) of ∞/95.0;

echo train length, 128; flip angle, 150 degrees; section thickness, 3.0 mm with no gap; field of view, 270 mm × 270 mm; number of signals acquired, 1; matrix, 240 × 256 and acquisition time, 20 s. Fat saturation and shim adjustments were used in all cases.

After MRCP, conventional axial MR imaging was conducted to examine the abdomen. MR imaging sequences included unenhanced T1-weighted breath-hold spoiled gradient echo (148/5 ms; flip angle, 70 degrees; section thickness, 10 mm; gap, 30%), unenhanced T2-weighted breath-hold fast SE (3500/138 ms; section thickness, 8 mm; gap, 25%), unenhnaced in phase and out-of-phase T1-weighted gradient recalled echo, unenhanced and double-phased dynamic contrast-enhanced T1-weighted fat suppression (200/4.4 ms; flip angle, 70 degrees; section thickness, 8 mm; gap, 20%) 30 and 60 s after beginning of intravenous administration of the contrast materials. Gadopentetate dimeglumine (Magnevist; Berlex Laboratories, Wayne, NJ, United States) was administered intravenously using an automatic injector at a dose of 0.1 mmol per kilogram of body weight as a bolus followed by a normal-saline flush.

Imaging analysis

All the images in this study were reviewed retrospectively using interactive picture archiving and communicating system (PACS) workstations by two experienced (10-15 years of practice) abdominal radiologists (Wang D and Yu J) in consensus. The disputes were resolved *via* consultation with a third experienced abdominal radiologist (Fulcher AS). During the reading, the following items were taken into account: (1) classification of PD, complete PD or incomplete PD; (2) distribution of pancreatitis in the pancreas (ventral, dorsal or ventral plus dorsal pancreas); (3) morphologic changes including pancreatic parenchyma (enlargement or atrophy of pancreas), and pancreatic duct changes (side branch ectasia, pancreatic ductal dilatation and strictures, and intraductal calculi); (4) signal intensity abnormalities on unenhanced or enhanced images, including necrosis or cystic changes in pancreas; (5) changes outside of the pancreas, *i.e.*, peripancreatic stranding, fluid collections, and involvement of the adjacent organs and vessels, *etc*; and (6) abscess inside pancreas and outside of pancreas. The classifications and distributions of pancreatitis were compared between the study group and control group. After careful analysis of the abovementioned findings, the imaging features of pancreatitis associated with PD were established.

Statistical analysis

Pearson χ^2 and Fisher exact probability test were introduced for classification and distribution of pancreatitis in patients with PD in the study group compared with those in the control group. A P value less than 0.05 was considered to indicate a statistically significant difference.

Table 1 Comparison of classification of pancreatitis with and without pancreas divisum *n* (%)

	Acute pancreatitis	Chronic pancreatitis	Recurrent acute pancreatitis	Total
Pancreatitis with PD	1 (2.63)	16 (42.11)	21(55.26)	38
Pancreatitis without PD	11 (8.14)	115 (85.19)	9 (6.67)	135
Total	12 (6.93)	131 (75.72)	30(17.34)	173

PD: Pancreas divisum.

RESULTS

Clinical features of the pancreatitis superimposed on PD

The classifications of pancreatitis in the 38 cases with PD in the study group included: recurrent acute pancreatitis in 21 cases (all cases with abdominal pain, 5 with gallstones, 1 with jaundice, and 16 with hyperlipasemia and hyperamylasemia), chronic pancreatitis in 8 cases (all cases with abdominal pain, 3 with mild serologic enzymes elevation, and 1 with gallstones) and the other 8 cases revealed with chronic pancreatitis incidentally at MRI and MRCP primarily for detecting hepatic lesions or biliary abnormalities, and acute pancreatitis in 1 case with worsening abdominal pain and serum lipase elevation of more than 1000 U/LH (normal, 23-300 U/LH). The gallstone pancreatitis was the dominant type in the control group (75.6%, 102/135). The other etiologies included intrapancreatic calculi (11.1%, 15/135), pancreatic ductal strictures (5.9%, 8/135), and autoimmune pancreatitis (4.4%, 6/135); and no distinct etiologic factors were found in 4 of the 135 cases (3%). The recurrent acute pancreatitis accounted for 55.26% (21 of 38) in pancreatitis with PD, which was higher in percentage than 6.67% (9 of 135) in the control group, whereas the chronic pancreatitis was a dominant type in the control group (85.19%, 115 of 135) when compared to the study group (42.11%, 16 of 38) (χ^2 = 40.494, P < 0.0001) (Table 1). The pancreatitis in patients with PD accounted for 21.96% (38 of 173) in the total population of pancreatitis in this study.

Imaging features of the pancreatitis superimposed on PD

Ductal and parenchymal changes of pancreas: Pancreatic duct in patients with PD and without PD both showed dilatation (6 *vs* 65), irregularity (16 *vs* 86), dilatation with irregularity (8 *vs* 80), focal stricture (6 *vs* 31), intrapancreatic duct calculi (2 *vs* 15), and side branch ectasia (36 *vs* 116) (Figure 1). In the study group, 7 cases of the isolated dorsal pancreatitis showed dilatation of main pancreatic duct all the way proximally to minor papilla. Two of recurrent cases each had a santorinicele of 5 mm (Figure 2) and 15 mm, respectively. Totally, 38 segments of duct of Santorini, 32 of duct of Wirsung, and 36 of duct in the body and in the tail as well were visualized

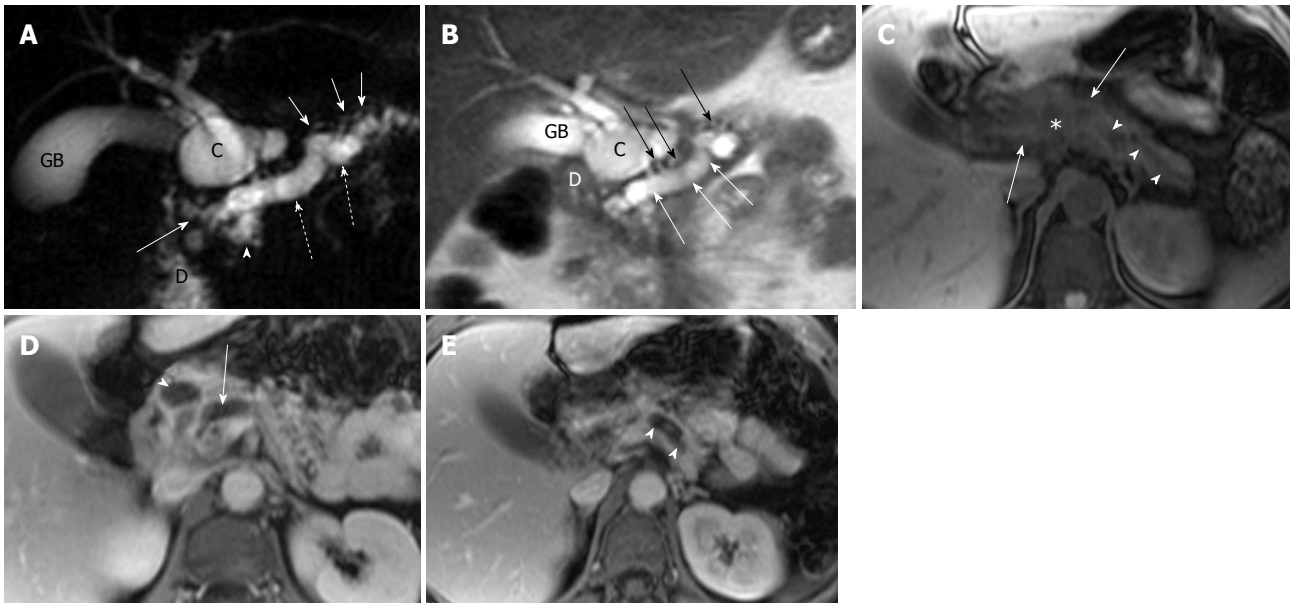

Figure 1 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of recurrent acute pancreatitis involving the entire pancreas in a 44-year-old woman with several episodes of abdominal pain. A: Coronal oblique thick-section rapid acquisition with relaxation enhancement magnetic resonance (RARE-MR) cholangiogram [infinite/1100 (effective), 40-mm section thickness] shows severe dilatation of pancreatic duct (dotted arrows) with stricture (solid arrow) just before entering the duodenum (D) and side branch ectasia (short arrows) as well. There is a pseudocyst (C) formation in pancreatic parenchyma. The gallbladder (GB) is distended and the common bile duct is dilated (arrowhead) as well; B: Thin-section half-Fourier RARE-MR cholangiogram [infinite/95 (effective), 3-mm section thickness] shows remarkable dilatation of dorsal pancreatic duct (white arrows) with severe side branch ectasia (black arrows). The pseudocyst (C) is formed in the pancreatic head region immediately adjacent to the duodenum (D) and GB; C: Axial precontrast T1WI SPGR shows the dilatation of pancreatic duct (arrowheads) and the pseudocyst (star) formation in the pancreatic neck were not easily appreciated. The signal intensity of the dorsal pancreas (arrows) is dramatically decreased; D: Axial postcontrast T1WI SPGR shows delayed enhancement of the dorsal pancreas and the wall of the pseudocysts (arrowhead). Dilatation of the pancreatic duct (arrow) was noted; E: Axial postcontrast T1WI SPGR shows delayed enhancement of the dorsal pancreas and the dilatation of the pancreatic duct (arrowheads).

Table 2 Caliber measurement in four portions of pancreatic duct at magnetic resonance-cholangiopancreatography

	Santorini	Wirsung	Body	Tail
Acute pancreatitis (mm)	4.5	2.4	3.5	2.2
Recurrent pancreatitis (mm)	3.81 ± 1.02	2.40 ± 1.360	4.62 ± 3.49	3.37 ± 1.99
Chronic pancreatitis (mm)	4.06 ± 1.26	2.11 ± 0.597	3.74 ± 2.01	3.24 ± 2.35
Mean (mm)	4.00 ± 1.17	2.22 ± 0.906	4.11 ± 2.72	3.27 ± 2.14

Data are expressed as absolute numbers or mean ± SD.

Table 3 Comparison of distribution of pancreatitis with and without pancreas divisum *n* (%)

	Dorsal	Ventral	Entire	Total
Pancreatitis with PD	17 (44.74)	0 (0)	21 (55.26)	38
Pancreatitis without PD	30 (22.22)	4 (2.96)	101 (74.81)	135
Total	47 (27.17)	4 (2.31)	122 (70.52)	173

PD: Pancreas divisum.

clearly enough to be measured. The mean duct diameter was 3.00 ± 1.17 mm (SD) for the duct of Santorini, 2.22 ± 0.906 mm for the duct of Wirsung, 4.11 ± 2.72 mm for the body, and 3.27 ± 2.14 mm in the tail segments (Table 2). There were 3 severe chronic cases and 3 recurrent cases of pancreatitis showing the maximum of dorsal pancreatic ductal dilatation measured from 5 mm to 13.5 mm.

In the study group, pancreatic edematous enlargement (n = 3), peripancreatic stranding (n = 13), atrophy with T1 signal intensity decrease (n = 12), atrophy with normal signal (n = 12), only T1 signal decrease (n = 3), and small intrapancreatic necrosis (n = 4) were detected in pancreatitis patients with PD. Six intrapancreatic pseudocysts were detected in 6 cases with a size ranging from 5 to 20 mm while 4 extrapancreatic fluid collections and pseudocysts were formed in the other 3 cases sized 35 to 76 mm. Thirty-two cases (recurrent acute, n = 21; chronic pancreatitis, n = 8) demonstrated delayed enhancement of pancreas (Figure 3). There were no adjacent organ and vessel involvement, and no hemorrhage or abscess formation due to pancreatitis in patients with PD.

Classification of pancreas divisum and distribution of pancreatitis: In the study group, 35 cases were classified as complete PD and 3 cases as incomplete PD. Totally, 16 cases with complete divisum and 1 with incomplete divisum comprised dorsal pancreatitis in patients with PD. In cases of pancreatitis with PD, the dorsal pancreatitis accounted for a much higher percentage than in pancreatitis without PD (17 of 38, 44.74% *vs* 30 of 135, 22.22%) (χ^2 =7.257, P < 0.05) (Table 3, Figures 4 and 5). Fifteen cases of dorsal pancreatitis were classified as recurrent acute pancreatitis. According to the anatomical involvement of lesion, the 17 cases of dorsal pancreatitis

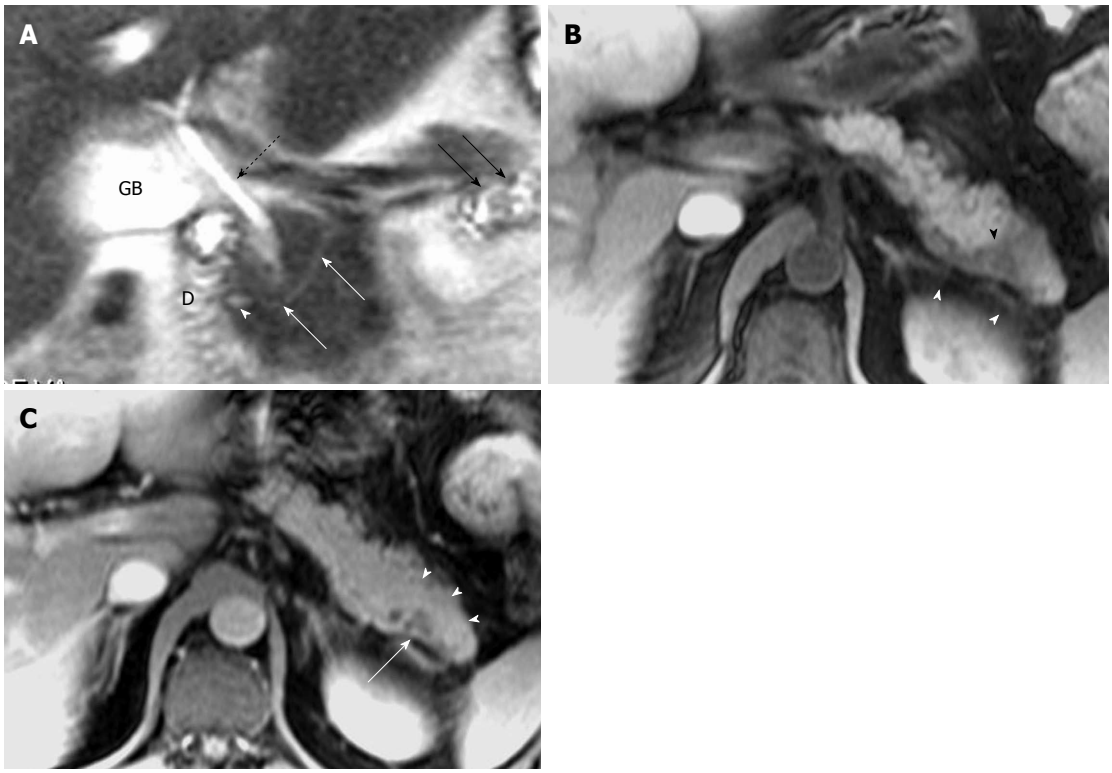

Figure 2 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of recurrent acute pancreatitis with pancreas divisum only involving the pancreatic tail with a small santorinicele in a 44-year-old man with several episodes of abdominal pain. A: Thin-section half-Fourier rapid acquisition with relaxation enhancement magnetic resonance (RARE- MR) cholangiogram [infinite/95 (effective), 3-mm section thickness] shows mild dilatation of the duct of Santorini (white arrows) with a focal enlargement consistent with santorinicele (arrowhead) at the entrance into the duodenum (D) *via* minor papilla. The side branch ectasia (black arrows) are noted in the pancreatic tail consistent with pancreatitis. Distended gallbladder (GB) and the CBD (dotted black arrow) are noted. B: Axial precontrast T1WI SPGR shows swelling and decrease of signal intensity in the pancreatic tail (black arrowhead), and thickening of the left anterior renal fascia (white arrowheads). C: Axial postcontrast T1WI SPGR shows mild delayed enhancement of pancreatic tail (arrowheads) with focal cystic changes (arrow).

could be classified as complete dorsal involvement (n = 7), suproanterior portion of head and neck involvement (n = 3), dominant body involvement (n = 2), and dominant tail involvement (n = 5) with ductal stricture at body-tail junction resulting in upstream ductal dilatation in the tail.

DISCUSSION

Classic PD (type 1) is defined as complete failure of fusion of the ducts of Santorini and Wirsung; other fusion anomalies with dominant dorsal drainage include absence of duct of Wirsung (type 2) and the presence of a filamentous or tiny caliber communication between the dominant dorsal duct of Santorini and the duct of Wirsung (type 3, incomplete PD)[15]. In patient with complete PD, a larger amount secretion from the dorsal pancreas could exert a significant burden on the relatively smaller orifice of minor duodenal papilla causing elevation of endoluminal pressure in pancreatic duct, resulting in subsequent pancreatitis. It has been reported that the clinical implications of incomplete PD may be similar to those of complete PD though the precise physiology may differ from each other[16,17].

A prior study with ERCP showed that the highest incidence of PD associated with idiopathic acute pancreatitis reached 50% in a total of 58 cases, which is significantly higher than in both controls and the whole population[18]. The recurrent acute pancreatitis in our study accounted for 55.26% (21 of 38) of pancreatitis in patients with PD, which is higher than in control group (6.67%, 9 of 135) (P < 0.0001, Table 1). The recurrent acute pancreatitis is a clinical entity that is characterized by repeated episodes of pancreatitis, which evolves over time with recurrent attacks of acute pancreatitis in otherwise normal pancreas until interventional procedures were performed in this clinical setting[19]. The pancreatitis in patients with PD accounted for 21.97% (38 of 173) in total population of pancreatitis in this study, which is higher than in the results based on ERCP by Bernard *et al*[18] and Kamisava *et al*[20].

In Western countries, the incomplete PD is uncommon with a reported incidence of 0.13%-0.9%. However, there was a much higher prevalence of incomplete PD in the recent reports from Japan and Korea, indicating 48% and 52% of PD[16,17]. In the present study, the incomplete PD occurred in 7.9% (3 of 38) among the pancreatitis patients with PD. Partially, the fluctuation of the frequency of incomplete PD could result from the different techniques employed for the detection of PD, *i.e.*, the ERCP or MRCP, even for the same imaging modality, the techniques may be different with time due to intrinsic advances resulting in improved resolution[16,21-24].

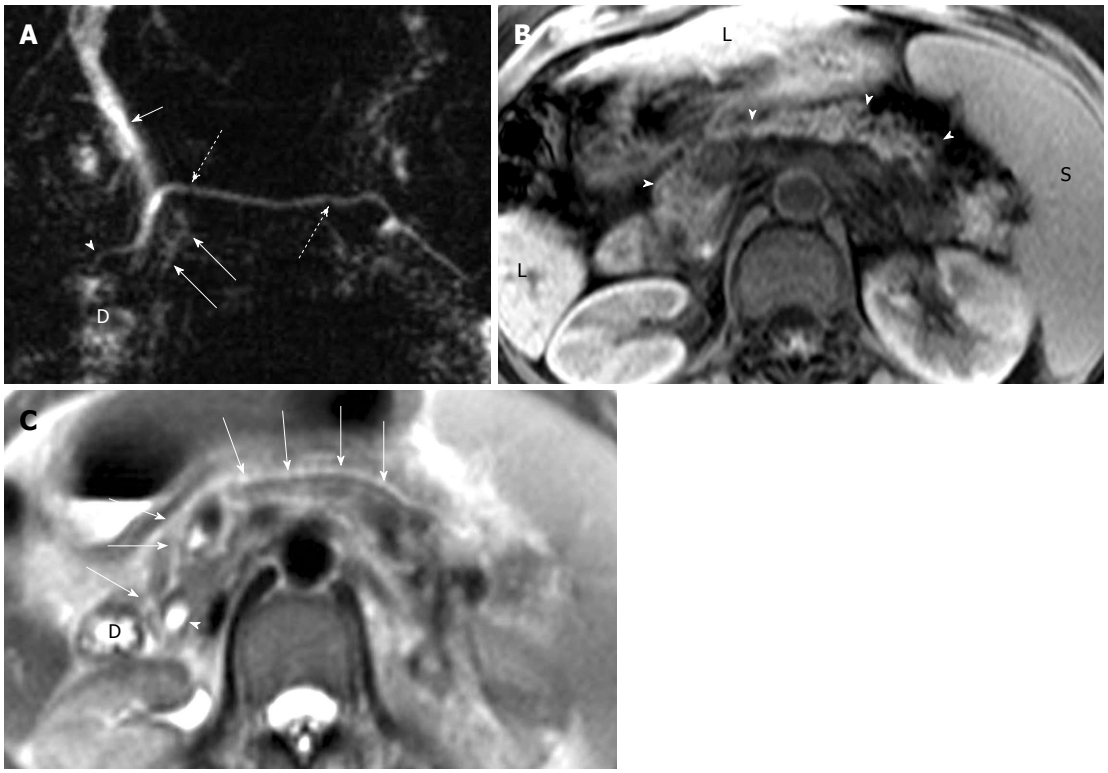

Figure 3 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of incidental chronic pancreatitis in a 47-year-old woman with a history of liver cirrhosis. A: Coronal oblique thick-section rapid acquisition with relaxation enhancement magnetic resonance (RARE-MR) cholangiogram [infinite/1100 (effective), 40-mm section thickness] of the pancreaticobiliary ducts shows slight dilatation with irregularity and strictures in the main pancreatic duct (dotted arrows) and in the duct of Santorini (arrowhead) just before entering the duodenum (D) *via* the minor papilla. Several side branch ectasia (long arrows) arising from the ventral duct and a normal common bile duct (short arrows) are demonstrated; B: Axial T1WI with fat saturation shows severe atrophy of the entire pancreas parenchyma (arrowheads). The liver (L) cirrhosis and splenomegaly (S) are noted; C: Axial T2WI shows main pancreatic duct (solid arrows) continued by duct of Santorini entering the duodenum (D) anterior to the common bile duct (arrowhead).

In our study, 94.1% (16 of 17) of dorsal pancreatitis were detected in cases with complete PD. According to the pathophysiology of PD, the majority of pancreatic juice drained through minor papilla can result in endoluminal pressure elevation or obstruction with subsequent pancreatitis likely involving the dorsal pancreas and sparing the ventral pancreas instead.

Although the incidence of dorsal pancreatitis (44.74%, 17 of 38) in patients with PD was significantly higher than in patients without PD (30 of 135, 22.22%) ($P < 0.05$, Table 3) in this study and in the study with ERCP conducted by Kamisava *et al*[20], it is lower than the prior study performed with ERCP by Morgan *et al*[4]. The presence of pancreas divisum may reduce the severity of acute gallstone pancreatitis, as stone impaction at the major papilla only affects the ventral pancreas, a smaller portion (about 10%) of pancreas compared to the dorsal pancreas[25].

Among the isolated dorsal pancreatitis cases, severe strictures at the duct junction of body and tail were responsible for upstream ductal dilatation in tail with atrophy of the affected pancreas, which presented with recurrent acute pancreatitis clinically. The pancreatic parenchyma in pancreatitis associated with PD demonstrated a spectrum of abnormalities including low-signal-intensity of pancreas on T1-weighted fat-suppressed images due to edema or fibrosis, decreased and delayed enhancement after intravenous contrast administration, parenchymal atrophy or enlargement, and pseudocysts. Inflammation and fibrosis can diminish the proteinaceous fluid content in the pancreas, leading to the loss of the usual high signal intensity on T1-weighted fat-suppressed images; therefore, the pancreatitis in patients with PD can have some findings similar to the pancreatitis without PD as indicated in literature[8].

Although the ERCP is still considered as a gold standard for diagnosing PD, it is an invasive technique and expensive, particularly it has several drawbacks including failure to cannulate minor papilla[12,26], a high rate of complications such as ERCP-induced pancreatitis[26], radiation, and use of iodinated contrast medium. It was reported that as high as 35% of patients with pancreatitis had no abnormalities on ERCP[27]. A recent article reported that the MDCT could detect the PD *via* visualization of the Santorini duct[13]. However, MRCP together with MRI is a non-invasive technique without radiation; MRCP can always be done together with MRI in a single study, which can delineate the parenchymal morphology in detail[28]. Comparing to ERCP and MDCT, MRCP and MRI can be repeated more safely in the follow-up of the patients of pancreatitis with PD since patients in this subgroup are likely younger and more sensitive to radia-

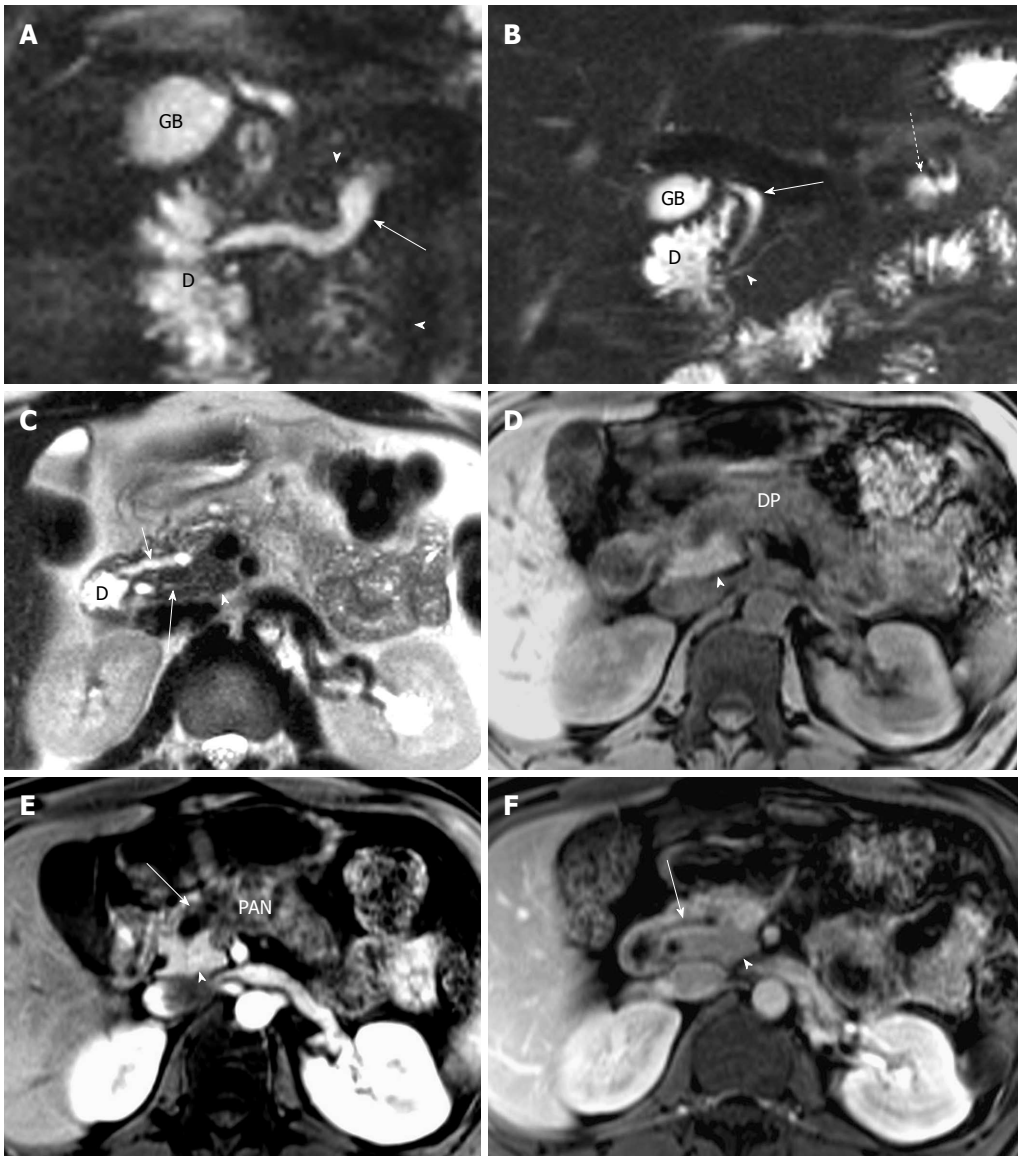

Figure 4 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of recurrent acute pancreatitis only involving the dorsal pancreas in a 43-year-old man. A: Coronal-oblique, thin-section half-Fourier rapid acquisition with relaxation enhancement magnetic resonance (RARE-MR) cholangiogram [infinite/95 (effective), 3-mm section thickness] shows marked dilatation (1 cm in diameter) of duct of Santorini (arrow) with apparent side branch ectasia (arrowheads). The gallbladder (GB) and the duodenum (D) are demonstrated clearly; B: Coronal-oblique, thin-section half-Fourier RARE MR cholangiogram [infinite/95 (effective), 3-mm section thickness] shows normal common bile duct (arrow) and ventral duct (arrowhead) of pancreas. The cystic changes secondary to pancreatitis in the pancreatic tail (dotted arrow) is shown while the GB and duodenum (D) appear normal; C: Axial T2WI shows dilatation and irregularity of duct of Santorini (short arrow) which enters the duodenum (D) *via* minor papilla. The ventral duct (solid arrow) and the pancreatic uncinate (arrowhead) are normal in size and signal intensity while the anterior portion of pancreatic head is abnormal with elevation of signal intensity; D: Axial precontrast T1WI SPGR shows that the pancreatic uncinate (arrowhead) is normal in size and signal intensity and the dorsal pancreas (DP) is abnormal with decreased T1 signal intensity and the swelling of the parenchyma; E: Axial T1WI SPGR after administration of Gd-DTPA at arterial phase shows normal enhancement of the pancreatic uncinate (arrowhead) and the enhancement of dorsal pancreas (PAN) is remarkably compromised with duct dilatation (arrow) and cystic changes; F: Axial T1WI SPGR at portal venous phase after administration of Gd-DTPA shows normal wash-out of contrast material in pancreatic uncinate (arrowhead) and delayed enhancement of the anterior portion of pancreatic head with dilatation of duct of Santorini (arrow).

tion. MRCP with secretin stimulation can provide better visualization of pancreatic duct, resulting in higher sensitivity and specificity for diagnosis of the pancreatic abnormalities[29,30]. MRI can have the same accuracy as CT for pancreatitis at present. Additionally, MRCP is thought to depict the pancreatic duct in more physiologic states than under exogenous pressure such as ERCP. Therefore, MRCP and MRI can serve as a comprehensive diagnostic tool without radiation for the pancreatitis associated with PD whereas the ERCP can be reserved for those who require interventional procedures for therapeutic purpose.

This study had several limitations. One limitation was that not all cases (21 of 38) underwent ERCP procedure for reference or interventional management. Owing to the resolution of MRCP without secretin stimulation at present, there could be some compromises resulting in possible false-negative consequences in detection and characterization of PD and pancreatitis associated with

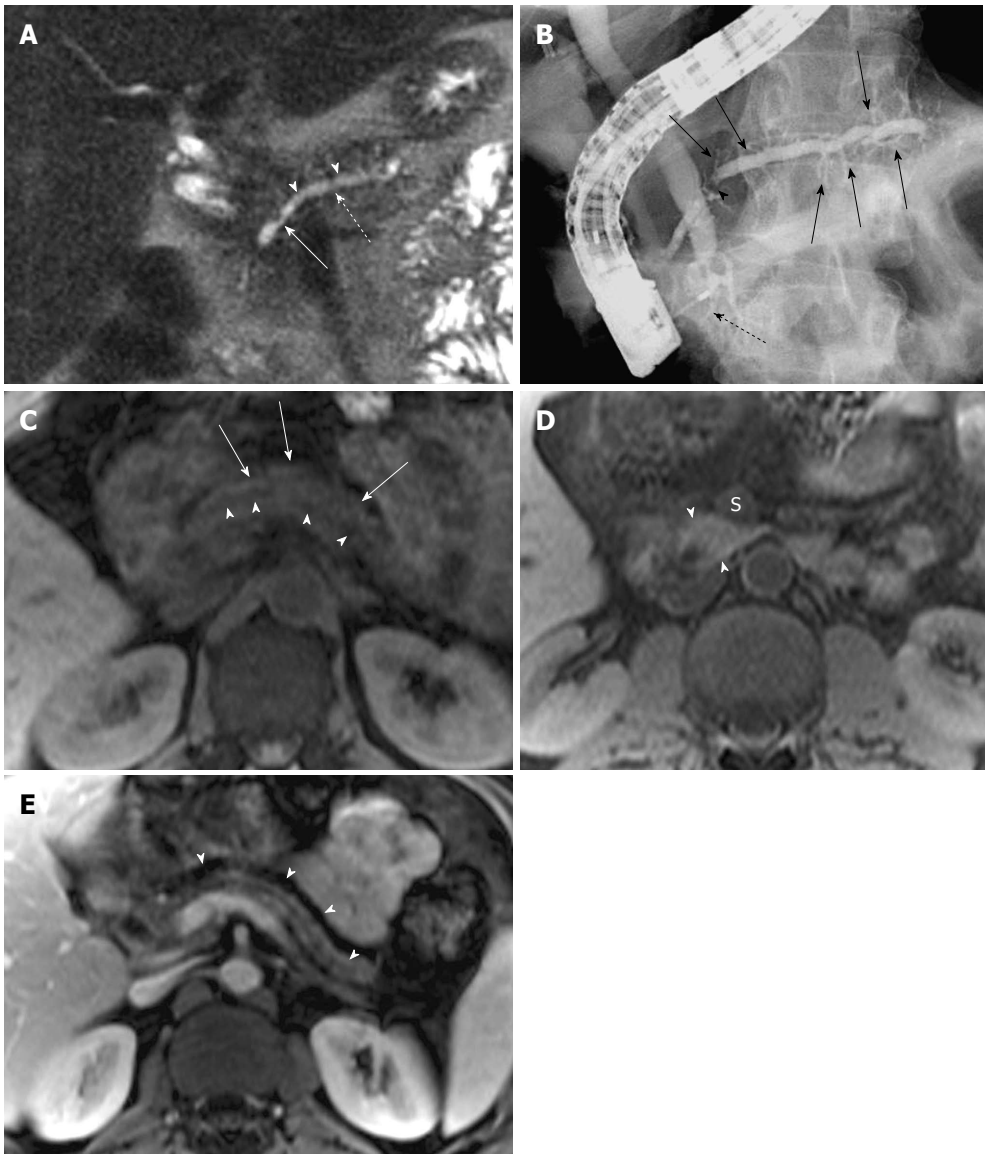

Figure 5 Magnetic resonance cholangiopancreatography and magnetic resonance imaging of recurrent acute pancreatitis only involving the dorsal pancreas, with endoscopic retrograde cholangiopancreatography correlation, in a 42-year-old man with several episodes of abdominal pain. A: Coronal-oblique, thin-section half-Fourier rapid acquisition with relaxation enhancement magnetic resonance (RARE-MR) cholangiogram [infinite/95 (effective), 3-mm section thickness] shows remarkable dilatation of the dorsal pancreatic duct (dotted arrow) with a conspicuous stricture (solid arrow) and severe side branch ectasia (arrowheads); B: Endoscopic retrograde cholangiopancreatography shows the dilatation of dorsal pancreatic duct and duct of Santorini with a well-seen stricture (arrowhead) and remarkable side branch ectasia (arrows) continual and proximal to the minor papilla. The intrapancreatic segment of common bile duct (C) is very narrow while the other part of the CBD is dilated. The ventral duct is normal in size (dotted arrow); C: Axial precontrast T1WI SPGR shows a marked decrease of signal intensity of dorsal pancreas (arrows) with ductal dilatation (arrowheads); D: Axial precontrast T1WI SPGR shows that the uncinate of pancreas is normal in size and signal intensity (arrowheads) is much higher than the superior mesenteric vein (S) and similar to the liver; E: Axial postcontrast T1WI SPGR shows the atrophy of dorsal pancreas with delayed enhancement (arrowheads) and dilatation of the allied pancreatic duct.

PD in some cases. Finally, the subjects enrolled into the study was based on the referring criteria for MRCP and MRI studies; the severity of pancreatitis or the classifications of pancreatitis might not exactly reflect the real profile of pancreatitis in patients with PD since most of severe and acute patients prefer CT because it is quicker than MRI in examination.

In conclusion, recurrent acute pancreatitis is more common in patients with divisum than in patients without divisum (21 of 38, 55.26% *vs* 9 of 135, 6.67%). In divisum patients, the dorsal pancreatitis accounts for a much higher percentage than in patients without PD (17 of 38, 44.74% *vs* 30 of 135, 22.22%). Therefore, MRCP and MRI could be a comprehensive diagnostic tool without radiation for the pancreatitis associated with PD whereas the ERCP can be reserved for those who require therapeutic interventions.

COMMENTS

Background

Pancreas divisum (PD) is the most common developmental anatomic variant

of pancreatic duct. Elevation of the intraluminal pressure of the pancreatic duct could result in pancreatitis. Magnetic resonance-cholangiopancreatography (MRCP) can always be performed together with magnetic resonance imaging (MRI), which can accurately detect both the ductal and the parenchymal abnormalities of the pancreas in detail.

Research frontiers

The pancreatitis in patients with PD could be different from the cases without PD both in clinical presentations and distribution of the abnormalities in pancreas since the congenital anomaly, however, the clinical significance remains controversial. To their knowledge, there is no published literature on imaging features of pancreatitis in patients with PD using MRI together with MRCP without secretin injection.

Innovations and breakthroughs

The pancreatitis associated with PD was usually distributed in dorsal pancreas and presented as recurrent acute type. MRCP in combination with MRI can accurately detect ductal and parenchymal abnormalities of pancreatitis in patients with PD.

Applications

The results of the present study indicated that repeated attacks of acute pancreatitis or isolated dorsal involvement of pancreas could imply a congenital PD in pancreas. MRCP and MRI should be referred to as a primary diagnostic tool for pancreatitis patients associated with PD whereas ERCP can be reserved for those who require therapeutic interventions.

Terminology

Recurrent acute pancreatitis was defined in this study as a condition in which the clinical or/and serologic features were characteristic of acute pancreatitis with a history of recurrence at least 2 times.

Peer review

Pancreas divisum is a common congenital anomaly of the pancreatic duct and possible cause of recurrent pancreatitis and chronic pancreatitis. But, there are still controversies in clinical significance as a cause of pancreatitis. This study revealed that recurrent acute pancreatitis is more common in divisum patients compared with those without divisum. The sample size was large to reach a conclusion and those results can help clinicians understand clinical significance of pancreas divisum. The imaging quality of presented figures is excellent and representative.

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ORIGINAL ARTICLE

Skp2-RNAi suppresses proliferation and migration of gallbladder carcinoma cells by enhancing p27 expression

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Abstract

AIM: To explore the role of S-phase kinase-associated protein-2 (Skp2) in gallbladder carcinoma and to identify whether depletion of Skp2 by Skp2-RNAi could attenuate proliferation and migration of gallbladder carcinoma.

METHODS: Skp2-RNAi was transduced into cells of the gallbladder carcinoma cell line GBC-SD, using a lentiviral vector. The effect of Skp2-RNAi on the proliferation, migration, invasion and cell cycle of GBC-SD cells was studied using *in vitro* assays for cell proliferation, colony formation, wound healing and cell cycle. The expression of Skp2 and p27 was detected by real-time polymerase chain reaction and Western immunoblotting. The effect of Skp2-RNAi on the proliferation of GBC-SD cells *in vivo* was investigated by tumorigenicity experiments in nude mice.

RESULTS: Lentivirus-mediated RNAi reduced the expression of Skp2 in cultured cells. The expression of the p27 protein increased along with the down-regulation of Skp2, although no significant difference was found in p27 mRNA expression. Flow cytometry revealed that Skp2-RNAi transfection significantly increased the proportion of cells in the S phase and significantly decreased the proportion of cells in the G_2/M phase. No significant difference in the frequency of cells in the G_0/G_1 phase was observed. The results from the cell proliferation, colony formation and wound healing assays revealed that Skp2-RNAi transfection markedly inhibited the proliferation and migration of GBC-SD cells *in vitro*. Additionally, tumorigenicity experiments showed that suppression of Skp2 significantly decreased the weights of the tumors (0.56 ± 0.11 and 0.55 ± 0.07 g in the control and Scr-RNAi groups *vs* 0.37 ± 0.09 and 0.35 ± 0.08 g in the Skp2-RNAi-L and Skp2-RNAi-H groups).

CONCLUSION: The expression of Skp2 in GBC-SD cells was inhibited following Skp2-RNAi transfection. Silencing of the *Skp2* gene inhibited proliferation, migration and invasiveness of GBC-SD cells by mechanisms dependent on enhanced expression of the p27 protein.

Key words: Gallbladder carcinoma; S-phase kinase-associated protein-2; p27; Gene therapy; Cell cycle

Core tip: The association between S-phase kinase-associated protein-2 (Skp2)/p27 and gallbladder carcinoma has rarely been reported. This study investigated the effects of Skp2-RNAi on *in vitro* and *in vivo* growth and the invasive potencies of gallbladder carcinoma cells. The authors proposed that the effects were due to the accumulation of the p27 protein following Skp2-depletion.

Zhang B, Ji LH, Liu W, Zhao G, Wu ZY. Skp2-RNAi suppresses proliferation and migration of gallbladder carcinoma cells by enhancing p27 expression. *World J Gastroenterol* 2013; 19(30): 4917-4924 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4917.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4917

INTRODUCTION

Primary gallbladder carcinoma is a common biliary malignancy. Its incidence is estimated to be approximately 1.2-10.6/100000, and this cancer accounts for almost 3% of all tumors[1]. Unfortunately, the majority of patients with primary gallbladder carcinoma have intermediate-advanced disease at presentation due, in part, to diagnostic difficulties and a high degree of malignancy. Thus, for these patients, the prognosis is extremely poor.

The cancer suppressor gene *p27* (wherein *p27* represents the gene and $p27^{(Kip1)}$ represents the protein) is a cyclin-dependent kinase inhibitor (CKI), which plays an important role in tumorigenesis and tumor development[2]. Altered expression of $p27^{(Kip1)}$ is closely associated with the prognosis in several types of human cancers[3,4]. It has been shown that the stability of $p27^{(Kip1)}$ can be enhanced by a specific proteasome inhibitor, which can further inhibit the growth of the tumor[5]. Over-expression of $p27^{(Kip1)}$ with an adenoviral vector (adenovirus-p27) can inhibit tumor growth and induce apoptosis[6,7]. In addition, the expression of p27 mRNA was determined to be constant during a normal cell cycle. The highest expression of $p27^{(Kip1)}$ was found during the G_0/G_1 phase of the cell cycle, and the lowest expression was throughout the S and M phases[8-10]. The expression of $p27^{(Kip1)}$ was found to be predominantly regulated by S-phase kinase-associated protein-2 (Skp2)[8,9].

Skp2 (wherein SKP2 represents the gene and Skp2 represents the protein) is an S-phase dependent protein kinase that was originally found by Rodriguez *et al*[11], constituting the F-box unit of the SCF-E3 ligase that specifically targets CKIs, such as $p21^{(Cip1)}$, $p27^{(Kip1)}$, $p57^{(Kip2)}$ and p130, for degradation[12]. Functional deletion of Skp2 leads to stabilization of CKIs, which can subsequently induce cell-cycle delay or arrest; conversely, the over-expression of Skp2 is frequently associated with a variety of human cancers[11,13]. Nelsen *et al*[14] reported that cotransfection of cyclin E and Skp2 synergistically promoted cell cycle progression in cultured primary hepatocytes in the absence of mitogen or in the presence of growth inhibitors. Furthermore, transfection of hepatocytes with cyclin E and Skp2 *in vivo* promoted abundant hepatocyte replication and hyperplasia of the liver. Hence, Skp2 is thought to be closely associated with cell cycle regulation, tumor emergence, tumor development and disease prognosis.

$p27^{(Kip1)}$ and Skp2 have been studied in many types of tumors[15-19]. The determination of an association between Skp2/$p27^{(Kip1)}$ and gallbladder carcinoma has been rarely reported[20,21]. In the current study, we constructed a lentiviral vector of Skp2-RNAi, and explored the role of Skp2/$p27^{(Kip1)}$ in the proliferation and metastasis of gallbladder carcinoma cells.

MATERIALS AND METHODS

Groups

The gallbladder carcinoma cell line (GBC-SD) cells (Shanghai Cell Library, China) were divided into four groups: (1) control group: without any treatment; (2) Scr-RNAi group (Scr-RNAi group): GBC-SD cells were transfected with a negative control RNA interference sequence (TTCTCCGAACGTGTCACGT) using lentivirus vectors (SunBio, United States); (3) for the Skp2-RNAi-Low group (Skp2-RNAi-L group); and (4) the Skp2-RNAi-High group (Skp2-RNAi-H group), the cells were transfected with an RNA interference sequence of Skp2 (AGGTC-TCTGGTGTTTGTAA) at a dose of 10 and 20 MOI, respectively.

Construction and identification of the RNAi lentivirus vector

The GBC-SD cells were plated and cultured in 24-well plates until cell fusion reached 40%-60%. Next, the appropriate amounts of lentivirus were added to the cells according to the different MOI values (2.5×10^4 TU/well in Skp2-RNAi-L group and 5×10^4 TU/well in Skp2-RNAi-H group). The transduction efficiency was assessed by fluorescence microscopy (Nikon, Japan) after 96 h. The cells were harvested 10 d following transduction.

The effect of the RNAi-Lentivirus on the expression of *Skp2* gene was assessed by determination of the mRNA and protein levels of Skp2 in the GBC-SD cells after infection with lentivirus for 5-7 d; real-time polymerase chain reaction (PCR) and Western immunoblotting were used for these assessments.

Cell proliferation assay

Cell proliferation ability was assessed with a methylthiazol tetrazolium (MTT) assay kit (SunBio, United States). The cells were inoculated into 96-well plates (1×10^4 cells per well). After incubation for 1, 2, 3, 4 and 5 d, 100 μL of sterile MTT (5 mg/mL, Sigma-Aldrich Corp, United States) was added to each well. The cells were further incubated at 37 ℃ for 4 h, and the reaction was stopped by adding 200 μL of dimethyl sulfoxide. After mixing for 10 min at room temperature, formazan production was determined by measurement of the optical density (OD) at 570 nm using an enzyme immunoassay analyzer (1420 multi-label counter).

Colony formation assay

Two hundred cells were prepared and plated into 35-mm culture plates for a period of 10 d. The resulting cellular clones were counted using an inverted microscope (BX45-72P15, Olympus, Japan). A cell clone was scored as positive following confirmation that the number of cells within the clone exceeded 50. The experiment was repeated 8 times.

Wound healing assay

Cells were inoculated into 6-well plates, and a 100-μL pipette tip was used to scribe a line across the cell monolayer. The cells that moved into the interspace of the wound line were counted 24 h later using a phase contrast microscope (BX45-72P15, Olympus, Japan). This assay was

repeated 8 times.

Cell cycle assay

Cells were seeded into a 6-well plate and harvested after infection for 10 d. After two washes in pre-cooled PBS, the cells were fixed in 70% alcohol. The percentage of cells in each stage of the cell cycle was determined by staining with propidium iodide (PI, Santa Cruz, United States). The cell cycle distribution was analyzed with a FAC-Scan Flow Cytometer (BD, United States), in accordance with the manufacturer's guidelines.

RNA extraction and real-time PCR

Total RNA (2 μg) was isolated and reverse-transcribed into cDNA. The cDNA samples (2 μL) were employed for real-time PCR in a total volume of 20 μL on a GeneAmp Thermal Cycler 9700 (ABI, United States). The reactions were incubated in 96-well optical plate at 94 ℃ for 4 min, followed by 35 cycles of 94 ℃ for 10 s, 57 ℃ for 15 s and 72 ℃ for 20 s, and a final extension reaction at 86.5 ℃ for 5 s. Melting-curve analysis was performed from 72 ℃ to 99 ℃ at a rate of 1 ℃ every 5 s. The average of the triplicate data obtained for each sample was employed to calculate the relative change in gene expression after normalization to β-actin mRNA. The primer sequences were as follows: Skp2: 5'-CCTAAGCAGCTGTCCCAGAC-3' (sense) and 5'-GTGTCAGTCGGCATTTGATG-3' (antisense); p27: 5'-ACCCAACAATACCACCGACC-3' (sense) and 5'-CCCGCCTAATCTGCACTGTG-3' (antisense); β-actin: 5'-CCAAGGCCAACCGCGAGAAGATGAC-3' (sense) and 5'-AGGGTACATGGTGGTGCCGCCAGAC-3' (antisense).

Western immunoblotting

After lysis with pre-cooled lysis buffer, 40 μg of protein extracted from the cells was loaded onto 10% SDS-PAGE gels, and the resolved proteins were transferred to a PVDF membrane over a 2-h period (Bio-Rad, United States). Next, the membrane was blocked in 5% non-fat milk for 1 h at room temperature and then probed overnight at 4 ℃ with antibodies against Skp2 (CST, United States, 1:1000 dilution) and p27 (CST, United States, 1:1000 dilution). After three washes with TBST (tris buffered saline with 0.5% Tween-20), the membrane was incubated with the appropriate secondary antibody (anti-mouse IgG, Santa Cruz, United States, 1:1000 dilution) for 2 h at room temperature. Following a further washing with TBST, the membrane's image was developed using enhanced chemiluminescence (ECL + plus™, Amersham, United Kingdom). β-actin was employed as an internal standard, and expressions of Skp2 and p27 were determined and normalized against the level of β-actin.

Tumorigenicity experiments in nude mice

Forty male nude mice weighing 18 to 21 g, provided by Shanghai Laboratory Animal Center (Chinese Academy of Science, China), were bred under aseptic conditions; the animals were housed in an area with a constant humidity of 60%-70% and a room temperature of 18 ℃-20 ℃. Animal maintenance, husbandry and experimental procedures were performed in accordance with the United States National Institute of Health Guidelines for the Use of Experimental Animals and approved by the Medical Animal Care and Use Committee of Renji Hospital (Shanghai, China). All of the mice were separated into four groups as described above: control, Scr-RNAi, Skp2-RNAi-L, and Skp2-RNAi-H groups. Lentivirus transfected cells from each group were administrated by subcutaneous injection (0.1 mL of a solution containing 1×10^4 cells/mL). The mice were examined every 4 d and were sacrificed 28 d after the initial subcutaneous injection. The tumors were resected and weighed.

Statistical analysis

All measurement data were expressed as the mean ± SD. The association analysis among the groups was performed using one-way analysis of variance with the SPSS17.0 statistical software package. Statistical significance was defined as having a P value less than 0.05.

RESULTS

Validation of Skp2-RNAi-lentivirus and expression of p27

The studies showed that Skp2-RNAi transfection could significantly reduce the level of Skp2 mRNA ($P < 0.05$, Figure 1A) and protein ($P < 0.05$, Figure 1B and C) in the Skp2-RNAi-L and Skp2-RNAi-H groups, compared with the control and Scr-RNAi groups, and that these alterations were closely related to the dosage of Skp2-RNAi. In addition, the expression of p27 was also detected in GBC-SD cells after infection with RNAi-Lentivirus. Expression of p27 mRNA did not change following the down-regulation of Skp2. Densitometric analysis of the immunoblot images showed that the ratios between the p27 protein in the Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups and the p27 protein in the control group were 0.99, 1.52 and 1.93, respectively ($P < 0.05$, Figure 1). This result suggests that p27 was increased at the protein level but not at the mRNA level following Skp2-RNAi transfection. Expression of cyclin D1 and E mRNA and protein was unaltered (data not shown).

Skp2-RNAi inhibited cell growth

The effect of Skp2-RNAi on cell growth was evaluated using an MTT assay kit. As shown in Figure 2, the A values in the Skp2-RNAi-L and Skp2-RNAi-H groups were significantly higher than the values in the control and Scr-RNAi groups (0.94 ± 0.12 and 0.87 ± 0.11 *vs* 0.48 ± 0.06 and 0.41 ± 0.05, respectively, $P < 0.01$). This result suggests that cell growth was significantly inhibited along with the down-regulation of Skp2 by Skp2-RNAi.

Skp2-RNAi inhibited colony formation of GBC-SD cells

Cell colony formation was significantly decreased in the

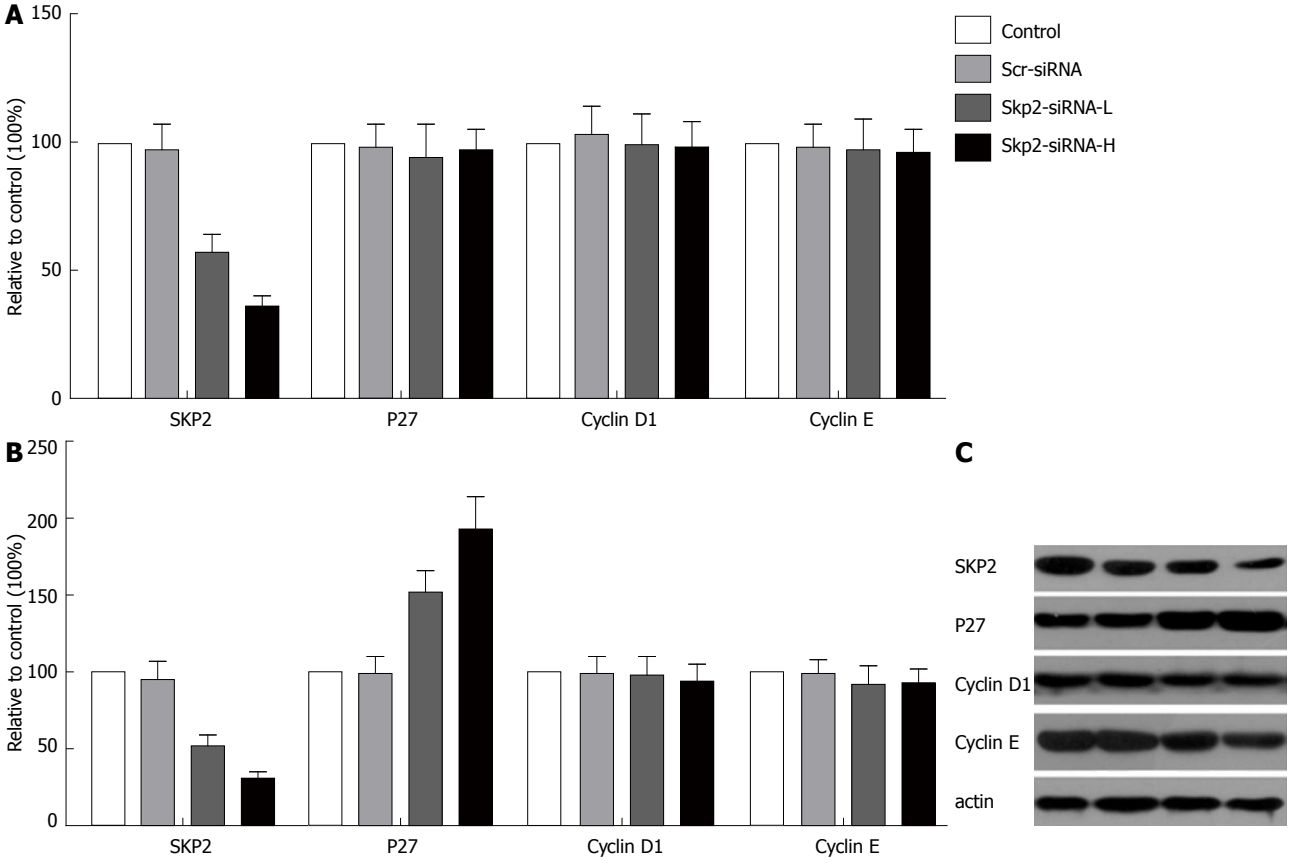

Figure 1 Detection of downstream gene and protein expression after kinase-associated protein-2-RNAi transfection. A: Expression of p27 mRNA did not change following down-regulation of S-phase kinase-associated protein-2 (Skp2); B and C: Protein expression of p27 was upregulated. Lane 1: Control; Lane 2: Scr-RNAi; Lane 3: Skp2-RNAi-L; Lane 4: Skp2-RNAi-H.

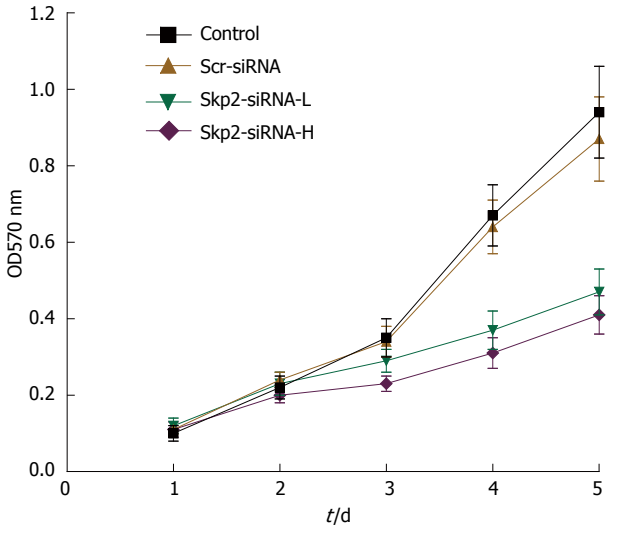

Figure 2 Proliferation curves for the gallbladder carcinoma cell line cells after the inhibition of kinase-associated protein-2 expression. Cell proliferation was significantly inhibited after down-regulation of S-phase kinase-associated protein-2 (Skp2) by Skp2-RNAi. GBC-SD: The gallbladder carcinoma cell line.

Skp2-RNAi-L and Skp2-RNAi-H groups, compared to that in the control and Scr-RNAi groups, (13.50 ± 5.90 and 7.25 ± 5.12 *vs* 51.25 ± 7.54 and 48.88 ± 11.93 cells per well, respectively, $P < 0.01$, Figure 3). Colony formation of GBC-SD cells was significantly inhibited after transfection with Skp2-RNAi, and those colonies formed were closely related to the dosage of Skp2-RNAi.

Skp2-RNAi suppressed migration ability of GBC-SD cells

The migrated cells in the control, Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups were found to be 111.75 ± 19.96, 101.38 ± 14.32, 76.50 ± 13.15 and 63.16 ± 11.00 cells per mm^2, respectively. The migrated cells in the Skp2-RNAi-L and Skp2-RNAi-H groups were markedly decreased compared with the control and Scr-RNAi groups ($P < 0.01$, Figure 4).

Cell cycle changes

No significant difference was observed in the proportion of cells in the G_0/G_1 phase following inhibition of Skp2. However, in the Skp2-RNAi-L and Skp2-RNAi-H groups, the proportion of cells in S phase increased. Nevertheless, inhibition of Skp2 decreased the proportion of cells in the G_2/M phase as compared with the control and Scr-RNAi groups ($P < 0.05$, Figure 5).

Skp2-RNAi inhibited tumor growth in nude mice

Twenty-eight days after the mice were injected with carcinoma cells, the weights of the tumors in the control,

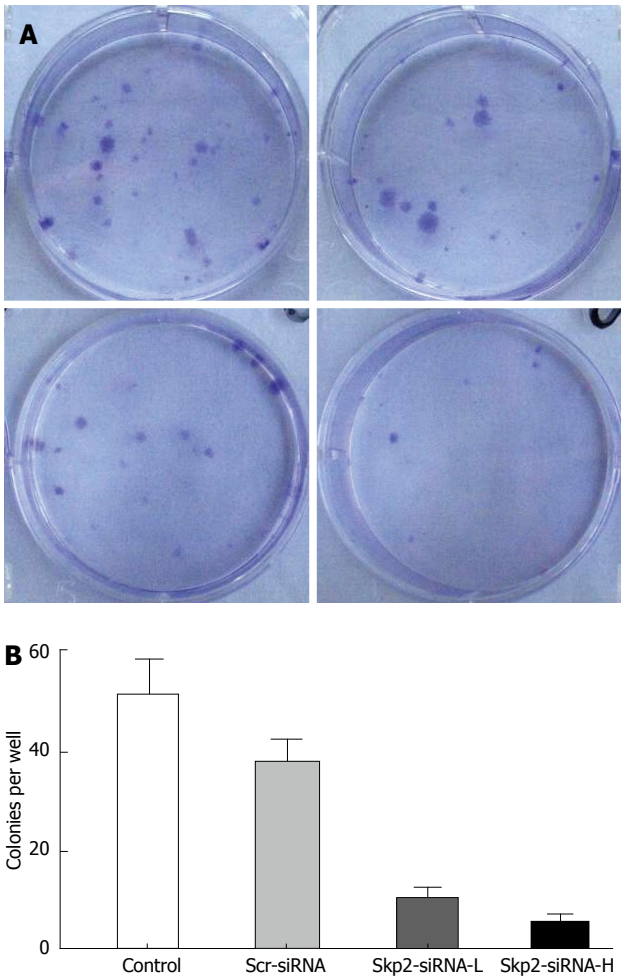

Figure 3 Colony formation assays after inhibition of kinase-associated protein-2 expression. A: Colony formation assays; B: Colony formation of the gallbladder carcinoma cell line (GBC-SD) cells was significantly inhibited after transfection with S-phase kinase-associated protein-2 (Skp2)-RNAi.

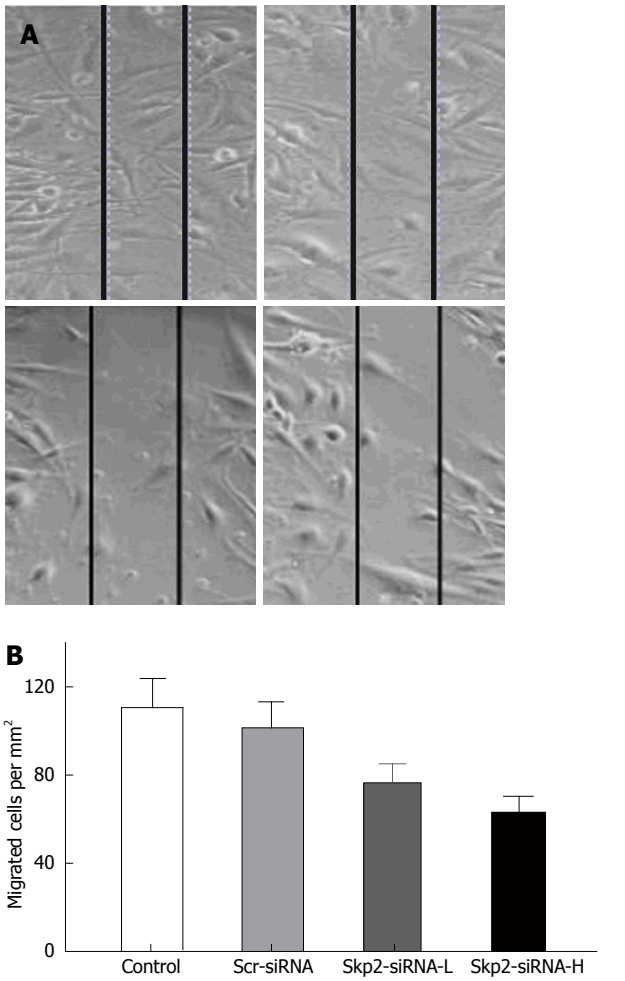

Figure 4 Results of the wound healing assay after inhibition of kinase-associated protein-2 expression. A: The wound healing assay after inhibition of kinase-associated protein-2 (Skp2) expression; B: The migrated cells in the two S-phase Skp2-RNAi groups were markedly decreased as compared with the two control groups.

Scr-RNAi, Skp2-RNAi-L and Skp2-RNAi-H groups were 0.56 ± 0.11, 0.55 ± 0.07, 0.37 ± 0.09 and 0.35 ± 0.08 g, respectively. Thus, treatment with Skp2-RNAi inhibited the growth of tumors as compared with both the control and Scr-RNAi groups ($P < 0.01$, Figure 6).

DISCUSSION

Gallbladder carcinoma was first described by Clemente *et al*[1]. Despite advances in hepatobiliary imaging techniques, the preoperative diagnosis of this condition remains a daunting task. Furthermore, the long-term survival remains dismal, not only because of the non-specific presentation of the disease and its similarity to benign biliary tract disorders, but also because of the malignant entity. Currently, the mean survival time of advanced stage gallbladder carcinoma is approximately 6 mo, and the 5-year survival rate is less than 5%[22]. Hence, the prognosis of gallbladder carcinoma remains poor despite improvements in surgical techniques. Moreover, the molecular mechanisms underlying the development of gallbladder carcinoma remain largely unknown.

Skp2 is an F-box substrate-recognition subunit of the SCF ubiquitin-protein ligase complex, which regulates progression of the cell cycle by targeting regulators such as $p27^{(Kip1)}$ for ubiquitin-mediated degradation. Decreased levels of $p27^{(Kip1)}$ are thought to be associated with highly aggressive tumors and related to a poor prognosis in a variety of cancers[21,23-25].

In the current study, Skp2 expression was inhibited in GBC-SD cells by transfection of a Skp2 specific vector, namely, Skp2-RNAi. Consequently, cells in the S-phase of the cell cycle were increased, whereas cells in the G_2/M phase were decreased. No significant difference was observed in the proportion of cells present in the G_0/G_1 phase; thereby, the cell cycle was blocked in the S phase. Cell growth was significantly decreased in several *in vitro* experiments, suggesting that silencing Skp2 could markedly reduce cell proliferation and the group-dependent capability to form colonies.

In most tumors, deletion or mutation of *p27* rarely occurs, and its transcription is negligibly changed. Our research found that suppression of Skp2 had no effect on the mRNA expression of p27, but it was found to upreg-

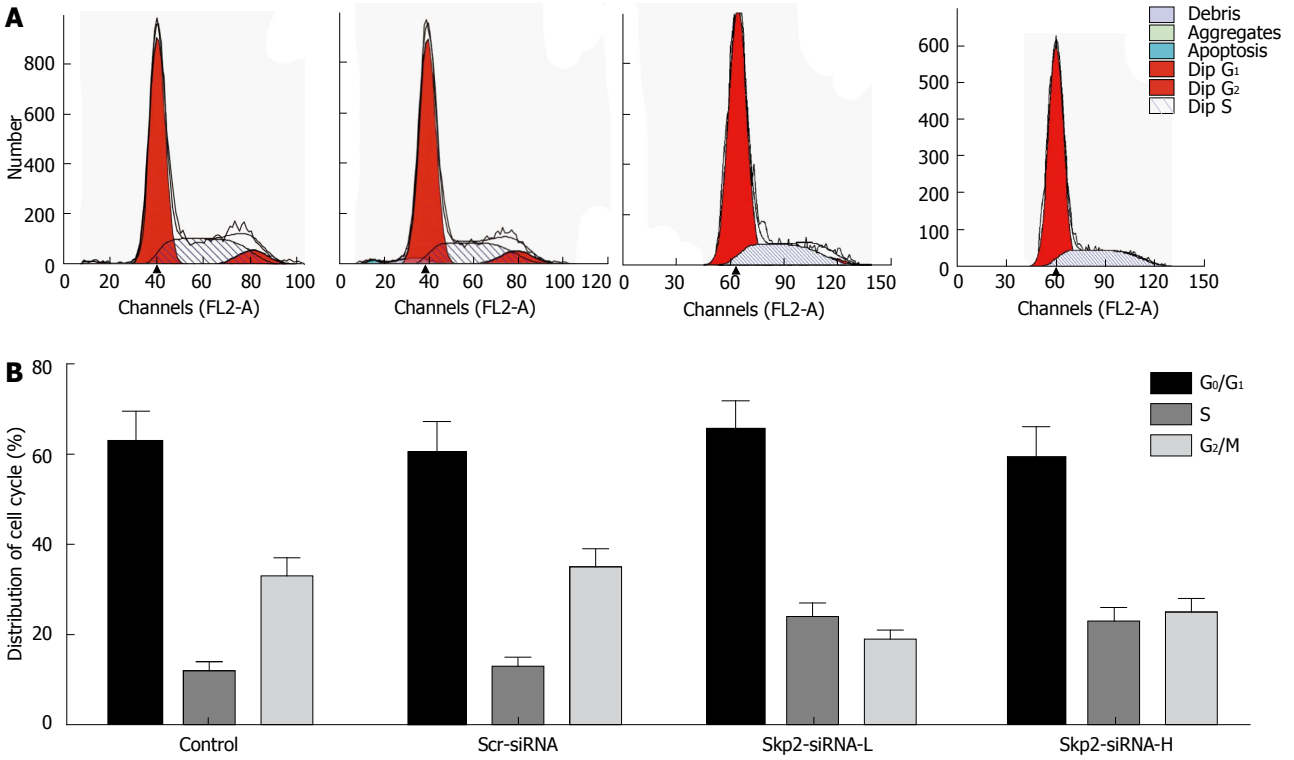

Figure 5 Proportion of cells in the cell cycle stages. A: Detection of the proportion of cells in the cell cycle stages after S-phase kinase-associated protein-2 (Skp2) expression was inhibited; B: The proportion of cells in the S phase of the cell cycle increased, and the proportion of cells in the G_2/M phase decreased in Skp2-RNAi-L and Skp2-RNAi-H groups.

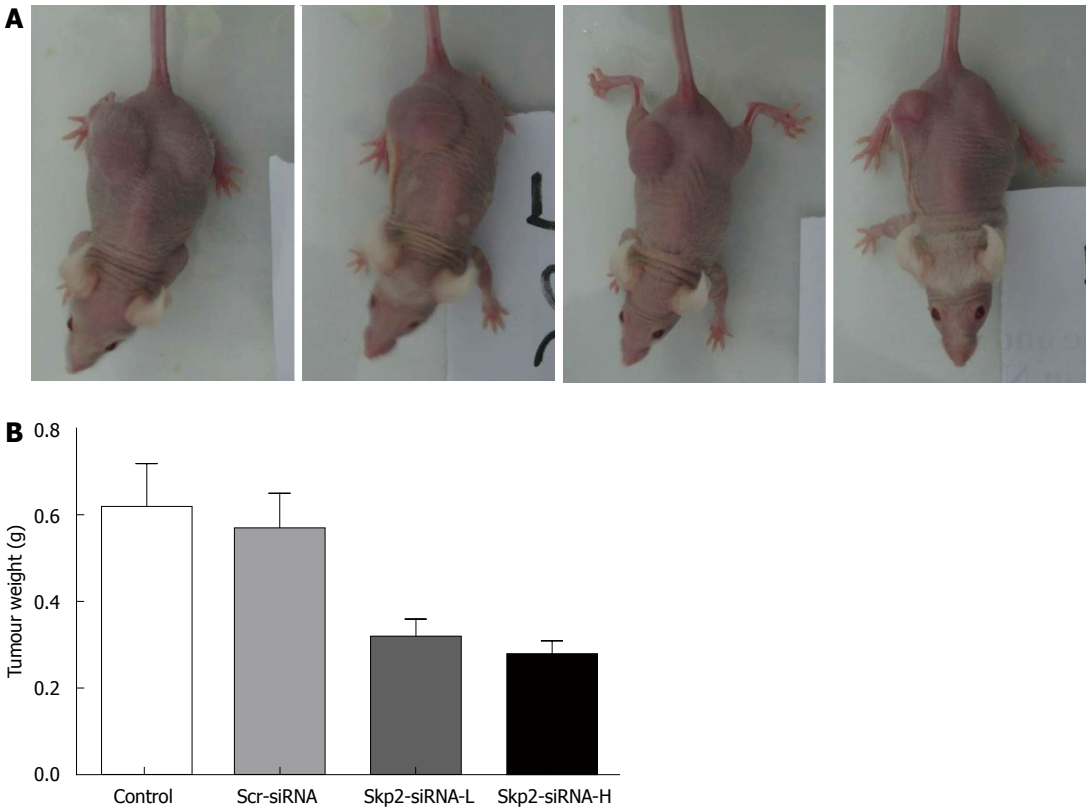

Figure 6 Tumorigenicity experiments in nude mice. A: Tumorigenicity experiments; B: Transfection with S-phase kinase-associated protein-2 (Skp2)-RNAi inhibited the growth of tumor cells.

ulate p27's protein expression. This observation suggested that regulation of gallbladder carcinoma proliferation by Skp2-siRNA is dependent on p27 protein expression, but not expression at the gene level. Nuclear polyubiquitination of $p27^{(Kip1)}$ is dependent on Skp2 and phosphorylation of $p27^{(Kip1)}$ at threonine 187. However, Hara *et al*[26] found that polyubiquitination activity was also detected in the cytoplasm of $Skp2^{(-/-)}$ cells, even with a threonine 187 to alanine 187 mutant of $p27^{(Kip1)}$ as the substrate. This outcome suggested that the polyubiquitination activity in the cytoplasm might contribute to an early phase of $p27^{(Kip1)}$ degradation in a Skp2-independent manner.

In addition to inducing the degradation of $p27^{(Kip1)}$ and promoting cellular proliferation, Skp2 also plays an important role in tumor invasiveness and metastasis. The numbers of migrated cells in the two Skp2-RNAi treated groups were found to be significantly fewer than those in the control groups. This observation suggested that Skp2-RNAi could inhibit the proliferation, migration and invasiveness of GBC-SD cells. We also studied the antitumor effect of Skp2-RNAi on nude mice in tumorigenicity experiments; the weights of the resulting tumors were decreased. This outcome suggested that treatment with Skp2-RNAi repressed the growth of metastatic tumors *in vivo*. Furthermore, the inhibition was shown to be positively associated with the dose of the lentivirus used. Hung *et al*[27] established Skp2-overexpressing stable transfectants in A549 human lung cancer cells and found that these stable transfectants exhibited increased migratory and invasive capabilities. Additionally, the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9 were up-regulated and neutralization of these two MMPs using antibody-mediated approaches reduced cellular invasion. These data suggest that Skp2 promoted both tumor growth and metastasis and that enhanced expression of both MMP-2 and MMP-9 may have provided a contributory mechanism.

Moreover, Skp2 and $p27^{(Kip1)}$ have been shown to be useful indicators of prognosis[28-31]. Sanada *et al*[21] reported that Skp2 and $p27^{(Kip1)}$ were independent predictors of poor prognosis in patients with biliary tract cancers (BTCs). Discrepancies between *SKP2* DNA copy number and the level of Skp2 protein were observed, although a correlation was found between copy number and protein expression in some primary BTCs. Therefore, it is formally possible that Skp2 protein expression could be considered a more accurate prognostic marker for BTCs than *SKP2* gene copy number. Hashimoto *et al*[32] revealed that low levels of protein expression of $p27^{(Kip1)}$ and high Skp2 were associated with aggressive tumor behavior and that both $p27^{(Kip1)}$ and Skp2 could be considered useful markers in predicting the outcome of patients with intrahepatic cholangiocarcinomas. In an immunohistochemical study of 62 cases using tissue microarray, Li *et al*[20] confirmed that Skp2 over-expression represented the most significant independent adverse prognostic indicator in gallbladder carcinoma. Beyond the prognostic importance of Skp2/$p27^{(Kip1)}$, the development of drugs targeting Skp2 may provide novel molecular therapeutic approaches.

In summary, the results from our studies support the idea that Skp2 inhibitors and/or Skp2 regulatory sequences could provide a useful therapeutic protocol for the treatment of gallbladder carcinoma. In the future, the role of Skp2/$p27^{(Kip1)}$ in gallbladder carcinoma can be expected to be gradually unveiled.

ACKNOWLEDGMENTS

We would like to thank Jing Zheng for technical and methodological support.

COMMENTS

Background

Primary gallbladder carcinoma is a common biliary malignancy, with a poor prognosis. p27 and S-phase kinase-associated protein-2 (Skp2) may play an important role in tumorigenesis and tumor development, and are closely associated with prognosis.

Research frontiers

Inhibition of Skp2 or over-expression of $p27^{(Kip1)}$ could inhibit tumor growth and induce apoptosis.

Innovations and breakthroughs

The authors explored the effect of Skp2-RNAi on GBC-SD cells, and found that suppression of the *Skp2* gene inhibited proliferation, migration and invasiveness of GBC-SD cells by mechanisms dependent on enhanced expression of p27 protein.

Applications

The results indicated that Skp2 inhibitors and/or Skp2 regulatory sequences such as Skp2-RNAi could provide a useful therapeutic protocol for the treatment of gallbladder carcinoma.

Peer review

The authors used several assays to explore the role of Skp2 in gallbladder carcinoma. The results indicated that Skp2-RNAi might provide a useful therapeutic protocol for the treatment of gallbladder carcinoma.

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ORIGINAL ARTICLE

Tumor necrosis factor-α mediates JNK activation response to intestinal ischemia-reperfusion injury

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Abstract

AIM: To investigate whether tumor necrosis factor-α (TNF-α) mediates ischemia-reperfusion (I/R)-induced intestinal mucosal injury through c-Jun N-terminal kinase (JNK) activation.

METHODS: In this study, intestinal I/R was induced by 60-min occlusion of the superior mesenteric artery in rats followed by 60-min reperfusion, and the rats were pretreated with a TNF-α inhibitor, pentoxifylline, or the TNF-α antibody infliximab. After surgery, part of the intestine was collected for histological analysis. The mucosal layer was harvested for RNA and protein extraction, which were used for further real-time polymerase chain reaction, enzyme-linked immunosorbent assay and Western blotting analyses. The TNF-α expression, intestinal mucosal injury, cell apoptosis, activation of apoptotic protein and JNK signaling pathway were analyzed.

RESULTS: I/R significantly enhanced expression of mucosal TNF-α at both the mRNA and protein levels, induced severe mucosal injury and cell apoptosis, activated caspase-9/caspase-3, and activated the JNK signaling pathway. Pretreatment with pentoxifylline markedly downregulated TNF-α at both the mRNA and protein levels, whereas infliximab pretreatment did not affect the expression of TNF-α induced by I/R. However, pretreatment with pentoxifylline or infliximab dramatically suppressed I/R-induced mucosal injury and cell apoptosis and significantly inhibited the activation of caspase-9/3 and JNK signaling.

CONCLUSION: The results indicate there was a TNF-α-mediated JNK activation response to intestinal I/R injury.

Key words: Tumor necrosis factor-α; Intestine; Mucosa; Apoptosis; c-Jun N-terminal kinase

Core tip: Ischemia-reperfusion (I/R) injury is a critical physiopathological phenomenon wherein further damage may occur when the blood supply of ischemic organs is recovered, and the mechanism of I/R remains unclear. This paper demonstrates that tumor necrosis factor-α (TNF-α) played a pivotal role in intestinal I/R injury, and pretreatment with the TNF-α inhibitor pentoxifylline or the TNF-α antibody infliximab remarkably attenuated I/R-induced injury by inhibiting TNF-α-mediated apoptosis and c-Jun N-terminal kinase (JNK) activation. The results of the study indicate there is a TNF-α-mediated JNK activation response to intestinal I/R injury.

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INTRODUCTION

Intestinal ischemia-reperfusion (I/R) not only damages the local intestinal mucosa but also induces remote organic injury. I/R injury may occur in numerous situations, such as small bowel transplantation, strangulated hernias, neonatal necrotizing enterocolitis, cardiopulmonary bypass surgery and hypovolemic/septic shock[1]. A series of factors, including reactive oxygen species (ROS) production, calcium overload, neutrophil infiltration and cytokines release, are involved in I/R injury. Among these mediators, tumor necrosis factor-α (TNF-α), as an initial factor of the inflammatory reaction in I/R injury, is thought to play a pivotal role[2]. Prophylactic anti-TNF-α treatment may be an effective therapeutic strategy for preventing I/R-induced injury, as has been demonstrated by some studies[3]. TNF-α is thought to initiate three signaling pathways involved in cell injury and apoptosis: the apoptotic signaling pathway, the c-Jun N-terminal kinase (JNK) signaling pathway and the nuclear factor kappa-B (NF-κB) signaling pathway[4]. However, the role of TNF-α in I/R-induced injury and its mechanisms remain to be elucidated. Previous studies[5,6] demonstrated that apoptosis is a major mode of cell death caused by I/R, whereas the effect of TNF-α-mediated signaling pathways in cell apoptosis requires further exploration.

Pentoxifylline, a TNF-α inhibitor, has been investigated for a long time in I/R injury, but its effects on I/R-induced intestinal apoptosis and apoptotic pathways remain to be evaluated. Recent studies also have suggested that infliximab, a TNF-α antibody, attenuates I/R-induced injury[7]. However, it is still poorly understood whether infliximab alleviates the intestinal mucosal injury by downregulating apoptotic signaling or has acts *via* some other mechanisms.

The aims of this study were to determine: (1) whether inhibition of TNF-α ameliorates I/R-induced intestinal mucosal injury by suppressing cell apoptosis; (2) whether TNF-α is involved in a caspase-dependent apoptotic signaling in intestinal I/R injury; and (3) whether the JNK signaling pathways are activated by TNF-α in response to cell apoptosis in intestinal I/R injury.

MATERIALS AND METHODS

Animals and surgery

The experimental protocol and design were approved by the Sun Yat-sen University Animal Experimentation Committee and performed according to the Sun Yat-sen University Guidelines for Animal Experimentation. Male Sprague-Dawley rats (approximately 200-250 g) were used in this study. The animals were housed in wire-bottomed cages placed in a room illuminated from 8:00 AM to 8:00 PM (12:12-h light-dark cycle) and maintained at 21 ℃ ± 1 ℃. The rats were allowed access to water and chow ad *libitum*. The rats were anaesthetized for 3 h by intraperitoneal injection of 4% chloral hydrate (200 mg/kg). A laparotomy was performed under chloral hydras anesthesia, and the superior mesenteric artery (SMA) was occluded with a micro bulldog clamp. At the end of the ischemic period, the clamp was released, and three drops of lidocaine were applied directly onto the SMA to facilitate reperfusion. After the experiment, the animals were euthanized, and then, the entire small intestine was carefully removed and placed on ice. The oral 10-cm segment (duodenum) was removed, and the rest of the intestine was divided into two equal segments, representing the proximal (jejunum) and distal (ileum) segments. Each segment was rinsed thoroughly with physiological saline. Jejunal and ileal pieces, approximately 2 cm in length, were removed from the middle portion of each segment and fixed in 10% neutral-buffered formalin for the measurement of mucosal injury, terminal deoxynucleotidyl transferase-mediated dUDP-biotin nick-end labeling (TUNEL) assay, and immunohistochemistry of caspase-3. The remainder of the segment was opened longitudinally on the antimesenteric border to expose the intestinal mucosa. The mucosal layer was harvested by gentle scraping using a glass slide.

Experimental design and animal pretreatment

To investigate mucosal injury after I/R, the SMA was occluded for 60 min followed by 60-min reperfusion (I/R), and pretreatment with vehicle (1 mL of physiological saline) for 60 min prior to I/R. To evaluate the effect of pentoxifylline, a TNF-α inhibitor, on mucosal injury in the small intestine after I/R, the animals were pretreated with 50 mg/kg pentoxifylline (Sigma, St Louis, MI, United States), dissolved in 1 mL of physiological saline, by intraperitoneal injection 60 min prior to I/R. Similarly, an infliximab (Janssen Biotech, Horsham, PA) dose of 5 mg/kg dissolved in 1 mL of physiological saline was administered by intraperitoneal injection 60 min prior to I/R to evaluate the effect of infliximab pretreatment. In sham-operated (SO) rats, pretreatment was performed with vehicle for 60 min, and then, the SMA was isolated in a similar manner but was not occluded. Six rats were studied in each group.

RNA extraction and real-time polymerase chain reaction

RNA was extracted from 100 mg of mucosal scrapings using TRIzol reagent (Invitrogen, Carlsbad, CA, United States) per the manufacturer's instructions. First-strand cDNA was synthesized from 1.5 μg of total RNA using a ReverTra Ace kit (Toyobo, Japan) per the manufacturer's instructions. An ABI Prism 7000 sequence detection system (Applied Biosystems, Bedford, MA) was then used for real-time polymerase chain reaction (PCR) experiments to quantitate the gene expression of TNF-α and β-actin for each sample. The reactions were performed in a 20-μL volume with TaKaRa Taq™ (TaKaRa, Japan).

The PCR conditions included a denaturation step at 94 ℃ for 5 min. Amplification was conducted for 35 cycles (denaturation at 94 ℃ for 30 s, annealing at 60 ℃ for 30 s, and extension at 72 ℃ for 30 s). The quantification was performed by using 7000 SDS instrument software (Applied Biosystems) for the relative quantification of gene expression. The primer sequences used were as follows: TNF-α forward primer, 5'-CACCACGCTCTTCTGTCT ACT-3'; TNF-α reverse primer, 5'-AGATGATCT-GAGTGTGAGGGTC-3'; β-actin forward primer, 5'-GAAATCGTGCGTGACATCAAAG-3'; and β-actin reverse primer, 5'-TGTAGTTTCATGGATGCCA-CAG-3'. The primers were supplied by Invitrogen. The results are expressed as the fold change in mRNA expression compared with the levels in sham-operated rats.

Purification of proteins

The mucosal scraping samples were immediately washed twice with ice-cold PBS (pH 7.4) and then homogenized in total protein extraction buffer. This extraction buffer consisted of 50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 0.1% SDS, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.5 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leuptin and 1.8 mg/mL iodoacetamide. The tissues were homogenized and lysed at 4 ℃ for 30 min and then centrifuged at 14000 *g* for 20 min at 4 ℃. The resulting supernatants were purified total proteins. The supernatants were divided into multiple samples and stored at -80 ℃. The protein concentrations were determined using a kit (Bio-Rad, Hercules, CA, United States).

Enzyme-linked immunosorbent assay

The TNF-α concentration of intestinal mucosa was measured using a commercial kit (eBioscience, San Diego, CA, United States), according to the manufacturer's instructions. Briefly, the enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 μL/well of capture antibody diluted in coating buffer and incubated overnight at room temperature (RT). The plates were washed with wash buffer and blocked for 1 h at RT with 200 μL/well assay diluent. Then, the TNF-α standard and samples (100 μL) were pipetted into appropriate wells. Next, the plates were sealed and incubated at RT for 2 h. After washing, 100 μL of detection antibody was added to each well, sealed, and incubated for 1 h at RT. After washing, 100 μL of substrate solution was added to each well and incubated for 30 min at RT in the dark. Stop solution (2 mol/L H_2SO_4, 50 μL/well) was added, and the plates were read at 450 nm (570 nm correction) on a MicroPlate Reader (BioTek, Seattle, WA, United States). The results are expressed as pg TNF-α/mg protein.

Morphological analysis and mucosal injury score

After the animals were sacrificed, the tissue samples removed from the jejunum and ileum were immediately fixed in 10% neutral-buffered formalin, embedded in paraffin and sectioned. The sample sections were processed with hematoxylin-eosin staining and examined by light microscopy, according to the criteria described by Chiu *et al*[8] as follows: grade 0, normal mucosa; grade 1, development of subepithelial (Gruenhagen) spaces near the tips of the villi with capillary congestion; grade 2, extension of the subepithelial space with moderate epithelial lifting from the lamina propria; grade 3, significant epithelial lifting along the length of the villi with a few denuded villous tips; grade 4, denuded villi with exposed lamina propria and dilated capillaries; and grade 5, disintegration of the lamina propria, hemorrhage, and ulceration. Twenty visual fields at × 100 magnification were evaluated for each sample slide, and the final injury scoring was a gross assessment of the degree of mucosal damage. All slides were evaluated by two examiners in a blinded fashion.

TUNEL assay and apoptotic index analysis

The sample sections were used to detect cell apoptosis. The fragmented DNA of apoptotic cells was stained via the TUNEL method by using an in situ cell death detection kit (Roche, Switzerland). The apoptotic index was calculated in a minimum of 20 randomly selected crypts and analyzed in six separate samples. The apoptotic index was determined by dividing the number of apoptotic cells by the total number of cells in the crypt column and multiplying by 100.

Immunohistochemistry

Sample sections were used for caspase-3 immunohistochemical staining. The sections were deparaffinized and rehydrated prior to antigen retrieval by using EDTA (pH 8.0) for 3 min at 130 ℃. The sections were incubated for 10 min with 5% BSA prior to incubation with caspase-3 antibody (1:400, Cell signaling technology, Danvers, MA, United States) at 4 ℃ overnight. Subsequently, the sections were incubated at 37 ℃ for 30 min with anti-rabbit IgG (1:400; Santa Cruz Biotechnology, Santa Cruz, CA). Antigen-antibody complexes were visualized by staining with a DAB kit (Dako, Denmark). The slides were then counter-stained with hematoxylin for 1 min and mounted. The negative controls were created by omitting the primary antibody.

Western blotting analysis

Apoptotic proteins (caspase-9 and caspase-3) were analyzed by Western blotting. Equal quantities (20 μg) of lysates were electrophoresed in an SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Bio-Rad). After blocking with PBS containing 0.1% polyoxyethylene sorbitan monolaurate (Tween 20, Sigma) and 5% skim milk for 1 h, the membrane was incubated with a rabbit polyclonal anti-TNF-α antibody (1:500; cell signaling technology), a mouse polyclonal anti-caspase-9 antibody (1:1000; cell signaling technology), a rabbit polyclonal anti-caspase-3 antibody (1:1000; cell signaling technology), a rabbit polyclonal anti-JNK antibody (1:1000; cell signaling technology), a rabbit polyclonal anti-p-JNK antibody (1:500; cell signaling technology), a rabbit polyclonal anti-c-Jun antibody (1:1000; Cell signaling technology), and a rabbit polyclonal anti-p-c-Jun antibody (1:500;

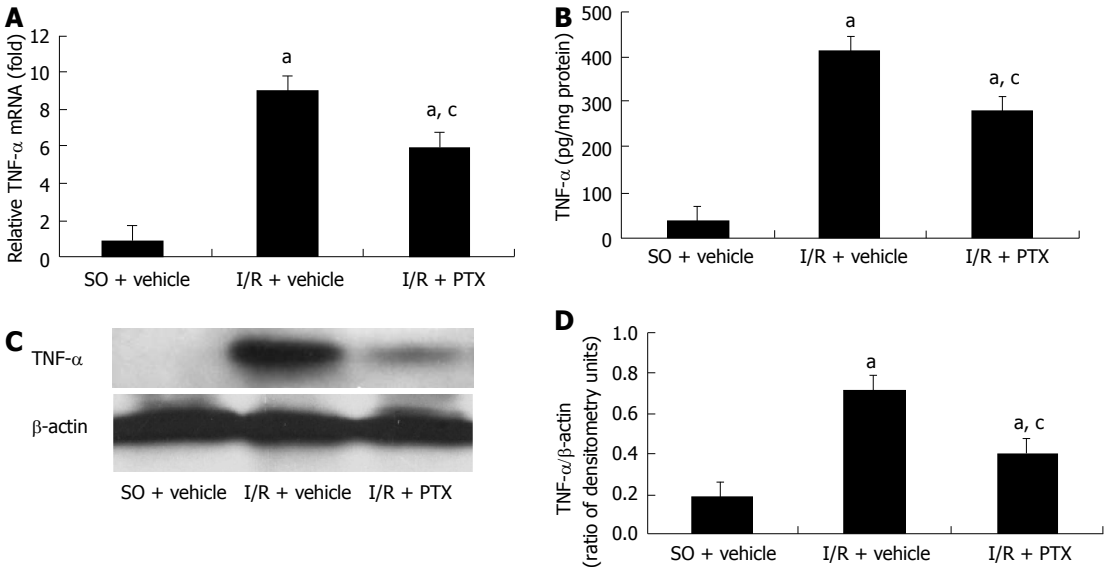

Figure 1 Ischemia-reperfusion induced intestinal mucosal tumor necrosis factor-α expression. A: Small intestinal mucosal tumor necrosis factor-α (TNF-α) mRNA expression; B: ELISA analysis of small intestinal mucosal TNF-α protein expression; C: Western blotting analysis of small intestinal mucosal TNF-α protein expression; D: The results of Western blotting analysis were expressed as a ratio to β-actin densitometry units. A TNF-α inhibitor, pentoxifylline (PTX), significantly suppressed the TNF-α mRNA and protein expression. Values are mean ± SE. Six rats were tested in each group. $^{a}P < 0.05$ *vs* sham-operation (SO) rats pretreated with vehicle (SO + vehicle), $^{c}P < 0.05$ *vs* ischemia-reperfusion (I/R) rats pretreated with vehicle (I/R + vehicle).

Cell signaling technology) at 4 ℃ overnight. Antigen-antibody complexes were detected with horseradish peroxidase-conjugated anti-rabbit IgG (1:6000; Santa Cruz Biotechnology) or anti-mouse IgG (1:6000; Santa Cruz Biotechnology). Detection of chemiluminescence was performed using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ, United States). The densitometric assessment of bands from the autoradiogram was performed using Image Gauge VDS (Fujifilm, Tokyo, Japan). Band intensities were quantified by measuring the absolute integrated optical intensity, which estimates the band in the lane profile. The results are expressed as the ratios of β-actin densitometry units.

Statistical analysis

Ranked data (mucosal injury scoring) are presented as the median and range, and the significance between groups was tested with the Wilcoxon rank test. Other results are expressed as the mean ± SE. Data were evaluated by one-way Analysis of Variance, and multiple comparisons were performed using the method of least significant difference *t* test. Differences were considered significant if $P < 0.05$.

RESULTS

I/R induced TNF-α expression in rat small intestines

Real-time PCR, ELISA and Western blotting analysis were conducted to evaluate the expression of TNF-α after intestinal I/R and the effect of pretreatment with pentoxifylline on the expression of TNF-α. The results are shown in Figure 1. A small amount of TNF-α was detected in sham-operated rats. Compared with the sham-operated rats, the amount of intestinal mucosal TNF-α at both the mRNA and protein levels was significantly increased after I/R, and this increase of TNF-α was markedly inhibited by pretreatment with pentoxifylline.

Suppression of TNF-α alleviated I/R-induced mucosal injury

The hematoxylin-eosin staining of jejunum sections is shown in Figure 2. Samples from sham-operated rats pretreated with the vehicle alone displayed an intact mucosal structure, whereas the intestinal I/R induced apparent mucosal damage, extensive epithelial layer damage, disintegration of lamina propria and hemorrhage. In contrast, pretreatment with pentoxifylline or infliximab attenuated the I/R-induced injury. In the ileum, the results were similar to those observed in the jejunum (data not shown). The mucosal injury score is shown in Table 1, and the results indicated that pretreatment with pentoxifylline or infliximab markedly attenuated I/R-induced intestinal mucosal injury, compared with I/R pretreatment with vehicle.

Repression of TNF-α attenuated mucosal cell apoptosis after intestinal I/R

The effect of TNF-α on mucosal cell apoptosis was investigated. The results of TUNEL staining of the jejunum indicated that few apoptotic cells were observed at the villus tips in the sham-operated rats, which is consistent with physiological apoptosis during the renewal of intestinal epithelium. Compared with the sham-operated rats, marked destruction of the jejunum structure and increased staining signal of apoptotic cells were observed in I/R rats. Pretreatment with either pentoxifylline or infliximab reduced the destruction of the structure in the jejunum and decreased the number of apoptotic cells (Figure 3A-D). The jejunum mucosal apoptotic index in each

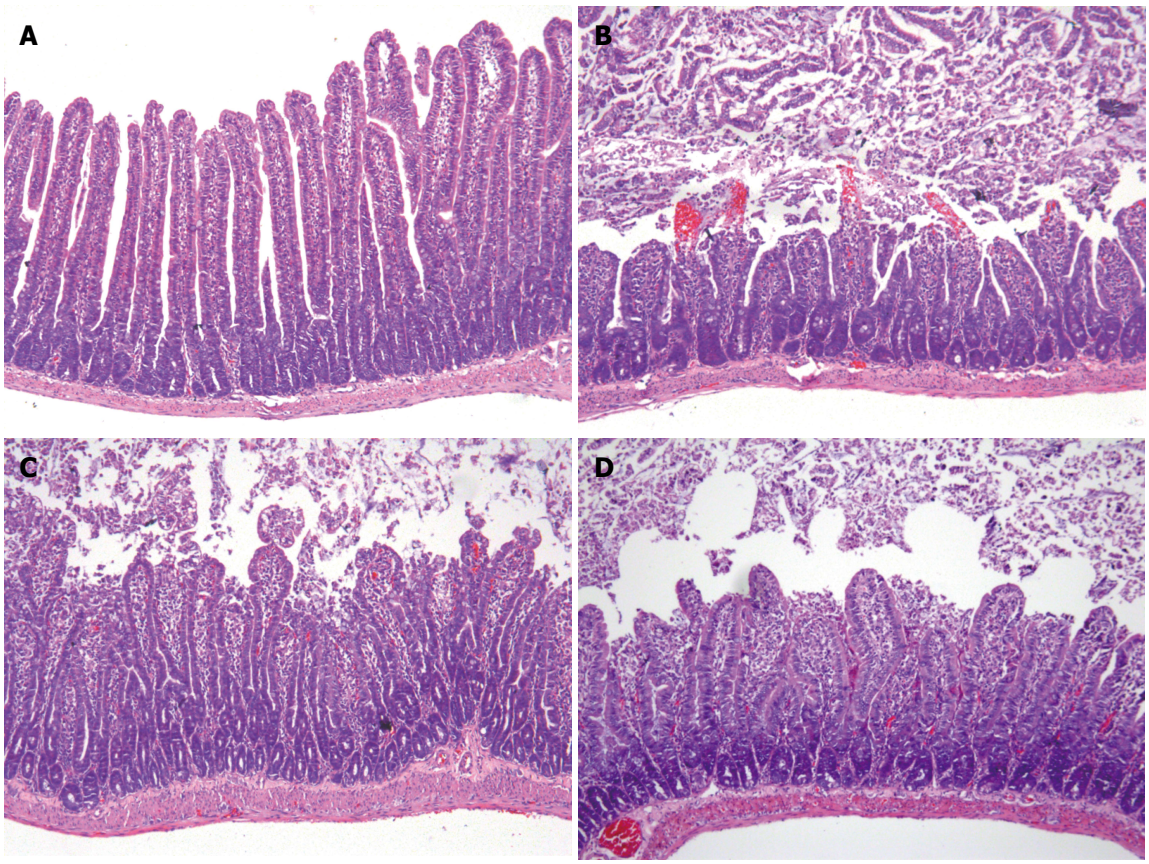

Figure 2 Suppression of tumor necrosis factor-α alleviated ischemia-reperfusion-induced small intestinal injury. A: Sham-operation pretreated with vehicle; B: Ischemia-reperfusion (I/R) pretreated with vehicle. C: I/R pretreated with a tumor necrosis factor-α (TNF-α) inhibitor pentoxifylline; D: I/R pretreated with a TNF-α antibody infliximab; Representative sections of jejunum for hematoxylin and eosin staining (× 100) were showed. Six rats were studied in each group, and a similar pattern was seen in six different rats in each group.

Table 1 Inhibition of tumor necrosis factor reduced mucosal injured scoring after intestinal ischemia-reperfusion

Group	Mucosal injured scoring	
	Jejunum	Ileum
SO rats: pretreated with vehicle	0.0 (0-1)	0.0 (0-1)
I/R rats: pretreated with vehicle	5.0 (4-5)[a]	4.5 (4-5)[a]
I/R rats: pretreated with PTX	3.0 (2-4)[a, c]	3.0 (2-4)[a, c]
I/R rats: pretreated with IFX	3.5 (2-4)[a, c]	3.0 (2-4)[a, c]

Values are expressed as the median (range). Six rats were tested in each group. [a]$P < 0.05$ *vs* sham-operated (SO) rats pretreated with vehicle, [c]$P < 0.05$ *vs* ischemia-reperfusion (I/R) rats pretreated with vehicle. PTX: Pentoxifylline; IFX: Infliximab.

group is shown in Figure 3E. Pretreatment with pentoxifylline or infliximab significantly attenuated the apoptotic index after intestinal I/R. The results in the ileum were similar to those observed in the jejunum (data not show).

To confirm the cell apoptotic death, immunohistochemistry staining of caspase-3 was performed. The results are shown in Figure 4. In the sections from the sham-operated rats, few caspase-3-positive cells were observed at the villus tips. Samples from I/R-treated rats displayed intense and extensive positive staining for caspase-3. The number of caspase-3-positive cells was markedly reduced in I/R rats pretreated with pentoxifylline or infliximab.

TNF-α mediated I/R-induced mucosal apoptosis via caspase activation in small intestine

Intestinal apoptotic proteins were analyzed by Western blotting assay, and the results are shown in Figure 5. In the sham-operated rats, only small amounts of cleaved caspase-9 and caspase-3 were detected in the small intestinal mucosa. In contrast, the expression of cleaved activated caspase-9 and caspase-3 were significantly increased after intestinal I/R. However, pretreatment with pentoxifylline or infliximab significantly suppressed the activation of those caspase proteins.

TNF-α mediated JNK activation response to intestinal I/R-induced injury

As shown in Figure 6, after intestinal I/R, the phosphorylation of JNK/c-Jun was significantly enhanced compared with that in sham-operated rats pretreated with the vehicle alone. Pretreatment with pentoxifylline markedly inhibited the phosphorylation of JNK/c-Jun, and pretreatment with infliximab also significantly decreased the activation of JNK/c-Jun. These results suggest that TNF-α mediated a JNK activation response to intestinal ischemia-reperfusion injury.

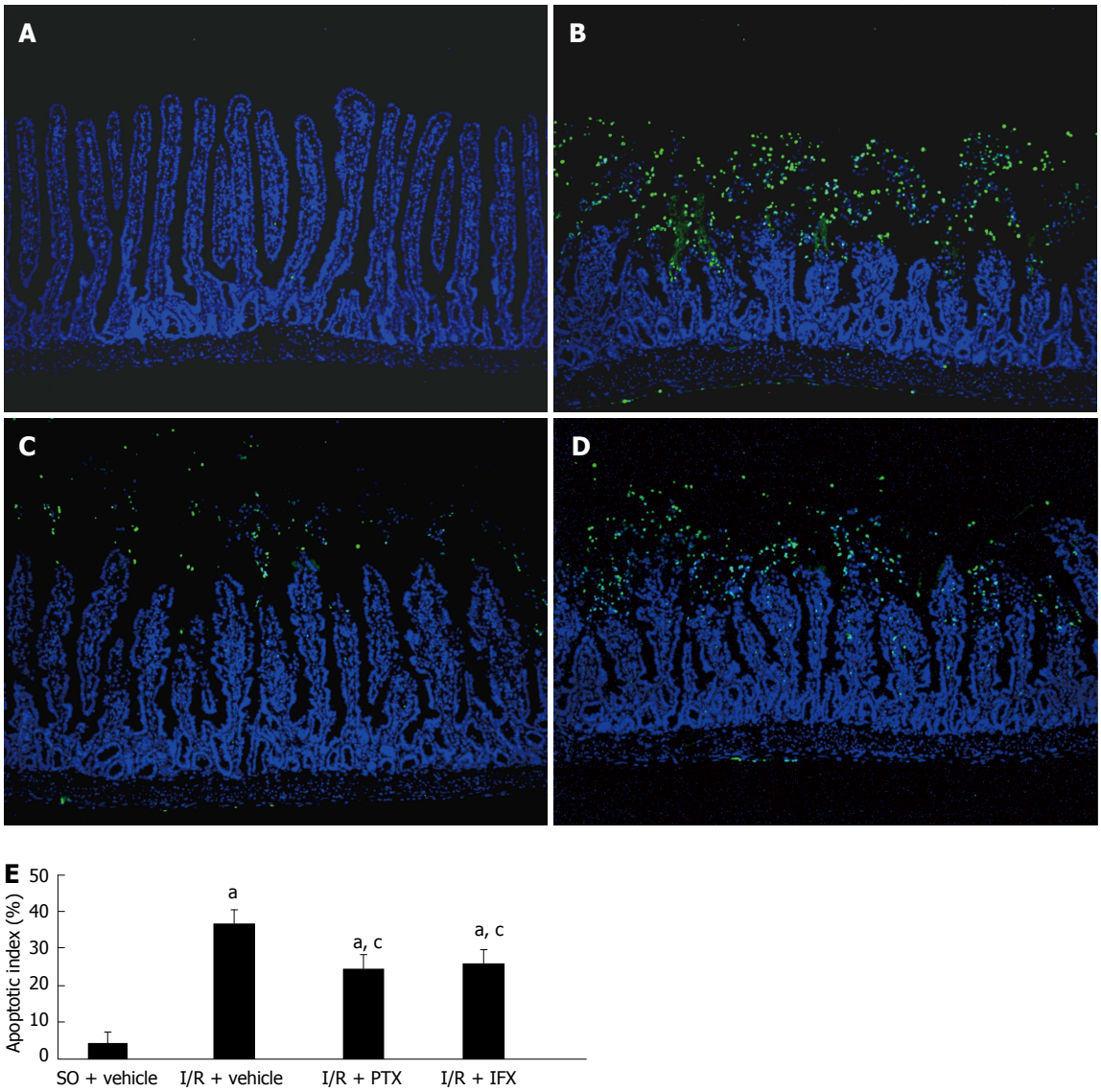

Figure 3 Suppression of tumor necrosis factor-α attenuated ischemia-reperfusion-induced intestinal mucosal apoptosis. A: Sham-operation (SO) pretreated with vehicle; B: Ischemia-reperfusion (I/R) pretreated with vehicle; C: I/R pretreated with a tumor necrosis factor-α (TNF-α) inhibitor pentoxifylline (PTX); D: I/R pretreated with a TNF-α antibody infliximab (IFX); E: The apoptotic index was calculated by counting a minimum of 20 randomly selected villi and crypts in the sections following terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining. The index was obtained by dividing the TUNEL positive cells by the total number of cells. Values are mean ± SE. Six rats were tested in each group. $^{a}P < 0.05$ *vs* SO rats pretreated with vehicle (SO + vehicle); $^{c}P < 0.05$ *vs* I/R rats pretreated with vehicle (I/R + vehicle). Apoptosis was assessed by TUNEL immunofluorescence staining. Mid-jejunum sections of rats were stained by TUNEL (green), with nuclei counterstained by 4',6-diamidino-2-phenylindole dihydrochloride (blue). Magnifications: × 100. Six rats were studied in each group, and a similar pattern was seen in six different rats in each group.

DISCUSSION

In tissue I/R injury, TNF-α is believed to be an early mediator. TNF-α is produced by a variety of cells, including macrophages, neutrophils, endothelia cells, nature killer cells and T/B lymphocytes[9,10]. The increased TNF-α may, in turn, augment the activation and action of those cells in I/R injury. TNF-α is involved in ROS production and the release of inflammation factors, such as interleukin-1, platelet-activating factor, and intercellular adhesion molecule[11]. TNF-α also mediates the injury of endothelia cells and the infiltration of neutrophils[12,13] and plays a pivotal role in I/R injury[14]. Subsequent studies have indicated that TNF-α mediates the injury induced by I/R, while inhibiting its function or expression supplied a protective effect[3]. However, some studies have demonstrated that TNF-α might be a protective factor in I/R-induced injury[15-17]. Because TNF-α has pleiotropic functions, researchers believe that after its interaction with TNF-receptor-1 and TNF-receptor-2, TNF-α activates pathways that favor both cell survival and apoptosis depending on the cell type and biological context.

Currently, two classic signal pathways are believed to mediate apoptotic signals: the caspase-8-mediated type- I apoptotic pathway and caspase-9-mediated type- II pathway. Caspase-3 ultimately executes the apoptotic signal. In a study on hepatic ischemia-reperfusion injury, the researchers demonstrated that TNF-α, but not Fas, medi-

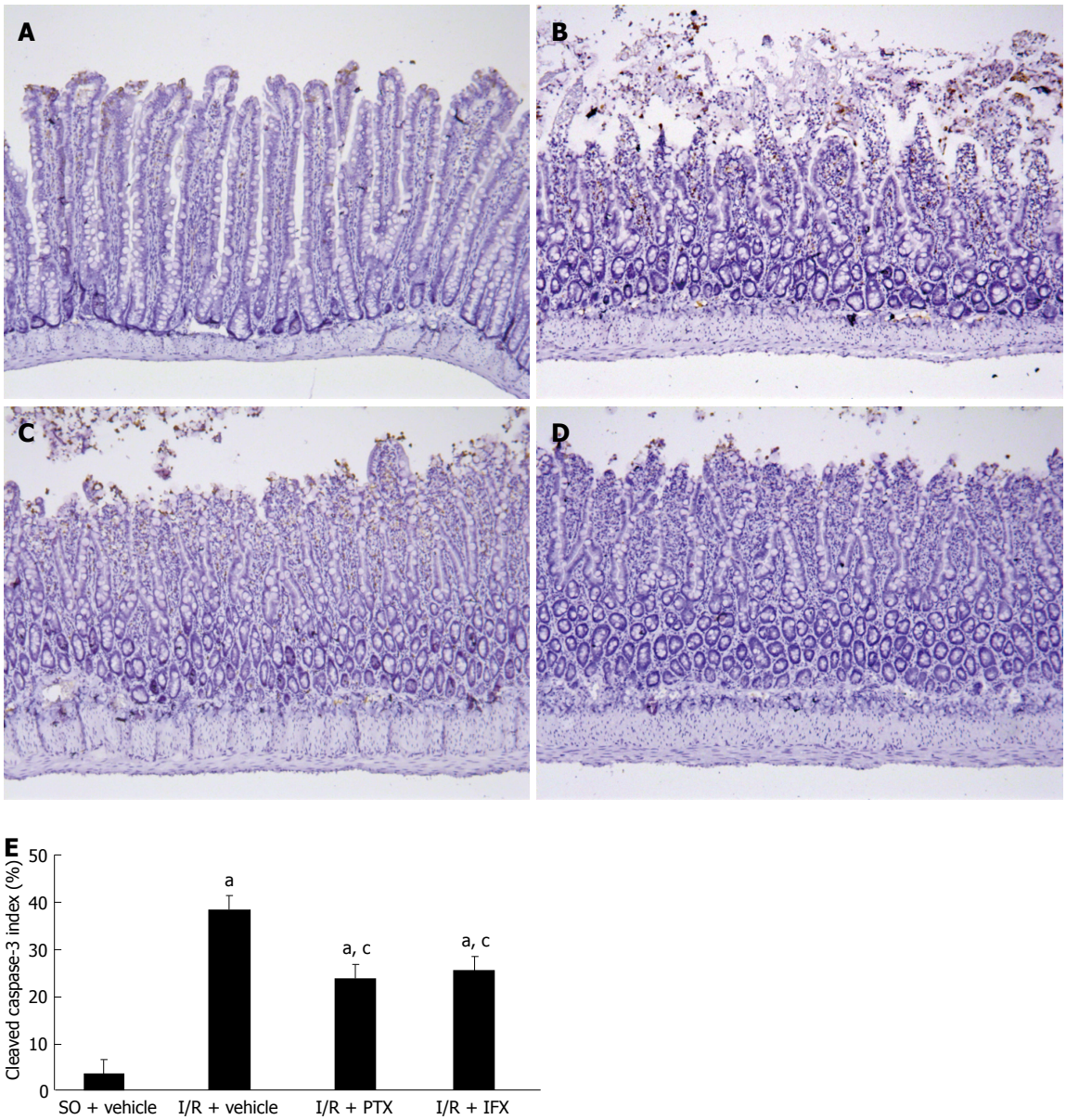

Figure 4 Suppression of tumor necrosis factor-α repressed caspase-3 activity in ischemia-reperfusion intestinal mucosa. A: Sham-operation (SO) pretreated with vehicle; B: Ischemia-reperfusion (I/R) pretreated with vehicle; C: I/R pretreated with a tumor necrosis factor-α (TNF-α) inhibitor pentoxifylline (PTX); D: I/R pretreated with a TNF-α antibody infliximab (IFX); Activated caspase-3 was assessed by immunohistochemical staining. Magnifications: × 100; E: The active caspase-3 index was calculated by counting a minimum of 20 randomly selected villi and crypts in the sections following cleaved caspase-3 staining. The index was obtained by dividing the cleaved caspase-3 positive cells by the total number of cells. Values are mean ± SE. Six rats were tested in each group. $^{a}P < 0.05$ *vs* SO rats pretreated with vehicle (SO + vehicle); $^{c}P < 0.05$ *vs* I/R rats pretreated with vehicle (I/R + vehicle). Six rats were studied in each group, and a similar pattern was seen in six different rats in each group.

ated apoptosis in hepatocytes[18]. Our previous study indicated that TNF-α mediated mucosal cell apoptosis in rat intestines that suffered venous congestion[19]. However, the effect of TNF-α is poorly understood in I/R-induced intestinal cell apoptosis.

TNF-α can activate both the stress-activated protein kinase cascade, including JNK[20,21], and the NF-κB signaling pathway[22,23]. Therefore, the stimulation of cells with TNF-α may lead to both pro-apoptotic and anti-apoptotic consequences. JNK is a member of the mitogen-activated protein kinases (MAPKs). It has been shown that JNK mediates inflammatory processes by inducing the expression of adhesion molecules and inflammatory chemokines. The activation of JNK is closely related to cell apoptosis in I/R-induced injury, and TNF-α is a strong stimulating factor[24,25]. Under I/R conditions, JNK is activated by the dual phosphorylation of threonine (Thr) and tyrosine (Tyr), and the phosphorylated-JNK translocates to the nucleus, where it phosphorylates and activates different transcription factors and transactivates target genes, including c-Jun[26]. Phosphorylation of c-Jun leads to the formation of AP-1, which is involved in the transcription of a wide variety of proteins, and some of them are known to be pro-apoptotic proteins[27].

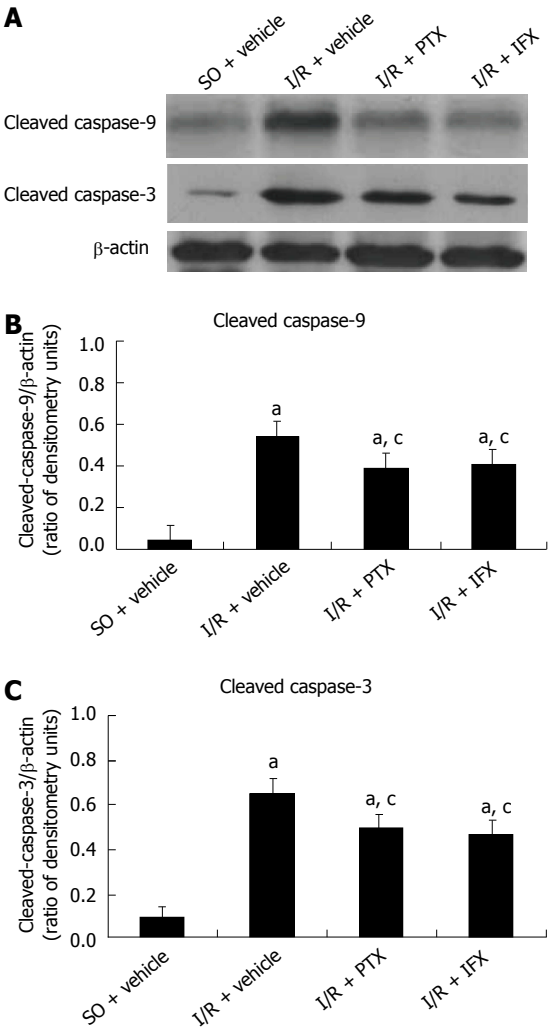

Figure 5 Tumor necrosis factor-α mediated ischemia-reperfusion-induced mucosal apoptosis *via* caspase activation in intestinal mucosa. Total protein was extracted from intestinal mucosa, and subjected to SDS-PAGE and Western blotting analysis. β-actin was used as the control for loading. The results were expressed as a ratio to β-actin densitometry units. A: Western blotting analysis; B: The ratio of cleaved caspase-9 and β-actin; C: The ratio of cleaved caspase-3 and β-actin. Values are mean ± SE. Six rats were tested in each group. $^{a}P < 0.05$ *vs* sham-operation (SO) rats pretreated with vehicle (SO + vehicle); $^{c}P < 0.05$ *vs* ischemia-reperfusion(I/R) rats pretreated with vehicle (ischemia-reperfusion + vehicle). PTX: Pentoxifylline; IFX: Infliximab.

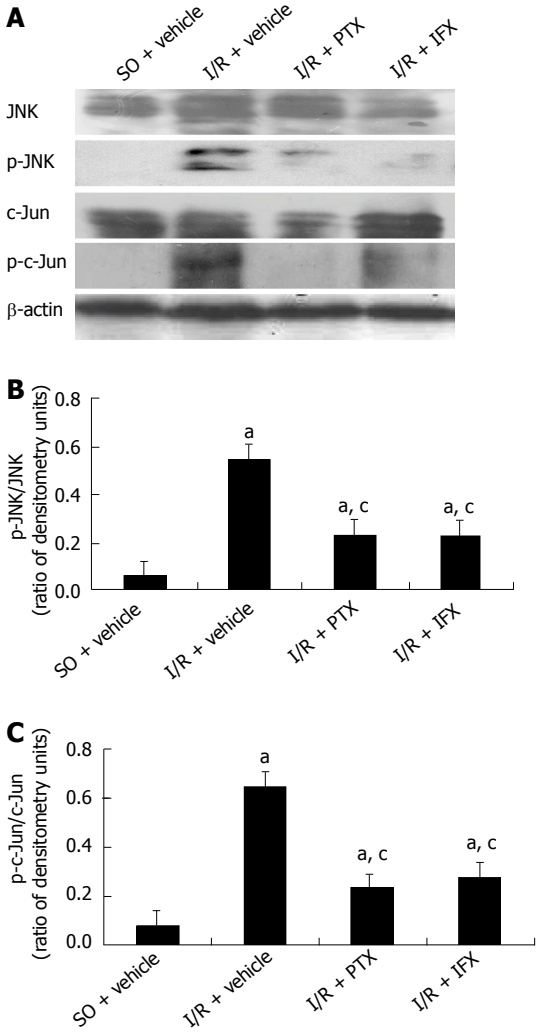

Figure 6 Tumor necrosis factor-α mediated c-Jun N-terminal kinase activation response to mucosal injury in ischemia-reperfusion-induced intestine. Equal quantities of protein were subjected to Western blotting analysis, and β-actin was used as the control for loading. The results were expressed as a ratio of densitometry units. A: Western blotting analysis; B: The ratio of p-c-Jun N-terminal kinase (JNK) and JNK; C: The ratio of p-c-Jun and c-Jun. Values are mean ± SE. Six rats were tested in each group. $^{a}P < 0.05$ *vs* sham-operation(SO) rats pretreated with vehicle (SO + vehicle); $^{c}P < 0.05$ *vs* ischemia-reperfusion (I/R) rats pretreated with vehicle (I/R + vehicle). PTX: Pentoxifylline; IFX: Infliximab.

Pentoxifylline has been proven to be a potent inhibitor of TNF-α production[28]. Recently, several studies have suggested that pretreatment with pentoxifylline in intestinal I/R not only attenuates the local intestinal injury but also improves the tolerance of the remote organ to I/R[29,30]. However, the specific effects of pentoxifylline on the cell apoptosis and apoptotic signal pathways in intestinal I/R are not clear. Infliximab, a chimeric TNF-α monoclonal antibody, has been shown to inhibit the function of TNF-α in a variety of studies[31]. Because infliximab is a potent antibody against TNF-α, capable of neutralizing all forms (extracellular, transmembrane, and receptor-bound) of TNF-α[32], it may also attenuate TNF-α-mediated injury in I/R. Few studies have been conducted that examine the effect of infliximab on I/R injury. Guven *et al*[33] suggested that infliximab exerted neuroprotective effects in a study involving an experimental spinal cord ischemic injury. A recent study indicated that infliximab might have protective effects in intestinal I/R injury because of its anti-inflammatory and antioxidant properties[7].

In this study, our data identified that I/R dramatically induced TNF-α expression at both the mRNA and protein levels using real time-PCR, ELISA and Western bloting analysis, and pretreatment with pentoxifylline significantly downregulated TNF-α expression. Morphological analysis indicated that I/R induced apparent intestinal mucosal injury, which was characterized by the appearance of hemorrhage, extensive epithelial disruption and disintegration of lamina propria. Pretreatment with pentoxifylline or infliximab significantly alleviated the injury, which was confirmed by mucosal injury scor-

ing. TUNEL assay and caspase-3 immunohistochemical staining demonstrated that I/R remarkably induced mucosal apoptosis, and pretreatment with pentoxifylline or infliximab suppressed cell apoptosis, thus contributing to the alleviation of injury. The observed apoptotic index values were also consistent with these results.

The Western blotting results showed that apoptotic proteins, including caspase-9 and caspase-3, were significantly activated after intestinal I/R, and the activation of caspases was suppressed by pretreatment with pentoxifylline or infliximab. The present data suggest that TNF-α mediates caspase-dependent mitochondrial apoptotic signaling. In addition, our data showed that the phosphorylation of JNK and c-Jun increased after intestinal I/R. When TNF-α was suppressed by pretreatment with pentoxifylline or infliximab, the phosphorylation of JNK and c-Jun, as well as intestinal apoptosis, were also significantly inhibited. The data suggest that the activation of the JNK signaling pathway is involved in TNF-α-mediated caspase-dependent apoptosis.

In summary, intestinal I/R dramatically induced TNF-α expression, and TNF-α activated the JNK signaling response to caspase-dependent apoptosis, resulting in intestinal injury. Clinically, a treatment involving inhibition of TNF-α by pentoxifylline or infliximab might ameliorate intestinal injury in I/R patients, and pentoxifylline has potential as a low-cost and efficacious anti-TNF-α compound.

COMMENTS

Background

Intestinal ischemia-reperfusion (I/R) injury may occur in numerous situations, such as small bowel transplantation, strangulated hernias, neonatal necrotizing enterocolitis, cardiopulmonary bypass surgery and hypovolemic/septic shock. A series of factors, including reactive oxygen species production, calcium overload, neutrophil infiltration and cytokine release, are involved in I/R injury. Among these mediators, tumor necrosis factor-α (TNF-α) acts as an initial inflammation factor in I/R injury and is believed to play a pivotal role. However, the exact mechanism of TNF-α in intestinal I/R is still not clearly understood.

Research frontiers

TNF-α is believed to initiate three signaling pathways: the apoptotic signaling pathway, c-Jun N-terminal kinase (JNK) signaling pathway and nuclear factor kappa-B signaling pathway. These pathways are all involved in cell injury and apoptosis. However, the role of TNF-α in I/R-induced injury and its mechanisms remain to be elucidated. The effect of TNF-α-mediated JNK signaling pathways on cell apoptosis requires further exploration.

Innovations and breakthroughs

The study demonstrated that I/R significantly enhanced expression of mucosal TNF-α at both the mRNA and protein levels, induced severe mucosal injury and cell apoptosis, and activated JNK. Pretreatment with the TNF-α inhibitor pentoxifylline or the TNF-α antibody infliximab dramatically suppressed I/R-induced mucosal injury and cell apoptosis and significantly inhibited the activation of caspase-9/3 and JNK. The results of this study indicate that TNF-α played a pivotal role in intestinal I/R injury, and TNF-α mediated a JNK activation response to intestinal I/R injury.

Applications

In the present study, the authors investigated the mechanism of TNF-α in I/R-induced intestinal injury, which will provide new insight into the pathogenesis of I/R injury. Moreover, the study indicated that treatment *via* inhibiting TNF-α by pentoxifylline or infliximab might ameliorate intestinal injury in I/R patients.

Terminology

TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. It is produced chiefly by activated macrophages (M1), although it can be produced by many other cell types such as CD4+ lymphocytes, NK cells and neurons. JNK were originally identified as kinases that bind and phosphorylate c-Jun on Ser-63 and Ser-73 within its transcriptional activation domain. They belong to the mitogen-activated protein kinase family and are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock. They also play a role in T-cell differentiation and the cellular apoptosis pathway.

Peer review

The authors have done some intriguing studies in rats in TNF-α mediated JNK activation response to intestinal ischemic reperfusion injury. This is very interesting and well written paper.

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BRIEF ARTICLE

Analysis of single nucleotide polymorphisms in the region of *CLDN2-MORC4* in relation to inflammatory bowel disease

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Abstract

AIM: To investigate a possible genetic influence of claudin (*CLDN*)*1*, *CLDN2* and *CLDN4* in the etiology of inflammatory bowel disease.

METHODS: Allelic association between genetic regions of *CLDN1*, *CLDN2* or *CLDN4* and patients with inflammatory bowel disease, Crohn's disease (CD) or ulcerative colitis were investigated using both a case-control study approach (one case randomly selected from each of 191 Swedish inflammatory bowel disease families and 333 controls) and a family-based study (463 non-Swedish European inflammatory bowel disease -families). A nonsynonymous coding single nucleotide polymorphism in *MORC4*, located on the same linkage block as *CLDN2*, was investigated for association, as were two novel *CLDN2* single nucleotide polymorphism markers, identified by resequencing.

RESULTS: A single nucleotide polymorphism marker (rs12014762) located in the genetic region of *CLDN2*

was significantly associated to CD (case-control allelic OR = 1.98, 95%CI: 1.17-3.35, *P* = 0.007). *MORC4* was present on the same linkage block as this CD marker. Using the case-control approach, a significant association (case control allelic OR = 1.61, 95%CI: 1.08-2.41, *P* = 0.018) was found between CD and a nonsynonymous coding single nucleotide polymorphism (rs6622126) in *MORC4*. The association between the *CLDN2* marker and CD was not replicated in the family-based study. Ulcerative colitis was not associated to any of the single nucleotide polymorphism markers.

CONCLUSION: These findings suggest that a variant of the *CLDN2-MORC4* region predisposes to CD in a Swedish population.

Key words: Crohn's disease; Genetic predisposition; Inflammatory bowel disease; Single nucleotide polymorphism; Tight junctions

Core tip: Tight junction proteins are key components in the regulation of paracellular permeability and therefore we considered claudin genes as candidate genes in the study. Association was identified between a single nucleotide polymorphism marker (rs12014762) in the *CLDN2-MORC4* region and the occurrence of Crohn's disease (CD) in a Swedish population. Additionally, a nonsynonymous coding single nucleotide polymorphism (rs6622126) in *MORC4* was associated to CD. Our findings add further support for a genetically impaired intestinal epithelial barrier as one predisposing factor in the etiology of CD.

Söderman J, Norén E, Christiansson M, Bragde H, Thiébaut R, Hugot JP, Tysk C, O'Morain CA, Gassull M, Finkel Y, Colombel JF, Lémann M, Almer S. Analysis of single nucleotide polymorphisms in the region of *CLDN2-MORC4* in relation to inflammatory bowel disease. *World J Gastroenterol* 2013; 19(30): 4935-4943 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4935.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4935

INTRODUCTION

Chronic inflammatory bowel disease (IBD) encompasses Crohn's disease (CD), ulcerative colitis (UC) and, in the absence of a confident diagnosis, unclassified colitis (IBD-U). Susceptibility to IBD and the broad spectrum of phenotypic expressions depends on contributions from environmental factors and a genetic predisposition. Several etiological factors have been suggested, including the presence of specific strains of commensal enteric bacteria, defective bacterial killing, aberrant regulation of innate and adaptive immune responses and an impaired intestinal barrier[1]. Consistent with this, several genetic associations in IBD have been described[2-8].

Both CD and UC have been associated with an increase in intestinal permeability[9-11]. Based on findings of an increased intestinal permeability among healthy first-degree relatives to CD patients, a role for a tight junction (TJ) based genetic contribution to permeability changes has been suggested as a predisposing susceptibility factor for CD[12]. This suggestion has been contradicted by other studies[13-15]. However, by defining a normal range of intestinal permeability in healthy controls, a subgroup of healthy first-degree relatives to CD patients with increased intestinal permeability has been identified[16]. An increased permeability response to acetylsalicylic acid has been observed in CD patients and their relatives, indicating hereditary factors underlying this responsiveness[14]. An increased permeability may be primary or a consequence of subclinical intestinal inflammation present as an inherited abnormality in relatives of CD-patients[17]. It is still unknown whether this disturbed permeability is caused by genetic or environmental factors, but several studies provide support for a genetic rather than environmental induced increase[18,19].

The barrier of epithelial cells, with their apical TJ-structure, is critical for the permeability properties of the intestine. The TJ-structure is a multicomponent protein complex that serves to seal and regulate permeability across the paracellular space between adjacent epithelial cells, with the family of membrane-spanning claudin-proteins as the major determinants[20,21]. Claudin-1 and claudin-4 have been associated with a tight TJ-structure whereas claudin-2 expression results in a more leaky epithelial layer[22-24]. Expressions of claudins, and other TJ-proteins, are subject to regulation by different cytokines[20]. Claudin-4 seems to be preferentially expressed in M-cells[25] and the dome area of the follicle-associated epithelium[26], which has been suggested to be the site of initial inflammation in ileal CD[27].

Our aim was to elucidate a possible genetic influence of tight junction-components to IBD-susceptibility and therefore we conducted genetic association studies using single nucleotide polymorphism (SNP) markers of the genetic regions of claudin 1 (*CLDN1*, chromosome 3q28-q29), claudin 2 (*CLDN2*, chromosome Xq22.3-q23) and claudin 4 (*CLDN4*, chromosome 7q11.23). Furthermore, in order to identify putative functional sequence variants of *CLDN2*, the promoter region, exon-intron boundaries and exons harbouring the 5' untranslated region and protein coding region were amplified and resequenced.

MATERIALS AND METHODS

Study subjects

The IBD-families in this study originated from the large European collaboration that lead to the discovery of *NOD2/CARD15* as a CD susceptibility gene[5,28]. Swedish IBD-patients were selected for inclusion in a case-control study, whereas the remaining non-Swedish families were used in a family based genetic association study (Table 1). Samples from an anonymized regional DNA bank con-

Table 1 General study outline

Study design[1]	Cohort and disease	Number of families	Number of individuals	Women
Case control study	Healthy unrelated controls		333	162
	Swedish IBD families	191		
	IBD		347	157
	CD		150	69
	UC		166	71
	IBD-U		31	17
Family based approach	Non-Swedish families	463		
	IBD		715	398
	CD		528	297
	UC		151	83
	IBD-U		36	18

[1]Including Claudin (*CLDN*)*1, CLDN2, CLDN4*. IBD: Inflammatory bowel disease; CD: Crohn's disease; UC: Ulcerative colitis; IBD-U: Unclassified colitis.

sisting of randomly selected individuals (n = 800) living in the southeastern part of Sweden were used as controls in the case-control studies. The study was conducted under approval by the ethics committees of Linköping University (DNR 97271) and Karolinska Institutet (DNR 97-327).

SNP selection for genetic association studies

Linkage blocks were defined using SNP data from the HapMap CEPH collection and the SNPbrowser software version 3.5 (Applied Biosystems, Foster City, CA, United States) and a default value of 0.3 linkage disequilibrium units (LDU)[29]. SNP markers were selected for *CLDN1*, *CLDN2* and *CLDN4* (Table 2). A distance less than 1 LDU has been considered useful for allelic association[30]. SNP markers were also chosen from adjacent linkage blocks.

Genotyping

Allele discrimination was carried out using TaqMan SNP genotyping assays (Table 2) and TaqMan Genotyping Master Mix or TaqMan Fast Universal polymerase chain reaction (PCR) Master Mix without AmpErase UNG (Applied Biosystems), using either a 7300 Real-Time PCR System or a 7500 Fast Real-Time PCR System (Applied Biosystems). All genotype data were analyzed using the 7500 Fast System SDS Software version 1.3.1.21 (Applied Biosystems).

In addition, a nonsynonymous coding SNP in *MORC4* (rs6622126; Applied Biosystems assay ID C_22273025_10) and two novel *CLDN2* SNP markers (this study; rs62605981 and rs72466477) were genotyped. The two novel *CLDN2* sequence variants were ordered as custom assays from Applied Biosystems. Primer and probe sequences are available from the authors upon request.

Resequencing of CLDN2

The promoter region, exon-intron boundaries and exons harbouring the 5' untranslated region and protein coding region of *CLDN2* were amplified by PCR and resequenced (Table 3). PCR amplifications were in accordance with the manufacturer's recommendations, using HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 2.0 mmol/L $MgCl_2$, and 0.2 μmol/L per PCR primer (Operon Biotechnologies GmbH, Cologne, Germany, and Scandinavian Gene Synthesis AB, Köping, Sweden). The following PCR cycle was repeated 45 times: 94 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 60 s. A total of 93 individuals (21 CD, 21 UC, 5 IBD-U, and 46 healthy; 60 females) were resequenced.

All PCR products were purified in accordance with the QIAquick PCR purification kit protocol (Qiagen), and analyzed using the DNA 1000 assay on the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, United States). Cycle sequencing was carried out using the CEQ8800 system and accompanying sequencing reagents from Beckman Coulter (Fullerton, CA, United States) and conducted using half concentrated CEQ DTCS quick start kit, purification with an ethanol-precipitation protocol. Sequence data were analyzed with the heterozygote detection option activated in the software (version 8.0.52) and relative a reference sequence. New sequence variants were deposited in the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) and provided with rs-numbers.

Prediction of transcription factor binding sites

Transcription factor binding sites were predicted using the Alibaba 2.1 software[31] available through Biobase (www.gene-regulation.com), in conjunction with the Transfac database public release version 7.0[32] at Biobase.

Statistical analysis

Allelic OR with accompanying 95%CI, and P values based on χ^2 statistics were calculated using a likelihood-based analysis for genetic association with the Unphased software version 3.0.13[33], both in the case-control approach and in the family-based study. In order to avoid bias due to genetic relatedness in the case-control study, one case per family was randomly selected from 191 Swedish IBD families. These random samplings of cases were repeated 15 times and the median OR was used as a representative measure of association. The case-control studies with respect to IBD, CD and UC were based on 191, 103 and 102 cases of IBD, CD, and UC, respectively, and 333 controls. No deviations from Hardy-Weinberg equilibrium were observed.

Since *CLDN2-MORC4* are located in a non-pseudo-autosomal region of the X-chromosome, males contribute one allele and females two alleles. Because the analysis did not identify sex as a confounder, males and females were analyzed jointly. Association to clinical features was tested for using a chi-square test for qualitative variables and one way ANOVA for quantitative variables, and carried out using the GraphPad Prism 4 software (GraphPad Software, La Jolla, CA, United States). For all statistical

Table 2 Single nucleotide polymorphism markers in the genetic regions of *Claudin-1*, *Claudin-2* and *Claudin-4*

Candidate gene	SNP rs number[1]	MAF[2]	Assay ID[3]	Position in kbp relative candidate gene and location[4]	Coverage[5]
CLDN1	rs1491991	0.25	C_7550365_10	-66.3 (5′-flanking region)	*CLDN16*
	rs3732923	0.41	C_27509271_10	5.5 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs3732924	0.29	C_8528578_10	5.6 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs9848283	0.49	C_2057729_10	6.4 (intron 1)	*CLDN1* (from promoter until first two thirds of intron 1)
	rs12629166	0.47	C_2057718_10	13.8 (intron 3)	*CLDN1* (from second intron until 3′-flanking region)
	rs7620166	0.41	C_8528273_10	45.9 (3′-flanking region)	*CLDN1* (from second intron until 3′-flanking region)
	rs567408	0.42	C_1587588_10	94.5 (3′-flanking region)	intergenic block
	rs536435	0.42	C_1587674_10	155.3 (3′-flanking region)	*LOC391603*
CLDN2	rs4409525	0.34	C_382795_10	-23.3 (5′-flanking region)	*RIPPLY1*, *TBC1D8B*
	rs5917027	0.48	C_11771710_10	-1.0 (5′-flanking region)	*CLDN2*, *MORC4*
	rs12014762	0.21	C_2013132_20	20.0 (3′-flanking region)	*CLDN2*, *MORC4*
CLDN4	rs4131376	0.43	C_26657639_10	-56.9 (5′-flanking region)	*ABHD11*, *CLDN3*, *CLDN4*, *WBSCR27*, *WBSCR28*
	rs8629	0.18	C_7493975_10	0.3 (exon 1)	*ABHD11*, *CLDN3*, *CLDN4*, *WBSCR27*, *WBSCR28*

[1]Initially all of the selected single nucleotide polymorphism (SNP) markers were evaluated, using the Hapolview software 4.0[52], for linkage disequilibrium (LD) and association to inflammatory bowel disease (IBD) in a case-control study using a subset of Swedish IBD patients (73, 39 and 42 individuals with IBD, Crohn's disease (CD) or ulcerative colitis, respectively). Because of significant associations to CD, SNP markers for claudin (*CLDN)1* (rs7620166) and *CLDN2* (rs12014762) were chosen for further evaluation on the complete case-control. Even though non of the *CLDN4* SNP markers showed evidence of association to any of the disease categories rs8629 was included for further evaluation; [2]Minor allele frequency (MAF) according to SNP data from the HapMap CEPH collection; [3]TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, United States); [4]With respect to each candidate gene, SNP positions are defined relative a genomic reference sequence. The first nucleotide of a reference cDNA sequence has been designated +1, with the preceeding genomic position being -1. The following reference sequences have been used for *CLDN1* NT_005612.15 (genomic) and NM_021101.3 (mRNA), *CLDN2* NT_011651.16 (genomic) and NM_020384.2 (mRNA) and *CLDN4* NT_007758.11 (genomic) and NM_001305.3 (mRNA); [5]Linkage blocks have been defined using SNP data from the HapMap CEPH collection and the SNP browser software with 0.3 LDU (linkage disequilibrium units) as threshold for linkage block computation.

Table 3 Primers for polymerase chain reaction amplification and sequencing of *Claudin-2*

Candidate gene	Exon	Forward primer[1]	Reverse primer[2]	Size of PCR-product
CLDN2	1	GTAGGACCTGCTCTTTGAACTC[2,3]	TGAATTTAAAAGGCAGCAACTA[2,3]	708 bp
	1	TCAATCTTTCCCAGCCTCCA[3]	TTTCTGTCAAAAACTCCACTCC[3]	
	2	TTGTAGAGAATGGGGAGGTGTGC[2,3]	TCAATTGCAGACTGAGGCCAAA[2]	599 bp
	2		TGCAGACTGAGGCCAAACTG[3]	
	2	CTAGGCCCCTGGAGATTCAAGA[2,3]	TGTGCCCAAAAGCCCCAGAA[2,3]	682 bp
	2	TTCCTTTCTCATGTGTTATTTCTAA[3]	GAGAAAGGAAAAAAAAAACAAC[3]	
	2	TGAGGGATTAGAGGTGTTCAA[2,3]	GCAGCACCTTCTGACACGA[2,3]	892 bp
	2	ATCCTACGGGACTTCTACTCA[3]	ACTCCACCTGCTACCGCCACT[3]	

[1]q, Q-solution for polymerase chain reaction (PCR)-amplification; [2]Primer used for PCR; [3]Primer used for sequencing. CLDN: Claudin.

tests, two-sided *P* values < 0.05 were considered significant. Correction for multiple testing was not performed.

RESULTS

Genetic association - case-control approach

Based on a subset of Swedish IBD patients (73, 39 and 42 individuals with IBD, CD or UC, respectively) one marker per gene was selected for analysis in the full study material (Table 2). Allelic association between any of the three markers for the genetic regions of *CLDN1*, *CLDN2* or *CLDN4* and patients with IBD, CD or UC were investigated using a case-control study approach (Table 4). Significant associations were observed between the *CLDN2* marker (rs12014762; associated C allele frequency of 0.776 among controls) and CD (*P* = 0.007), and the *CLDN1* marker (rs7620166; associated T allele frequency of 0.470 among controls) and IBD (*P* = 0.025). No associations were observed for the *CLDN4* marker (rs8629; C allele frequency of 0.772 among controls), neither to IBD, CD nor UC.

Genetic association - family-based approach

The same three SNP markers were further investigated using a family-based approach in non-Swedish families (Table 4). The significant associations identified in the case-control study were not confirmed (*P* = 0.126 and *P* = 0.177 for the *CLDN2* and *CLDN1* marker, respectively). The *CLDN2* marker rs12014762 was not related to any demographic or disease characteristics in CD-patients (Table 5).

Resequencing of CLDN2

Based on the most significant association (CD and the *CLDN2* marker), *CLDN2* was chosen for resequencing in an approach to identify novel sequence variants of putative importance for the risk of developing CD. Two novel non-coding sequence variants were identified for

Table 4 Inflammatory bowel disease-phenotypes were investigated by performing family-based and case-control approach in genetic association studies

		rs7620166 (*CLDN1*)		rs12014762 (*CLDN2*)		rs8629 (*CLDN4*)	
		allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value
IBD	Swedish case-control	1.33 (1.04-1.72)	0.025	1.39 (0.95-2.01)	0.083	1.21 (0.89-1.65)	0.225
	Non-Swedish families	0.87 (0.72-1.06)	0.177	1.25 (0.89-1.77)	0.195	1.09 (0.88-1.33)	0.432
CD	Swedish case-control	1.17 (0.86-1.60)	0.319	1.98 (1.17-3.35)	0.007	1.25 (0.84-1.85)	0.258
	Non-Swedish families	0.80 (0.64-1.00)	0.052	1.37 (0.91-2.07)	0.126	1.14 (0.89-1.46)	0.287
UC	Swedish case-control	1.35 (0.98-1.84)	0.064	1.27 (0.80-2.02)	0.304	1.18 (0.80-1.73)	0.409
	Non-Swedish families	1.19 (0.77-1.84)	0.436	0.91 (0.39-2.14)	0.827	1.15 (0.75-1.77)	0.512

The family-based association studies included a total of 463 families. For the single nucleotide polymorphism -markers rs7620166 [claudin (*CLDN*)*1*], rs12014762 (*CLDN2*) and rs8629 (*CLDN4*) genotyping failed for 5 [including 2 Crohn's disease (CD)], 9 (including 3 CD) and 7 (including 2 CD) samples, respectively. Results were based on 191, 103 and 102 cases of inflammatory bowel disease (IBD), CD and ulcerative colitis (UC), respectively and 333 controls. OR (and its associated 95%CI) and *P* values (based on log likelihood ratio χ^2 statistics) were calculated for the T allele of rs7620166, the C allele of rs12014762, and the C allele of rs8629.

Table 5 Genotype-phenotype correlation between *Claudin-2* marker (rs12014762) polymorphism and clinical characteristics of 677 patients with Crohn's disease (all families)

rs12014762 Crohn's disease	At least one C	T	*P* value
Sex			
Male	265	47	
Female	354	11	
Age at diagnosis (yr)			
Mean (SD)	24.48 (12.11)	24.36 (11.46)	0.90
Median (range)	22 (3-70)	22 (8-63)	
Location at onset:	*n* = 583	*n* = 54	
Pure colonic disease	78	6	0.64
Pure ileal disease	117	14	0.31
Ileocolonic disease	274	309	0.87
Any colonic disease	414	37	0.70
Any ileal disease	424	42	0.42
Upper digestive tract	118	12	0.73
Perineal disease	147	11	0.53
Granuloma	237/478	26/43	0.12
Penetrating disease	218/478	22/43	0.48
Strictures	221/478	15/43	0.15
Extra-digestive symptoms	190/619	15/58	0.44
Smoking habits	*n* = 505	*n* = 51	
Non-smoker	272	21	0.08
Smoker	157	21	0.14
Ex-smoker	76	9	0.62

CLDN2 (Table 6).

Prediction of transcription factor binding sites in the CLDN2 promoter and analysis of genetic association to the newly identified sequence variants

The two novel *CLDN2* polymorphisms were located in a region corresponding to the *CLDN2* promoter, as described by Sakaguchi *et al*[34] Analyzing the promoter region of *CLDN2* for the presence of possible transcription factor binding sites, revealed that these new variants were located in a putative specificity protein 1 (Sp1) binding site/GC-box (rs62605981) and a putative upstream stimulatory factor (USF) binding site/E-box (rs72466477). The genotyping results of these two novel SNPs were based on 166, 89 and 89 cases of IBD, CD and UC, respectively, from the Swedish families and their 333 non-related controls (Table 1). Neither rs62605981 (C allele frequency of 0.860 among controls) nor rs72466477 (AT allele frequency of 0.842 among controls) showed any significant associations to IBD overall or to any sub-entities (Table 7).

MORC4 and genetic association

In addition to *CLDN2*, *MORC4* is located on the same linkage block as the CD associated marker (rs12014762). A nonsynonymous coding SNP (rs6622126) was identified in the *MORC4* gene using the NCBI SNP database, for which a high minor allele frequency (A allele = 0.440) was present in the non-related Swedish control samples. This SNP was investigated for genetic association to IBD overall and to sub-entities, using the same individuals as used for the two novel *CLDN2* variants. A significant association was observed between the G allele (frequency of 0.560 among controls) and CD ($P = 0.018$), but not to UC or IBD (Table 7).

DISCUSSION

TJ proteins are considered key components in the regulation of paracellular permeability of both epithelial and endothelial cell linings[20,21], and due to their barrier-forming properties we considered claudin genes as candidate genes affecting a leaky gut phenotype of IBD.

Using the case-control approach, an association was identified between the *CLDN2-MORC4* region (rs12014762) and the occurrence of CD. This SNP marker was associated with an overall increased risk of having CD, but not to any distinctive phenotypic pattern. This association was not confirmed in the family-based approach, where non-Swedish families were included. Such a regional heterogeneity may in part be explained by different genetic backgrounds. Substantial genetic heterogeneity due to geographic stratification has been demonstrated in genome-wide association studies[6]. A geographically homogenous population (this study) should be advantageous for unveiling regionally restricted genetic risk factors. The case of underrepresented *NOD2* mutations in CD patients from the Nordic countries[35-37] together with our data on the presence of *CLDN2* vari-

Table 6 Sequence variants identified by resequencing of selected gene regions

Gene[1]	Position of SNP	rs-number	Part of gene/predicted consequence	VAF/control[2]	VAF/patients[2]
CLDN2	c.-178-678C>G (g.29466911)	rs62605981	Intron/5′ gene flanking region[3]	0.175	0.139
	c.-178-104_-103delAT (g.29467485_29467486delAT)	rs72466477	Intron/5′ gene flanking region[3]	0.175	0.083

[1]The following reference sequences have been used for claudin (*CLDN*)2 NT_011651.16 (genomic) and NM_020384.2 (mRNA); [2]Variant allele frequencies (VAF) are defined with respect to the corresponding reference sequence; [3]The intronic region of *CLDN2*, according to the *CLDN2* reference mRNA (NM_020384), correspond to the promoter region described by Sakaguchi *et al*[34]. SNP: Single nucleotide polymorphism.

Table 7 Genetic association between inflammatory bowel disease-phenotype and either newly discovered *Claudin-2* promoter polymorphisms or a nonsynonymous coding polymorphisms in *MORC4* was analyzed by performing case-control studies

	rs62605981 (*CLDN2*)		rs72466477 (*CLDN2*)		rs6622126 (*MORC4*)	
	allelic OR (95%CI)	*P* value	allelic OR (95%CI)	*P* value	allelic OR(95%CI)	*P* value
IBD	1.11 (0.71-1.73)	0.659	1.23 (0.79-1.91)	0.350	1.24 (0.91-1.70)	0.179
CD	0.83 (0.49-1.41)	0.501	1.16 (0.68-2.00)	0.584	1.61 (1.08-2.41)	0.018
UC	1.24 (0.69-2.26)	0.463	1.19 (0.68-2.06)	0.543	0.903 (0.61-1.33)	0.606

Results were based on 166, 89 and 89 cases of inflammatory bowel disease (IBD), Crohn's disease (CD) and Ulcerative colitis (UC), respectively, available from the Swedish families and 333 controls (Table 1). OR (and its associated 95%CI) and *P* values (based on log likelihood ratio chi-square statistics) were calculated for the C allele of rs62605981, the AT allele of rs72466477, and the G allele of rs6622126. CLDN: Claudin.

ants in Swedish patients add support for such an assumption taking into account that IBD, and especially CD, are polygenic disorders[2,3]. When we investigated a possible interaction between *NOD2* and the *CLDN2-MORC4* region (rs12014762) with individuals stratified on the basis of carrying none or at least one *NOD2* mutant allele, no significant association was identified between these two genetic regions (data not shown).

Both CD and UC have been associated with an increase in intestinal permeability[9-11]. Experimental data reveal an altered expression of claudin-2, and other claudin proteins, in the intestinal epithelium of IBD patients, and such alterations affect the permeability characteristics[38]. Claudin-2 expression was increased in a cell culture of human colonic epithelial cells (HT-29/B6) in response to tumor necrosis factor-α (TNF-α)[38], a finding consistent with an increased expression of claudin-2 in the inflamed epithelium of CD patients[38,39]. Using another human colonic epithelial cell line (T84), Prasad *et al*[39] demonstrated an increase both in paracellular permeability and claudin-2 expression in response to interleukin-13, but not in response to interferon-γ/TNF-α treatment.

Within the resequenced parts of *CLDN2* we identified two novel polymorphisms in the promoter region. These two sequence variants were located in different putative transcription factor binding sites, a Sp1 binding site/GC-box (rs62605981) and an USF binding site/E-box (rs72466477). It has been shown that the *CLDN2* transcription is affected by several transcription factors[34], and both E-box binding[40-42] and Sp1 binding site[43,44] transcription factors have been identified as important determinants of claudin gene expression. However, neither of the two novel promoter variants were associated to CD.

MORC4 is present on the same linkage block as *CLDN2* and the CD associated marker (rs12014762). It is therefore possible that the C allele of rs12014762 is a marker for a functional variant of *MORC4* that results in an increased overall risk of developing CD. A significant association was observed between CD and the G allele of the nonsynonymous coding SNP in the *MORC4* gene (rs6622126), resulting in a substitution of the more hydrophobic amino acid isoleucine at position 473, located immediately outside the region predicted to be a zinc finger (420-472), with threonine.

MORC4 encodes a member of the MORC family of CW-type zinc finger proteins, which contain a number of predicted domains and motifs suggestive of being transcription factors[45]. *MORC4* exhibits a low-level mRNA expression in a variety of normal tissues, including the intestine. Using the SKI-like protein as bait in a two-hybrid screen, MORC4 has been identified as a putative interacting protein, linking MORC4 to the transforming growth factor-β (TGF-β) signaling pathway and the SMAD family of signal transduction proteins[46]. Increased intestinal expression of TGF-β has been observed in patients with CD[47] and, in monolayers of intestinal epithelial cells, TGF-β has been shown to preserve or enhance the paracellular barrier[48,49]. These two findings seem to contradict the increased intestinal permeability that has been associated to both CD and UC[9-11]. Possibly a genetic variant in the *CLDN2-MORC4* region could disturb a TGF-β mediated signal that preserves or enhances the paracellular barrier, or exert an effect on *CLDN2* expression that dominates a TGF-β mediated effect on paracellular permeability. In addition, a SMAD4-dependent, but TGF-β-independent, repression of *CLDN1* transcription[50] and a ZEB2-mediated repression of *CLDN4*[42] support a role for the SMAD signal transduction pathway in the regulation of claudin genes.

An increased intestinal permeability has been associated with the CD susceptibility allele CARD15 3020insC[18], and TJ associated genes have been suggested as suscepti-

bility genes for UC (*e.g.*, *GNA12*[2]) and for both UC and CD (*MAGI2*[51]).

In conclusion, our findings add further support for a genetically impaired intestinal epithelial barrier as one predisposing factor in the etiology of CD, either directly through *CLDN2* or indirect *via* a tentative link between *MORC4*, TGF-β/SMAD signalling and an effect on paracellular permeability. This putative genetic link between the *CLDN2-MORC4* region, intestinal epithelial integrity and the risk of developing CD needs to be further explored.

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COMMENTS

Background

For chronic inflammatory bowel disease (IBD) - Crohn's disease (CD), ulcerative colitis - a number of studies have demonstrated a substantial genetic predisposition. These inflammatory conditions have been associated with an increased intestinal permeability. Consistent with the phenotype of these diseases, several genetic association studies have implicated mainly components of the immune response, but also factors implicated in intestinal permeability. In order to further investigate intestinal permeability as a predisposing genetic risk factor for IBD, the authors have conducted genetic association studies on claudin genes (*CLDN1*, *CLDN2* and *CLDN4*), key components in the regulation of permeability.

Research frontiers

Although large-scale genome-wide association studies have uncovered a large number of genetic susceptibility loci in relation to IBD these factors still explain only a minority of the total genetic risk for IBD. The genetic background regulating intestinal barrier functions largely remains unknown.

Innovations and breakthroughs

The barrier of epithelial cells is critical for the permeability properties of the intestine. The tight junction structure is a multicomponent protein complex that serves to seal and regulate permeability across the space between adjacent epithelial cells, with significant contribution from members of the claudin family. This is the first study to report genetic link between claudin gene (*CLDN2*) and the risk of developing CD.

Applications

In this study the authors have identified a genetic link between the *CLDN2-MORC4* region and the risk of developing CD, and thereby highlighted claudins as therapeutic targets.

Terminology

The tight junction structure is critical for the permeability properties of the intestine. The structure is a multicomponent protein complex that serves to seal and regulate permeability across the paracellular space between adjacent epithelial cells.

Peer review

This is a small study on the importance of claudins (*CLDN1*, *CLDN2* and *CLDN4*) in Swedish and non-Swedish case-control and family-based approach. A weak suggestive association was reported in the case-control setting, while this was not replicated in the non-Swedish sample. The paper is interesting and well written: its main limitation lies in the study design, comparing Swedish and non-Swedish individuals using two different approaches.

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BRIEF ARTICLE

Treatment of hemorrhagic radiation-induced proctopathy with a 4% formalin application under perianal anesthetic infiltration

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Abstract

AIM: To evaluate the results of hemorrhagic radiation proctopathy treatment with a 4% formalin application.

METHODS: A prospective study was performed. Over a three-year period, 38 patients underwent 4% formalin application under perianal anesthetic infiltration for hemorrhagic radiation proctopathy. All patients included in the study were irradiated for prostate cancer. The patients ranged in age from 56-77 years (average 70 ± 5 years). All of the patients were referred for formalin therapy after noninvasive management had failed. Twenty-four (63.2%) patients underwent a single application, 10 (26.3%) patients underwent 2 applications, and 4 (10.5%) patients underwent 3 applications.

RESULTS: Two to 36 mo (average 12 ± 3 mo) following treatment, 34 patients were interviewed (four were lost to follow-up). Twenty (58.8%) subjects reported complete cure, 8 (23.5%) subjects reported significant improvement, and 6 (17.7%) subjects reported no change. One patient (who underwent a colostomy at a regional hospital with no specialized services available for previous bleeding episodes from radiation proctopathy) was cured, and the colostomy was closed. One patient (2.6%) developed rectal mucosal damage after the second application.

CONCLUSION: A 4-min application of 4% formalin for hemorrhagic radiation-induced proctopathy under perianal anesthetic infiltration in patients who have received external radial radiation therapy for prostate cancer is simple, reasonably safe, inexpensive, generally well tolerated, and effective.

Key words: Formalin application; Radiation proctopathy; Rectal bleeding; Prostate cancer

Core tip: In a prospective study conducted from 2006 to 2009, 38 patients underwent 4% formalin application under perianal anesthetic infiltration for hemorrhagic radiation proctopathy. Based on the rectal-telangiectasia density classification, eight (21.1%) patients had grade Ⅰ proctitis, 23 (60.5%) patients had grade Ⅱ proctitis, and seven (18.4%) patients had grade Ⅲ proctitis. A piece of gauze soaked with 4% formalin was applied to the entire diseased rectal mucosa and remained for 4 min under perianal anesthetic infiltration. Twenty patients (58.8%) reported complete cure, eight patients (23.5%) reported significant improvement, and

six patients (17.7%) reported no change. Application of 4% formalin under perianal anesthetic infiltration in patients who received external radial radiation therapy for prostate cancer was simple, safe, and effective.

Samalavicius NE, Dulskas A, Kilius A, Petrulis K, Norkus D, Burneckis A, Valuckas KP. Treatment of hemorrhagic radiation-induced proctopathy with a 4% formalin application under perianal anesthetic infiltration. *World J Gastroenterol* 2013; 19(30): 4944-4949 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4944.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4944

INTRODUCTION

Radiotherapy is a common treatment modality for carcinoma of the female genital tract, prostate, and urinary bladder[1]. Because of its fixed position in the pelvis and because of its proximity to the treated organs, the anorectal area is the most common site of bowel injury following pelvic radiotherapy. Chronic hemorrhagic radiation proctopathy occurs in 1%-5% of patients following radiotherapy for pelvic malignancy[2].

The gross pathologic changes can be acute, subacute, or chronic. Acute changes occur during and immediately after radiotherapy in the form of hyperemia, edema, and extensive inflammatory cell infiltration of the mucosa. To a variable extent, subacute and chronic changes begin after 2 to 12 mo of regeneration. In the vessels, there may be endothelial swelling leading to fibrosis of the connective tissues (intima) and endarteritis. Damage of the vessels increases the formation of arteriovenous shunts, *i.e.*, telangiectatic neovasculature that is fragile and prone to bleeding. Ulcers, strictures, and fistulae may also develop[3]. More often, patients will experience functional symptoms of proctopathy, such as urgency, tenesmus, mucoid rectal discharge, abdominal pain, and sphincter irritability[4].

No standard treatment exists for this condition. The primary treatment of radiation proctopathy is medical (or non-invasive). If non-invasive treatment is ineffective, then invasive treatment is considered. One such treatment is formalin application. Formalin is a solution of formaldehyde mixed with methanol that is commonly used to fix tissue samples for histological examination. Applied topically, formalin acts as a chemical cautery of telangiectatic mucosal vessels, and its main action is the sclerosing and sealing of fragile neovasculature[4]. In 1969, Brown *et al*[5] were the first to use formalin to treat radiation-induced hemorrhagic cystitis. Inspired by this experience, in 1986, Rubinstein *et al*[6] were the first to apply formalin to treat hemorrhagic radiation proctopathy. However, this treatment modality did not become popular until 1993, when Seow-Choen *et al*[4] reported their data, which indicated a high success rate. Rectal instillation of various concentrations of formalin solution has been used by several groups to control severe or refractory bleeding from radiation proctopathy, with encouraging results[1-16]. The local application of 4% formalin is safe and highly effective in both radiation cystitis and radiation proctopathy. We evaluated the use of a 4% formalin gauze (surgical swab) in patients with radiation proctopathy as an outpatient procedure under perianal anesthetic infiltration[17].

MATERIALS AND METHODS

This study was approved by the Lithuania Bioethical Committee in 2006.

We conducted a prospective study from July 2006 to July 2009 (3 years). Thirty-eight patients were included. The following inclusion criteria were applied: male patients older than 18 years who had undergone external beam radiotherapy for prostate cancer and developed rectal bleeding as the main symptom of proctopathy. The rectal bleeding occurred not more than two years post-radiotherapy. Proctopathy (classified using the rectal telangiectasia density score) was diagnosed with colonoscopy. Patients were excluded from the study if they met any of the following criteria: the bleeding occurred more than two years after radiotherapy; other symptoms dominated, such as tenesmus, pain, ulceration, or impaired defecation; the patient had impaired coagulation; or the patient was using anticoagulants. All patients provided written informed consent prior to the formalin application.

All patients were referred for formalin therapy after the failure of noninvasive management (peroral sucralfate and topical cortisone were used). The subjects received radical treatment for prostate cancer, including 3D conformal external radiotherapy to the prostate and the base of the seminal vesicles, up to a total dose of 74 Gy (70-74 Gy) over a 7.5-wk period. The patients ranged in age from 56-77 years, with an average of 70 ± 5 years.

Bleeding occurred for all patients during the first two years following treatment. The mean timepoint for the onset of symptoms was 9 ± 4 mo (range one week to 24 mo). In one case, hemorrhage occurred one week after treatment; in two cases, during treatment; and in the remainder of cases, three to 24 mo after treatment. Nineteen patients reported daily blood in their stools, and 19 patients reported bleeding two or three times per week. Two patients received blood transfusions for severe anemia, and one patient was treated with a colostomy for severe rectal bleeding at a regional hospital before coming to our institution.

A total colonoscopy was performed in all of the patients to exclude other synchronous causes of hemorrhage and to determine the extent of the radiation-induced damage. We used the rectal telangiectasia density score[18], in which the radiation proctopathy was graded into the following four grades: normal mucosa (Grade 0), fewer than 10 discrete telangiectasias within a luminal view (Grade I), a single coalescing patch of telangiectasias and/or greater than or equal to 10 discrete telangiec-

tasias (Grade Ⅱ), and the presence of two or more coalescing telangiectatic patches (Grade Ⅲ). Based on this classification, eight (21.1%) patients had grade I proctitis, 23 (60.5%) patients had grade Ⅱ proctitis, and seven (18.4%) patients had grade Ⅲ proctitis.

The formalin application was performed on an outpatient basis in an operating theater. All of the procedures were conducted with the patient in the prone, jack-knife position under perianal anesthetic infiltration, which was performed by injecting a mixture of lidocaine and bupivacaine solution. Vaseline (petroleum jelly) was applied to the perineum and upper anal canal up to the level of the dentate line, both to serve as a lubricant and to protect the skin from unnecessary exposure to formalin. A piece of gauze (surgical swab) soaked with 4% formalin was applied to the entire diseased rectal mucosa and left in place for four minutes. A Fansler proctoscope was used to visualize the radiation-induced rectal lesions and to avoid formalin application to the healthy rectum. At the end of the procedure, the anal canal and the rectum were abundantly rinsed with water.

A complete response was recorded if there were no further episodes of bleeding. Significant improvement was recorded if there was less than one bleeding episode per month. No response was recorded if the bleeding continued as prior to treatment. The patients were treated repeatedly if they exhibited no improvement after four weeks. All of the patients were interviewed using a questionnaire administered by mail or by telephone at 1, 2, 3, 6, 9, 12, 18, 24 and 36 mo after the treatment. Colonoscopy was not repeated after the treatment.

All of the statistical analyses were performed using SPSS version 17 was used (*SPSS* Inc., Chicago, IL, United States) for Windows.

RESULTS

Two to 36 mo after treatment (average 12 ± 3 mo), 34 patients were interviewed (four were lost to follow-up). Twenty-four (63.2%) patients were treated with only one formalin application, and 10 (26.3%) patients required a second application because of persistent bleeding. Four patients (10.5%) required three applications. The treatment was effective in 28 cases (82.3%); of these cases, 20 (58.8%) patients reported complete cessation of the bleeding, and eight (23.5%) patients reported significant improvement. Six patients (17.7%) reported no change in the bleeding. One patient, who underwent a colostomy for previous episodes of bleeding due to radiation proctopathy at another hospital, was cured, and the colostomy was closed. One patient (2.6%) developed rectal mucosal damage after the second application and underwent prolonged conservative management (*i.e.*, topical sucralfate, sucralfate enemas, cortisone enemas, analgesics, and mesalazine suppositories); in this case, the bleeding was controlled completely. No other complications occurred.

DISCUSSION

Currently, no "best" treatment exists for hemorrhagic radiation proctopathy. Non-invasive therapy includes a low-residue diet, laxatives and retention enemas with steroids, rebamipide[19] or hyperbaric oxygen therapy[20], oral antibiotics with colonic irrigation[21,22], short-chain fatty acids, pentoxifylline[23], hormonal therapy[24], antioxidants[25], and retinol palmitate[26]. However, these treatment modalities have not been proven effective in all cases of chronic hemorrhagic radiation proctopathy.

Studies of hyperbaric oxygen therapy suggest a clear benefit of this modality in the control of bleeding. Unfortunately, this procedure is expensive and requires additional prospective randomized studies to determine its efficacy in cases of rectal bleeding[16,20]. In a randomized placebo-controlled trial, retinol palmitate was proven effective in significantly reducing rectal functional symptoms[26]. One comparative study recently demonstrated that oral antibiotics combined with colonic irrigation was superior to 4% formalin application in reducing rectal functional symptoms but yielded the same results in controlling bleeding[22].

If non-invasive treatment is ineffective, then invasive procedures may be used. Successful results using endoscopic therapy have been reportedly achieved in controlling bleeding and providing symptomatic relief by reducing the frequency of hematochezia and the necessity for transfusion[16,27-31]. Initially, endoscopists used cryoablation[27] and heater and bipolar probes[30], followed by neodymium/yttrium aluminum garnet and potassium titanyl phosphate lasers[29,32], which were beneficial. Argon plasma coagulation (APC) is an innovative, no-touch electrocoagulation technique that is used to treat hemorrhagic digestive malformations. Studies have demonstrated the superior efficacy and safety of APC in treating hemorrhagic radiation proctopathy[28,33].

The treatment of hemorrhagic radiation proctopathy with formalin was first reported by Rubinstein *et al*[6] in 1986. The concentration of the formalin solution used, the treatment method (application *vs* instillation[12]), and the mucosal contact time vary largely, as reported by different authors. Diverse techniques have been used by different investigators with varying success rates; examples include irrigation of the rectum with a large volume of formalin for 15 min[33], insertion of a formalin-soaked gauze for 2 to 3 min[4] or up to cessation of the symptoms[7,14], and repeated instillation of 50 mL of formalin for 30 s[8]. Cullen *et al*[2] used 20 mL of a 5% formalin instillation for two or three minutes, with success rates of up to 85%. We used a piece of gauze (surgical swab) soaked with 4% formalin solution, which was applied to the entire diseased rectal mucosa for 4 min.

Several studies have indicated that systemic toxicity arises after more prolonged contact with formalin[31]. Systemic toxicity also increases when formalin instillation is used. The optimal concentration of formalin for the

procedure is unknown. Varying concentrations of formalin solution, ranging from 2% to 10%, have been used[1-4]. However, a 4% formalin solution has been used most widely. A lower concentration may be safer but is associated with a lower response rate[1]. In a study in which 2% formalin was used, the overall response rate was 78.2%, while the complete success rate was only 47.5%[1]. The use of 10% formalin has resulted in an overall success rate of 93%[3], which is comparable to 4% formalin, for which the success rates ranges from 70% to 100%. A higher concentration of formalin may result in a higher incidence of complications[4].

Formalin usually causes cessation of bleeding within a short period by acting as a local chemical cautery. It stops the bleeding by sealing the sites of leakage from the neovascularized telangiectatic spots and ulcers. Multiple sessions of formalin application were required in some of the nonresponsive or relapsed patients. In the study by Seow-Choen *et al*[4], 17 of 29 patients experienced the complete cessation of bleeding one month after a single application; 11 patients experienced only minor bleeding, and one patient continued to experience major rectal bleeding.

Repeated formalin applications resulted in further success in this study. In the investigation by Parikh *et al*[14], the number of formalin treatments ranged from 1 to 13, with a mean of 3.4. The response rates in other studies have been similar, ranging from 81% to 100%[1-4]. We used 4% formalin with a success rate of 82.3%. This response rate is comparable to that of previous studies. Twenty-four of 38 patients in our study were treated with only one formalin application, and 10 patients required a second application because of persistent bleeding. Four patients needed three applications. Decreased cost is a major advantage of formalin over APC and the other treatment modalities. However, APC poses the advantage of reaching lesions beyond the rectum[33].

One patient, who underwent a colostomy at another institution for previous episodes of bleeding from radiation proctopathy, was cured, and the colostomy was closed. One patient (2.6%) developed rectal mucosal damage after the second application. No other complications were observed. Several published reports have also shown no serious complications of local formalin therapy[7,8,13,14]. However, a higher incidence of local complications (*e.g.*, anorectal strictures, incontinence, anal ulcers and/or stenosis) has been reported[10]. These events may not be entirely caused by formalin, as a higher proportion (36%) of patients in the latter case series had anorectal malignancies. Other complications that have been reported in certain studies include the proximal migration of formalin, which is caused when a rigid sigmoidoscope is used for instillation. Overdistension of the distal rectum with subsequent proximal migration of the formalin should be avoided. In the present study, we used a Fansler proctoscope to visualize the damaged rectal mucosa.

One recently published randomized trial has compared formalin dab treatment with a sucralfate-steroid retention enema; in that investigation, Nelamangala Ramakrishnaiah *et al*[15] concluded that a 4% formalin dab was superior to a sucralfate-steroid retention enema for treating hemorrhagic proctopathy caused by radiotherapy. Surgery should be reserved for patients who have intractable symptoms, such as strictures and/or fistulas. However, surgery may be technically demanding because of adhesions and other radiation damage in the pelvis. Another surgical concern is that anastomoses involving radiated tissue may break down. Abdominoperineal resection may be the only reliable option in some patients.

The present study had the following limitations: colonoscopy was not repeated after the treatment, we could not relate our results to possible endoscopic changes in the rectum, and the follow-up time was markedly different for our patients in our study.

In conclusion, radiation-induced hemorrhagic proctopathy is a frequent complication following pelvic radiation. In our experience, formalin application therapy was an inexpensive, simple and highly effective therapy for radiation-induced hemorrhagic proctopathy and yielded few complications. We reported a clinical response rate of 82.3%. Therefore, we recommend 4% formalin application as a low-cost treatment for chronic hemorrhagic radiation proctopathy.

COMMENTS

Background

Radiation-induced hemorrhagic proctopathy is a common late complication that manifests after irradiation treatment for pelvic malignancies. The condition may present with signs and symptoms ranging from clinically insignificant bleeding during defecation to a debilitating disorder requiring blood transfusions, repeated admissions to the hospital and even surgery, thereby reducing the patient's quality of life. Preventing radiation-induced hemorrhagic proctopathy *via* different agents during the radiotherapy course has not been successful, and newer external radiation techniques or brachytherapy have not precluded this complication in all cases. Other types of radiotherapy-related damage to the rectum, such as ulcers, strictures or fistulas, are reported much more rarely. This article focuses on formalin applications for hemorrhagic radiation-induced proctopathy in patients undergoing radical external radiotherapy for prostate cancer.

Research frontiers

Conservative treatment (peroral drugs, suppositories or enemas) may be useful in certain patients suffering from radiation-induced hemorrhagic proctopathy. However, non-responders require other options to treat this condition. Formalin application, argon plasma coagulation, and hyperbaric oxygen therapy appear to be the most effective modalities after failed conservative treatment. Argon plasma coagulation requires specific equipment, hyperbaric oxygen (which is not accessible everywhere), multiple sessions, and a long treatment period, whereas formalin application is a simple and low-cost method that is available in most hospitals.

Innovations and breakthroughs

Despite the many published articles on formalin therapy for radiation-induced hemorrhagic proctopathy, several questions remain unanswered. What percentage of formalin solution should be used? Which method (application or instillation) is safer and more effective? How long should the mucosal contact period last? How should the application be performed? What type of anesthesia should be used? In this article, the authors attempt to standardize formalin application to address the above-mentioned considerations.

Applications

The perspectives regarding the future use of the method described in this article are as follows. After failed conservative treatment, other treatment modalities are not universally available. Because of the simplicity of this method, formalin

application for radiation-induced hemorrhagic proctopathy under perianal anesthetic infiltration may be performed in most hospitals by general surgeons who do not necessarily have extensive colorectal experience. Therefore, the standard technique described by their group may be useful for achieving the best-possible results with an existing approach.

Terminology

The authors used the term "radiation proctopathy" rather than "radiation proctitis," both of which describe the same condition. "Perianal anesthetic infiltration" was a better definition of the anesthesia method employed by their group, although it exhibits certain similarities with the "pudendal block" technique described a few decades ago.

Peer review

There is bot much new things, but it is ok to report these clinic data.

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BRIEF ARTICLE

Validation of the chronic liver disease questionnaire in Serbian patients

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Abstract

AIM: To translate into Serbian and to investigate the validity of the cross-culturally adapted the chronic liver disease questionnaire (CLDQ).

METHODS: The questionnaire was validated in 103 consecutive CLD patients treated between October 2009 and October 2010 at the Clinic for Gastroenterology, Clinical Centre of Serbia, Belgrade (Serbia). Exclusion criteria were: age < 18 years, psychiatric disorders, acute complications of CLD (acute liver failure, variceal bleeding, and spontaneous bacterial peritonitis), hepatic encephalopathy (grade > 2) and liver transplantation. Evaluation of the CLDQ was done based on the following parameters: (1) acceptance is shown by the proportion of missing items; (2) internal reliabilities were assessed for multiple item scales by using Cronbach alpha coefficient; and (3) in order to assess whether the allocation of items in the domain corresponds to their distribution in the original questionnaire (construction validity), an exploratory factor analysis was conducted. Discriminatory validity was determined by comparing the corresponding CLDQ score/sub-score in patients with different severity of the diseases.

RESULTS: The Serbian version of CLDQ questionnaire completed 98% patients. Proportion of missing items was 0.06%. The total time needed to fill the questionnaire was ranged from 8 to 15 min. Assistance in completing the questionnaire required 4.8% patients, while 2.9% needed help in reading, and 1.9% involved writing assistance. The mean age of the selected patients was 53.8 ± 12.9 years and 54.4% were men. Average CLDQ score was 4.62 ± 1.11. Cronbach's alpha for the whole scale was 0.93. Reliability for all domains was above 0.70, except for the domain "Activity" (0.49). The exploratory factor analysis model revealed 6 factors with eigenvalue of greater than 1, explaining 69.7% of cumulative variance. The majority of the items (66%) in the Serbian version of the CLDQ presented the highest loading weight in the domain assigned by the CLDQ developers: "Fatigue" (5/5), "Emotional function" (6/8), "Worry" (5/5), "Abdominal symptoms" (0/3), "Activity"

(0/3), "Systemic symptoms" (3/5). The scales "Fatigue" and "Worry" fully corresponded to the original. The factor analysis also revealed that the factors "Activity" and "Abdominal symptoms" could not be replicated, and two new domains "Sleep" and "Nutrition" were established. Analysis of the CLDQ score/sub-score distribution according to disease severity demonstrated that patients without cirrhosis had lower total CLDQ score (4.86 ± 1.05) than those with cirrhosis Child's C (4.31 ± 0.97). Statistically significant difference was detected for the domains "Abdominal symptoms" [F (3) = 5.818, P = 0.001] and "Fatigue" [F (3) = 3.39, P = 0.021]. *Post hoc* analysis revealed that patients with liver cirrhosis Child's C had significantly lower sub-score "Abdominal symptoms" than patients without cirrhosis or liver cirrhosis Child's A or B. For domain "Fatigue", patients with cirrhosis Child's C had significantly lower score, than non-cirrhotic patients.

CONCLUSION: The Serbian version of CLDQ is well accepted and represents a valid and reliable instrument in Serbian sample of CLD patients.

Key words: Chronic liver disease; Quality of life; Questionnaire; Validation; Factor analysis

Core tip: The Serbian validation of the chronic liver disease questionnaire (CLDQ) confirmed the 6-domain structure of the original United States version. However, in our investigation the original structure was only partially reproduced. The most prominent changes are related to the fact that the factors "Activity" and "Abdominal symptoms" could not be replicated, and two new domains "Sleep" and "Nutrition" were established Moreover, the domain "Nutrition" has been introduced for the first time. Our results of factors analysis gave the evidence that at list some items from the original version of CLDQ should be allocated or eliminated from the questionnaire because of the multiple loadings.

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INTRODUCTION

The concept of health-related quality of life (HRQoL) incorporates many aspects of an individual's experience, the general well-being, satisfaction, social and physical functioning[1]. Chronic liver disease (CLD) includes a wide range of disorders that are characterized by chronic inflammation and often progress to the cirrhosis. This group of diseases has a significant impact on HRQoL, and therefore its assessment is widely used as important outcome in clinical trials[2,3]. The most widely used general questionnaire is the short form health survey-36[4]. Furthermore, the liver disease-specific instruments comprise items that are specific for patients with CLD, and therefore they are more sensitive for capturing all relevant disease-burdened quality of life domains than a generic measure. These disease-specific questionnaires such as the CLD questionnaire (CLDQ)[5], liver disease quality of life instruments[6] and hepatitis quality of life questionnaire[7] are more sensitive and responsive to changes in HRQoL.

The CLDQ is a specific quality of life instrument designed for patients with liver disease, regardless of the underlining severity and etiology of CLD[5]. Its original version was developed by Younossi *et al*[5] and has demonstrated appropriate validity and reliability. The CLDQ has already been cross-culturally adapted and validated into different languages in previously published studies[8-18].

Up to now, there is no CLD-specific quality of life instruments adapted for Serbian patients. Therefore, the aim of this study was to investigate the validation of the translated and culturally adapted CLDQ questionnaire on a group of Serbian CLD patients.

MATERIALS AND METHODS

A cross-sectional study has been performed at the Clinic for Gastroenterology, Clinical Centre of Serbia, Belgrade. Between October 2009 and October 2010, consecutive inpatients and outpatients with CLD were considered for inclusion. Inclusion criteria were chronic hepatitis or liver cirrhosis. Diagnosis of liver disease was made by medical doctor-specialist in hepatology. Chronic hepatitis was defined as elevation of aminotransferases for 1.5 times greater than the upper limit of the reference interval, for more than 6 mo duration, and/or presence of histopathologic criteria for chronic hepatitis. The diagnosis of cirrhosis was based on clinical, laboratory, echo sonographic, endoscopic and histopathological criteria[9,19]. Ascites was diagnosed by ultrasound. Hepatic encephalopathy was assessed clinically, and patients were graded on a scale from 1 to 4. The presence of hypersomnia indicated grade 1, somnolentia grade 2, severe somnolence or stupor grade 3 and severe stupor or coma grade 4[8]. Exclusion criteria were: age < 18 years, psychiatric disorders (psychosis or dementia), acute complications of CLD (acute liver failure, variceal bleeding, and spontaneous bacterial peritonitis), hepatic encephalopathy (grade > 2) and liver transplantation. We also excluded the patients undergoing antiretroviral therapy because of a very small number of these subjects.

Severity of liver cirrhosis was determined by the Child-Pugh classification[20,21]. According to the severity of the diseases, patients were categorized into the following groups: non-cirrhotic, cirrhotic Child's A, cirrhotic Child's B and cirrhotic Child's C. According to the etiol-

ogy of the diseases, patients were categorized into the following categories: alcoholic, viral (viral hepatitis B and viral hepatitis C), autoimmune (autoimmune hepatitis, primary biliary cirrhosis and primary sclerosing hepatitis) and other (non-alcoholic steatohepatitis, Wilson's disease, hereditary hemochromatosis and cryptogenic).

This study was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade. All subjects gave written consent to participate in the study. Permission to use and validate CLDQ questionnaire was obtained by author of the original version (Younossi ZM).

The demographic data (age, gender, education, occupation, employment, marital status), clinical information (duration of the liver disease, haematemesis, ascites, hepatic encephalopathy), as well as the results of hematological, biochemical, virological and immunological analyses, were obtained from medical records.

The CLDQ was developed in 1999, by Younossi *et al*[5]. The questionnaire consists of 29 questions, which are divided into 6 domains as follows: "Fatigue", "Activity", "Emotional function", "Abdominal symptoms", "Systemic symptoms" and "Worry". Scores for each question were ranked from 1 (the worst quality of life - "All of the time") to 7 (the best quality of life - "None of the time"), for to the period of 2 wk ago. These scores were created using the Likert method. Domain scores are the means of the items contained. A summary score is calculated by the mean value of all subscale scores. The scores range from 1 to 7, whit higher values indicating better quality of life[5]. The CLDQ questionnaire was self-administered for all types of patients and filled in by the patients. In case of help in understanding and/or writing, the physician provided assistance when necessary.

The CLDQ adaptation was based on internationally accepted methodology for cultural adaptation of HRQoL questionnaires[22,23]. We used a standard methodology for the production of the Serbian version and it's included: (1) "Foward translation" - translation of the original version from English to Serbian language, so that the Serbian's version, semantically and conceptually corresponds to the original questionnaire. Translation was conducted by two independent, professional translators. Following review and editing by translators and experts, one single translation was formed; (2) "Backward translation" implied translation of the Serbian's version of CLDQ into English. Conducted by two translators, one an expert in quality of life and another one a clinician, with discussion on controversial items, it resulted in the final version of CLDQ culturally corresponding with Serbian's patients with CLD chronic disease liver; (3) Serbian version CLDQ questionnaire was tested on five patients with CLD who have had the opportunity to present their comments and suggestions. Test results are discussed by the group of experts, who created the final Serbian's version of the CLDQ (CLDQ-S); and (4) the final version was tested in 15 patients with CLD. During adaptation and pretesting of the CLDQ, there were no disputed items and any change from the original questionnaire items. Patients had no difficulty in understanding and completing the questionnaire.

Statistical analysis

In the data analysis, descriptive and analytical statistics were used. Continuous variables were described as mean ± SD, while the categorical variables were presented as proportions (percentages). For comparison of continuous variables between groups one-way Analysis of variance was used, including Bonferroni post hoc test for multiple comparisons.

Evaluation of the CLDQ was done through the following parameters: (1) acceptance is shown by the proportion of missing items; (2) internal reliabilities of Serbian version CLDQ were assessed for multiple item scales by using Cronbach alpha coefficient, ranges from 0-1, latter meaning perfect reliability; (3) in order to assess whether the allocation of items in the domain corresponds to their distribution in the original questionnaire (construction validity), an exploratory factor analysis (principal component analysis with varimax rotation) was conducted. A factor was considered as important if its eigenvalue exceeded 1.0; and (4) discriminatory validity was determined by comparing the corresponding CLDQ score/sub-score in patients with different severity of the diseases.

RESULTS

Out of 107 patients who met the inclusion criteria, 96.2% (n = 103) patients agreed to participate in the study. The reason for not accepting participation was a lack of interest or time. The mean age of the selected patients was 53.8 ± 12.9 years (range 21-79 years) and 54.4% were men (Table 1). According to the etiology of CLD the largest proportion was alcoholic liver disease (35%), and then autoimmune liver disease (28.2%). CLD in the stage of cirrhosis had 77.6% (n = 80) patients (Table 1).

The Serbian version of CLDQ questionnaire was completed by 98% (n = 101) patients. Proportion of missing items was 0.06% (2/2987). Two patients filled the questionnaire, but did not answered to all questions, for the "Systemic symptoms" domain (one for Question No.6 and one for No.27). The total time needed to fill the questionnaire ranged from 8 to 15 min. Assistance in completing the questionnaire was required by 4.8% (n = 5) patients, while 2.9% (n = 3) needed help in reading, and 1.9% (n = 2) involved writing assistance.

Analysis of distribution characteristics and reliability of the Serbian version of CLDQ showed that the average CLDQ score was 4.62 ± 1.11 and varied from 1.90 to 6.78. Cronbach's alpha for the whole scale (items 1-29) was 0.93. Reliability for all domains was above 0.70, except for the domain "Activity" (0.49) (Table 2).

In our validation study the exploratory factor analysis model revealed 6 factors with eigenvalue of greater than 1, explaining 69.7% of cumulative variance (Table 3). The majority of the items (66%) in the Serbian version of the

Table 1 Demographic and clinical characteristics of patients with chronic liver disease *n* (%)

Characteristics	Statistics
Age[1] (yr)	53.8 ± 12.9
Gender	
Male	56 (54.4)
Female	47 (45.6)
Education	
Unqualified[2]	6 (5.8)
Primary school	18 (17.5)
Secondary school	43 (41.7)
High school	17 (16.5)
University	18 (17.5)
Missing data	1 (1.0)
Current employment status	
Employed	29 (28.2)
Unemployed	25 (24.3)
Retired	49 (47.5)
Profession	
Housewife	13 (12.6)
Peasant	3 (2.9)
Worker	40 (38.8)
Official	21 (20.4)
Expert	21 (20.4)
Missing data	5 (4.9)
Marital status	
Single	15 (14.6)
Married/cohabiting	68 (66.0)
Separated/divorced	13 (12.6)
Widowed	7 (6.8)
Alcohol consumption	56 (54.4)
Smoker	32 (31.1)
Disease severity	
Non cirrhotic	23 (22.3)
Cirrhotic Child's A	25 (24.3)
Cirrhotic Child's B	30 (29.1)
Cirrhotic Child's C	25 (24.3)
Etiology	
Alcoholic	36 (35.0)
Viral	16 (15.5)[3]
Autoimmune/cholestatic	29 (28.2)
Other	22 (21.3)

[1]mean ± SD; [2]Without primary school; [3]Four patients with hepatitis B surface antigen positive chronic liver disease (CLD) and 12 patients with anti-hepatitis C virus positive CLD.

Table 2 Distribution and reliability of the chronic liver disease guestionnaire

Scale	*n*	mean ± SD	Min value	Max value	Missing items[1]	Cronbach alpha
Abdominal symptoms	103	4.75 ± 1.63	1.33	7	0%	0.82
Fatigue	103	4.20 ± 1.60	1.60	7	0%	0.90
Systemic symptoms	101	5.27 ± 1.60	1.60	7	1.94%	0.74
Activity	103	4.47 ± 1.33	1.33	7	0%	0.49
Emotional function	103	4.61 ± 1.62	1.62	7	0%	0.89
Worry	103	4.24 ± 1.61	1.00	7	0%	0.85
CLDQ total	101	4.62 ± 1.11	1.90	6.78	1.94%	0.93

[1]Proportion of patients with missing any item on the subscale. CLDQ: Chronic liver disease questionnaire.

CLDQ presented the highest loading weight in the domain assigned by the CLDQ developers: "Fatigue" (5/5), "Emotional function" (6/8), "Worry" (5/5), "Abdominal symptoms" (0/3), "Activity" (0/3), "Systemic symptoms" (3/5). The scales "Fatigue" and "Worry" corresponded fully to the original. An important difference compared to the original version was inclusion of two new factors, "Sleep" and "Nutrition". A new factor named "Sleep" was derived from the two items, No. 16 ("difficulty sleeping") and No. 20 ("incapable to fall asleep"), of the original subscale "Emotional function". An additional new factor "Nutrition" consisted of two items, No. 7 ("not able as much as would like") and 14 ("restriction of diet"), belonging to the "Activity" domains in original version of CLDQ. Furthermore, the factor "Activity", which consists of three items (No. 7, 9 and 14), could not be reproduced at all. Items No. 7 and 14 constructed the new factor "Nutrition", and item No. 9 had highest loading on "Fatigue". Also, the factor "Abdominal symptoms", which consists of three items (No. 1, 5 and 17) was not be replicated in the form like in the original version. Namely, in the Serbian version of CLDQ all of these three items had the highest loading in the same group and jointly with questions 3, 21 and 23 constituted a factor called "Systemic symptoms". In the original version of the questionnaire items No. 3, 21 and 23 are also part of the domain "Systemic symptoms", with the difference that in Serbian CLDQ questionnaire the two issues (No. 6 and 27) from the original version showing higher loadings on more than one other factors rather than the factor "Systemic symptoms". Explicitly, the item No. 6 ("shortness of breath in daily activities") showed higher loadings on "Fatigue", and "Nutrition", while the question No. 27 ("itching") revealed a higher degree of belonging to the domains of "Nutrition", "Worry" and "Sleep" (Table 3).

The analysis of etiology-specific scores of CLDQ have shown that the lowest total quality of life score (4.45 ± 1.11) was registered in the group of autoimmune/cholestatic origin of CLD, while the highest total score (4.84 ± 0.91) was observed in the CLD subcohort with the causes different from alcoholic, viral and autoimmune/cholestatic. However, there were no statistically significant differences between etiology-specific total quality of life scores, as well, as, among etiology-specific domain scores of CLDQ (data was not shown).

Analysis of the CLDQ scores distribution according to disease severity demonstrated that patients without cirrhosis had lower the total CLDQ score than those with cirrhosis Child's C, but without statistic significance [F (3) = 0.97, P = 0.402]. Statistically significant difference was detected for the domains "Abdominal symptoms" [F (3) = 5.818, P = 0.001] and "Fatigue" [F (3) = 3.39, P = 0.021]. *Post hoc* analysis revealed that patients with liver cirrhosis Child's C had significantly lower sub-score "Abdominal symptoms" than patients without cirrhosis or liver cirrhosis Child's A or B. For domain "Fatigue", patients with cirrhosis Child's C had significantly lower

Table 3 Exploratory factor analysis of the serbian version of the chronic liver disease questionnaire

Original CLDQ items	Factor 1 Systemic symptoms	Factor 2 Emotional function	Factor 3 Fatigue	Factor 4 Worry	Factor 5 Sleep	Factor 6 Nutrition
Fatigue						
(2) tired or fatigued	0.542	0.173	0.636[1,2]	0.271	0.018	0.140
(4) sleepy during the day	0.105	0.200	0.868[1,2]	0.004	0.156	-0.012
(8) reduced strength	0.489	0.119	0.595[1,2]	0.310	-0.019	0.220
(11) decreased level of energy	0.374	0.159	0.600[1,2]	0.385	0.055	0.282
(13) drowsy	0.168	0.287	0.824[1,2]	0.114	0.172	-0.085
Emotional function						
(10) anxious	0.313	0.521[1,2]	0.354	0.358	0.022	0.267
(12) unhappy	0.034	0.677[1,2]	0.283	0.284	0.096	0.264
(15) irritable	0.118	0.823[1,2]	0.157	0.082	0.036	0.091
(16) difficulty sleeping	0.256	0.184	0.175	0.229	0.745[1,3]	0.096
(19) mood fluctuations	0.127	0.794[1,2]	0.178	0.248	0.139	-0.042
(20) incapable to fall asleeP	0.252	0.183	0.123	0.155	0.822[1,3]	0.046
(24) felt depressed	0.162	0.815[1,2]	-0.003	0.288	0.203	0.092
(26) problem concentrating	0.161	0.697[1,2]	0.078	0.240	0.089	0.224
Worry						
(18) impact on family	0.237	0.269	0.037	0.760[1,2]	0.081	0.036
(22) symptoms developing into major problems	0.153	0.361	0.170	0.696[1,2]	0.229	0.015
(25) condition getting worse	-0.028	0.269	0.188	0.791[1,2]	0.240	0.152
(28) never feeling any better	0.005	0.293	0.162	0.659[1,2]	0.151	0.232
(29) availability of a liver	-0.142	-0.018	0.103	0.599[1,2]	0.460	0.127
Abdominal symptoms						
(1) abdominal bloating	0.763[1,3]	0.098	0.242	0.063	-0.041	0.074
(5) abdominal pain	0.785[1,3]	0.050	0.136	-0.054	0.207	0.110
(17) abdominal discomfort	0.811[1,3]	0.228	0.138	0.205	0.072	-0.049
Activity						
(7) not able to eat as much as would like	0.186	0.406	0.175	0.066	-0.079	0.543[1,3]
(9) trouble lifting or carrying heavy objects	0.389	-0.105	0.481[1,3]	0.162	0.116	0.236
(14) restriction of diet	-0.073	0.182	0.100	0.212	0.199	0.703[1,3]
Systemic symptoms						
(3) bodily pain	0.798[1,2]	0.103	0.116	0.097	0.121	-0.053
(6) shortness of breath in daily activities	0.367	0.296	0.433[1,3]	-0.047	0.083	0.389
(21) muscle cramps	0.470[1,2]	0.223	0.102	0.046	0.422	0.350
(23) dry mouth	0.556[1,2]	0.305	0.261	-0.065	0.316	0.172
(27) itching	0.252	0.011	-0.193	0.370	0.358	0.481[1,3]

[1]Highest factor loadings for each factor; [2]Factor loadings corresponding to the factors in the original version; [3]Factor loadings indicate highest loadings on other factors than the original ones. The factors "activity" and "abdominal symptoms" could not be reproduced; a new factors "sleep" and "nutrition" were found. CLDQ: Chronic liver disease questionnaire.

score, than non-cirrhotic patients. Significant difference was not detected for the following domains: "Systemic symptoms", "Activity", "Emotional function" and "Worry" (Table 4).

DISCUSSION

The CLDQ is a disease-specific instrument for assessment HRQoL in patients with CLD. It is reliable, reproducible, valid, short, easy to administer and economic questionnaire, which is validated and cross-culturally adapted into many different languages[8-18].

According to internationally accepted methodology for the validation of HRQoL questionnaires, we developed a Serbian version of CLDQ. Patients had no difficulty in understanding and completing the questionnaire. Only 4.8% of the patients required assistance in filling the questionnaire. The frequency of missing items is 0.06%, although this parameter seen in other studies varied from 0.4% to 23.5%[8,24].

In all validation studies CLDQ questionnaire shows outstanding reliability which ranged up to 0.96[9]. In our study, Cronbach's alpha is 0.93, for the overall scale which is the same as in Lithuanian[14], Greece[11], and the Spanish[10] versions. For all domains, internal reliability is acceptable, except for "Activity" where it is 0.49. However, this finding is in accordance with those obtained in Spanish[10] and Germany[8] validation study, where the Cronbach's alpha is 0.57 and 0.69, respectively. High reliability for this domain was found in Thais[9] and Pakistani[15] study.

The domain "Activity" includes three questions: No. 7 ("Not able to eat as much as you would like"), No. 9 ("Trouble lifting or carrying heavy objects") and No. 14 ("Limitation of diet"). The reason for the low internal reliability of this domain could be a cultural relationship between diet and disease in our population.

Exploratory factor analysis was carried out to establish whether the changes introduced in the Serbian version of CLDQ affected the structure of the questionnaire. The Serbian validated version confirmed the 6-domain structure of the original United States ver-

Table 4 Distribution of chronic liver disease questionnaire-S score/sub-score according disease severity

	n	Abdominal symptoms	Fatigue	Systemic symptoms	Activity	Emotional function	Worry	Total score
Non cirrhotic	23	5.37 ± 1.39[1]	4.81 ± 1.38[1]	5.54 ± 1.14	4.80 ± 1.32	4.40 ± 1.52	4.23 ± 1.54	4.86 ± 1.05
Child's class A	25	5.08 ± 1.65[1]	4.35 ± 1.30	5.34 ± 1.38	4.40 ± 1.39	4.62 ± 1.31	4.36 ± 1.70	4.69 ± 1.22
Child's class B	30	4.88 ± 1.58[1]	4.18 ± 1.51	5.25 ± 1.34	4.37 ± 1.59	4.77 ± 1.30	4.27 ± 1.61	4.63 ± 1.17
Child's class C	25	3.68 ± 1.46	3.52 ± 1.45	4.95 ± 0.99[2]	4.40 ± 1.37	4.60 ± 1.24	4.09 ± 1.65	4.31 ± 0.97
P value[3]		0.001	0.021	0.442	0.68	0.808	0.952	0.402

[1]Significantly better score compared with "cirrhosis Child's C" score (*post hoc* analysis); [2]For domain "systemic symptoms" (n = 23); [3]*P* value for Analysis of variance.

sion[5]. Such composition of the questionnaire has also been supported by the Hamburg[20] and Chinese (Hong Kong)[17]. The Italian version has five factors versions[13], while Spanish[10] and Greek[11] validated CLDQ revealed 7 factors. However, in our investigation the original structure was only partially reproduced. The most prominent changes are related to the fact that the factors "Activity" and "Abdominal symptoms" could not be replicated, and two new domains "Sleep" and "Nutrition" were established. In the validation studies of the CLDQ carried out in Italy[13], Spain[10], Germany[24] and Chinese (Hong Kong)[17] a new factor described as "Sleep" has already been found and composed of the same two items as in our analysis. Ferrer *et al*[10] pointed out that sleeping habits could vary among cultures (napping habits and bed-times) and therefore influenced cluster potential of sleeping-related items. Moreover, in Serbian version of CLDQ, the domain "Nutrition" has been introduced for the first time. This domain consisted of two items ("Not able to eat as much as would like" and "Restriction in diet") belonging to the "Activity" in original version of CLDQ. Keeping in mind the fact that the original factor "Activity" contains one additional question ("Trouble lifting or carrying heavy objects") that is not strictly related to the previous two, special allocation of the domain of nutrition makes the assessment of quality of life more sensitive. In accordance with our findings, the factor "Activity" could not also be reproduced in investigations conducted in Germany[24] and Italy[13]. Furthermore, majority of the studies dealing with the validation of this questionnaire have shown that the factor "Systemic symptoms" was difficult to be fully reproduced[10-13,24]. In our exploratory analysis the factor "Systemic symptoms" was also partially confirmed (3/5). In Serbian version two of five items in this original domain revealed a higher degree of belonging to the other factors. Namely, the item No. 6 ("Shortness of breath in daily activities") showed higher loadings on "Fatigue", and "Nutrition", while the question No. 27 ("Itching") revealed a higher degree of belonging to the domains of "Nutrition", "Worry" and "Sleep". Ferrer *et al*[10] found that three questions (No. 3, 6 and 23) derived from original "Systemic symptoms" had considerably higher loadings on more than one other factor. Additionally, in Spanish validated version, two items (No. 3 and 6) showed multiple loading on different factors. The other studies also confirmed the hypothesis that the original domain of "Systemic symptoms" consisted of items that could not be assigned clearly and strictly to any particular dimension[11,13].

Our results of factors analysis gave the evidence that at least some items from the original version of CLDQ should be allocated or eliminated from the questionnaire because of the multiple loadings. However, we do not yet want to recommend a change of domains because direct comparisons between the validated versions of CLDQ in different populations would no longer be possible.

The decreasing in total CLDQ score with increasing disease severity was shown in several studies[5,9-11,14,16,25-28]. Reduction of the total CLDQ-S score, between patients without cirrhosis and those with cirrhosis Child's C is 0.55 points, but without statistically significance. However, Younossi *et al*[5] described that a change of 0.5 points on the 1 to 7 point scale approximates the important difference in questionnaire score. Significant reduction of the CLDQ-S sub-score, with severe CLD is detected for the subscales "Abdominal symptoms" and "Fatigue".

In Germany validation study[8] a significant reduction of the CLDQ sub-score was detected for the domains "Abdominal symptoms", "Systemic symptoms", "Activity" and "Worry", while in Spanish validation[10] this finding was obtained for "Fatigue", "Activity" and "Worry". The US validation study reported a significant reduction of the CLDQ score and sub- score for domains: "Fatigue", "Systemic symptoms" and "Activity"[5]. Additionally, Sobhonslidsuk *et al*[9] has shown that severity of CLD affecting the quality of life in all domains of CLDQ, while Ray *et al*[16] confirmed these results for all subscales except for "Worry".

In our research, the etiology of CLD did not significantly affect the HRQoL, which is consistent with previously published results[14,28-30]. In patients with early stages of CLD, etiology does not affect HRQoL, while in patients with cirrhosis, cholestatic etiology is associated with better HRQoL, than hepatocellular CLD[25]. Ray *et al*[16] described that the etiology of CLD did not affect the overall score and most CLDQ sub-score, but had effect on sub-score "Abdominal symptoms" as well as the average scores for some questions. Etiology associated with a worse HRQoL are: chronic viral hepatitis C[16,31], nonalcoholic etiology[27] and non-alcoholic fatty liver disease[32]. In our validation sample, these results could not be reproduced, probably due to the small number of patients with chronic viral hepatitis C included in our study. Besides the impact of chronic hepatitis C and interferon therapy

has an impact on HRQoL[33]. However, data on its effects are controversial[7,33,34].

In conclusion, our results provide considerable support to the appropriate metric properties of the Serbian version of CLDQ. Therefore, it could be emphasized that the questionnaire might be reliable and valid instrument for indentifying HRQoL among liver disease patients and it can be used by health professionals in their clinical practices to improve assessment of patients, especially those with low scores of quality of life. Furthermore, the results reconfirmed psychometric characteristics of the questionnaire observed in other CLD patients populations.

COMMENTS

Background

Chronic liver disease (CLD) has a significant impact on health-related quality of life (HRQoL), and therefore its assessment is widely used as important outcome in clinical trials. The CLD questionnaire (CLDQ) is a specific quality of life instrument designed for patients with liver disease, regardless of the underlining severity and etiology of CLD. Its original version was developed by Younossi *et al* and has demonstrated appropriate validity and reliability.

Research frontiers

The CLDQ has already been cross-culturally adapted and validated into different languages in previously published studies. Up to now, there is no CLD-specific quality of life instruments adapted for Serbian patients. Therefore, the aim of this study was to investigate the validation of the translated and culturally adapted CLDQ questionnaire on a group of Serbian CLD patients.

Innovations and breakthroughs

The Serbian validation of the CLDQ confirmed the 6-domain structure of the original United States version. However, in our investigation the original structure was only partially reproduced. The most prominent changes are related to the fact that the factors "Activity" and "Abdominal symptoms" could not be replicated, and two new domains "Sleep" and "Nutrition" were established Moreover, the domain "Nutrition" has been introduced for the first time. Their results of factors analysis gave the evidence that at list some items from the original version of CLDQ should be allocated or eliminated from the questionnaire because of the multiple loadings.

Applications

The authors' results provide considerable support to the appropriate metric properties of the Serbian version of CLDQ. Therefore, it could be emphasized that the questionnaire might be reliable and valid instrument for indentifying HRQoL among liver disease patients and it can be used by health professionals in their clinical practices to improve assessment of patients, especially those with low scores of quality of life.

Terminology

Cross-cultural adaptation and validation procedures create a version of the original questionnaire in a target language that is conceptually equivalent to the origin instrument and psychometrically valid to allow for data pooling and cross-national comparisons.

Peer review

The manuscript investigated the validation of a CLDQ in Serbian CLD patients. The study is of clinical significance. The manuscript from Popovic *et al* reports the validation of a CLDQ in Serbian patients. Evaluation of health related quality of life is a very relevant issue as life expectancy for several chronic diseases has increased significantly. Adaptation of a preexisting questionnaire instead of proposing an alternative procedure allows comparison with results from other patient communities allowing cross-cultural validations and future refinements. Congratulations on this article which is well written and is a good initiative to better understnd the repercussions of CLD on the quality of life of these patients.

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BRIEF ARTICLE

Low-dose amitriptyline combined with proton pump inhibitor for functional chest pain

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Abstract

AIM: To investigate the efficacy of amitriptyline with proton pump inhibitor (PPI) for the treatment of functional chest pain (FCP).

METHODS: This was a randomized, open-label trial investigating the addition of low dose amitriptyline (10 mg at bedtime) to a conventional dose of rabeprazole (20 mg/d) (group A, *n* = 20) *vs* a double-dose of rabeprazole (20 mg twice daily) (group B, *n* = 20) for patients with FCP whose symptoms were refractory to PPI. The primary efficacy endpoints were assessed by global symptom score assessment and the total number of individuals with > 50% improvement in their symptom score.

RESULTS: The between-group difference in global symptom scores was statistically significant during the last week of treatment (overall mean difference; 3.75 ± 0.31 *vs* 4.35 ± 0.29, the between-group difference; $P < 0.001$). Furthermore, 70.6% of patients in group A had their symptoms improve by > 50%, whereas only 26.3% of patients in group B had a similar treatment response (70.6% *vs* 26.3%, $P = 0.008$). Specifically, patients in group A had a significantly greater improvement in the domains of body pain and general health perception than did patients in group B (52.37 ± 17.00 *vs* 41.32 ± 12.34, $P = 0.031$ and 47.95 ± 18.58 *vs* 31.84 ± 16.84, $P = 0.01$, respectively).

CONCLUSION: Adding amitriptyline to a PPI was more effective than a double-dose of PPI in patients with FCP refractory to a conventional dose of PPI.

Key words: Functional chest pain; Proton pump inhibitor; Amitriptyline

Core tip: Hypersensitivity and psychological problems have an important role in the pathogenesis of functional chest pain (FCP). In this regard, the principal treatment of FCP has moved towards hypersensitivity modulation and antidepressant agents on the basis that the underlying mechanisms were increased pain perception or visceral hyperalgesia in addition to psychologic causes. This is the first study to report that adding low-dose amitriptyline to a conventional dose of proton pump inhibitor (PPI) is more effective than a double-dose of PPI in patients with FCP resistant to a conventional dose of PPI treatment.

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INTRODUCTION

Noncardiac chest pain (NCCP) is a common condition that affects up to one third of the general population. Moreover, the effect of NCCP on an individual's quality of life and use of health care resources is considerable because evaluation of new patients with NCCP may require a variety of costly tests. Gastroesophageal reflux disease (GERD) is the most common cause of NCCP and is present in up to 60% of patients with NCCP in Western countries[1]. In addition, some patients with NCCP are regarded as having functional chest pain (FCP) by Rome Ⅲ criteria[2-4].

Despite extensive evidence indicating that the causes of FCP are visceral hypersensitivity and psychiatric pathology[5], the underlying mechanism for FCP has not been fully understood. This problem makes the treatment of FCP quite difficult. Indeed, therapeutic gains with a conventional dose of empirical PPI treatment may be obtained in only 9%-39% of patients with FCP[6,7]. A Cochrane review suggests that doubling the PPI dose is associated with greater relief of symptoms for those with NCCP. However, there is no clear PPI dose-response relationship for symptom resolution[8].

Reflecting recent interest, several authors have confirmed an important role for hypersensitivity in the pathogenesis of FCP[9,10]. Furthermore, psychological evaluation of patients with FCP has been suggested because a significant proportion may meet the criteria for panic disorder and depressive symptoms[11,12]. In this regard, several studies have assessed the psychological treatment of FCP with tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors, and serotonin-norepinephine reuptake inhibitors[11,13,14]. Hence, the principal treatment of FCP has moved towards hypersensitivity modulation and antidepressant agents on the basis that the underlying mechanisms are increased pain perception or visceral hyperalgesia in addition to psychologic causes[15,16]. Consequently, it would be reasonable to assume a beneficial effect of amitriptyline (a TCA) on the symptoms of FCP. As mentioned earlier, these drugs could reduce the severity of psychological manifestations which are thought to exacerbate the symptoms of FCP. In addition, amitriptyline has central analgesic actions, in addition to local pharmacological actions on the upper gut which specifically alter transit and gastric accommodation[16,17]. Although widely used for FCP, the combined therapy of PPI and antidepressant agents is not evidence-based. The purpose of this study is to investigate whether adding low-dose amitriptyline to a conventional dose of PPI is more effective than a double-dose of PPI in patients with FCP resistant to conventional dose of PPI treatment.

MATERIALS AND METHODS

Patients and study protocol

This was a single-center, prospective, randomized, open-label trial. Over 8 wk we investigated the addition of a subtherapeutic dose of amitriptyline to a conventional dose of PPI (group A) *vs* a double-dose of PPI (group B) for the treatment of refractory FCP. Consecutive patients were recruited for the study who presented to the Yonsei University Medical Center with persistent unexplained midline chest pain for a minimum of 3 mo, and who had a normal upper endoscopy, 24 h impedance esophageal pH monitoring, and esophageal manometry. Patients were only considered eligible for enrollment if they were free from cardiac, musculoskeletal, and pulmonary diseases, and if they had < 50% improvement of their global symptom scores after treatment with a conventional dose of PPI (rabeprazole 20 mg/d) for at least 1 mo. Patients were excluded if they had erosive esophagitis, Barrett's esophagus, other GERD-related disorders, or peptic ulcer disease during upper endoscopy. In addition, patients were excluded if they were unable to complete 24 h impedance esophageal pH monitoring or esophageal manometry, and if the results of these tests indicated GERD or a definite motility disorder. Finally, patients were excluded if they had a depressive disorder [Beck Depression Inventory (BDI) score > 19] or if they refused all procedures of this study. After signing a written informed consent patients were asked to complete a baseline Short-Form (SF-36) to generate quality of life (QOL) data, a global symptom score, and a BDI score. Enrollees were randomized by an independent investigator using a computer-generated random number table to one of two groups, whereby those in group A were treated with the combination of amitriptyline (10 mg at bedtime) and rabeprazole (20 mg/d), and those in group B were treated with a double-dose of rabeprazole (20 mg twice daily).

Efficacy was assessed by patient evaluation of global symptom relief scores using a daily symptom diary each week. At each visit the symptom diary was checked, side effects were reported, and compliance was assessed. At the end of the 8 wk study patients were asked to complete a final QOL questionnaire, global symptom and BDI scores were generated, and any side effects were reported. Primary efficacy endpoints were assessed by the subjective global symptom relief score and the total number of individuals with > 50% improvement in their symptom score. Secondary endpoints were related to QOL indices and the BDI score. The study described in this report was approved by the ethics committee of Yonsei University School of Medicine, Seoul, South Korea.

Demographics

All subjects completed a demographic questionnaire including age, gender, residence area, smoking and alcohol history, and body mass index (BMI).

Symptom assessment

The overall clinical assessment (global symptom score) was made using an analogue scale ranging from 0 (no symptoms) to 10 (intolerable), carried out immediately before treatment[18]. All patients were also questioned regarding symptoms possibly related to complications of the treatment. Subsequently, patients were contacted every week and re-evaluated for the presence of clinical symptoms. In addition, patients were asked to report to the center if any additional symptoms occurred during the study period. In both study groups the treatment of FCP was considered effective if the global symptom score improvement was > 50%[19].

SF-36

Health-related QOL was assessed with the SF-36, which contains 36 items that, when scored, yield eight domains. This approach was chosen because of its reliability and validity among both diseased and general populations, and given its usefulness in comparing the health burden of different conditions and the benefits of treatment. Specifically, the physical functioning domain (10 items) assesses limitations of physical activities, such as walking and climbing stairs. The physical role (4 items) and emotional role (3 items) domains measure problems with work or other daily activities as a result of physical health or emotional problems. The body pain domain (2 items) assesses limitations due to pain, and the vitality domain (4 items) measures energy and tiredness. The social functioning domain (2 items) examines the effect of physical and emotional health on normal social activities, and the mental health domain (5 items) assesses happiness, nervousness, and depression. Finally, the general health perception domain (5 items) evaluates personal health and the expectation of changes in health. All domains were scored on a scale from 0 to 100, with 100 representing the best possible health state.

Depression

We assessed depression using the BDI instrument. The BDI is a self-administered 21-item self-reported scale measuring supposed manifestations of depression. In particular, a BDI score between 9 and 18 implies mild-to-moderate depression, a score between 19 and 30 signifies moderate-to-severe depression, and a score > 30 implies severe depression. This score was measured before and after treatment.

Statistical analysis

On the basis of a previous meta-analysis with antidepressants[20], a two-sided comparison of the primary outcome variable with 17 patients per group, at the end of the treatment period, had the required 90% power and 5% type I error rate to detect a difference of 40% between the groups receiving additional amitriptyline and high dose PPI in the number of patients reporting > 50% improvement in symptoms (MedCalc® Version 12.3; MedCalc Software: Mariarkerke, Belgium). To allow for possible dropouts, defined as patients who failed to present or failed to follow the medication instructions, 20 subjects were required for each group.

To account for missing data, analysis of the primary and secondary endpoints was performed according to intention to treat (ITT) and per-protocol (PP) analyses. Descriptive statistics were provided for the binary and continuous variables using the incidence frequency (%) and the mean (standard distribution). The χ^2 test or Fisher's exact test was used to compare binary variables, and the two sample *t* test was used to compare continuous variables. Between-group differences of global symptom scores over time were analyzed using a linear mixed model with an unstructured residual covariance matrix. There were also two fixed effects that were assessed, including a between-subjects treatment effect (group A: amitriptyline + rabeprazole, group B: double dose of rabeprazole) and a within-subject time effect (time: week 0 to week 8). A possible group difference across time was analyzed by the group and time interaction effect. We also evaluated treatment effects of the drug on each SF-36 domain measured at baseline and during follow-up using a two sample *t* test. Two-sided *P* values were calculated with significance accepted at the 5% level. When necessary, the *P* value was adjusted by Bonferroni correction for multiple pair-wise comparisons. We primarily report the outcomes evaluated by ITT analysis since there were no differences for test results between ITT and PP analyses. All of the statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, United States).

RESULTS

Baseline characteristics

Figure 1 shows the progress of patients throughout the study. A total of 73 patients were enrolled in the study, all of whom had persistent unexplained midline chest pain for a minimum of 3 mo, a normal upper endoscopy, 24 h impedance esophageal pH monitoring, and esophageal manometry, and an improvement of their global symptom score by < 0% after treatment with a conventional dose of PPI (rabeprazole 20 mg/d) for at least 1 mo. The random assignment of patients into two arms resulted in 20 patients in group A designated to receive the addition of amitriptyline 10 mg once daily to rabeprazole 20 mg/d, and 20 patients in group B designated to receive a rabeprazole dose of 20 mg twice daily. Overall, 4 patients dropped out of the study, including three patients because of mild medication side effects (all of whom belonged to group A) and one who was lost to follow-up (this patient belonged to group B). Of the 36 patients who completed the 8-wk trial 17 were assigned to group A and 19 to group B. The ITT population consisted of 40 patients.

Baseline characteristics for each treatment group in the ITT population are summarized in Table 1. The mean age of individuals in group A was 51.1 ± 8.5 years *vs* 49.7 ± 9.59 years for those in group B. There was a slight

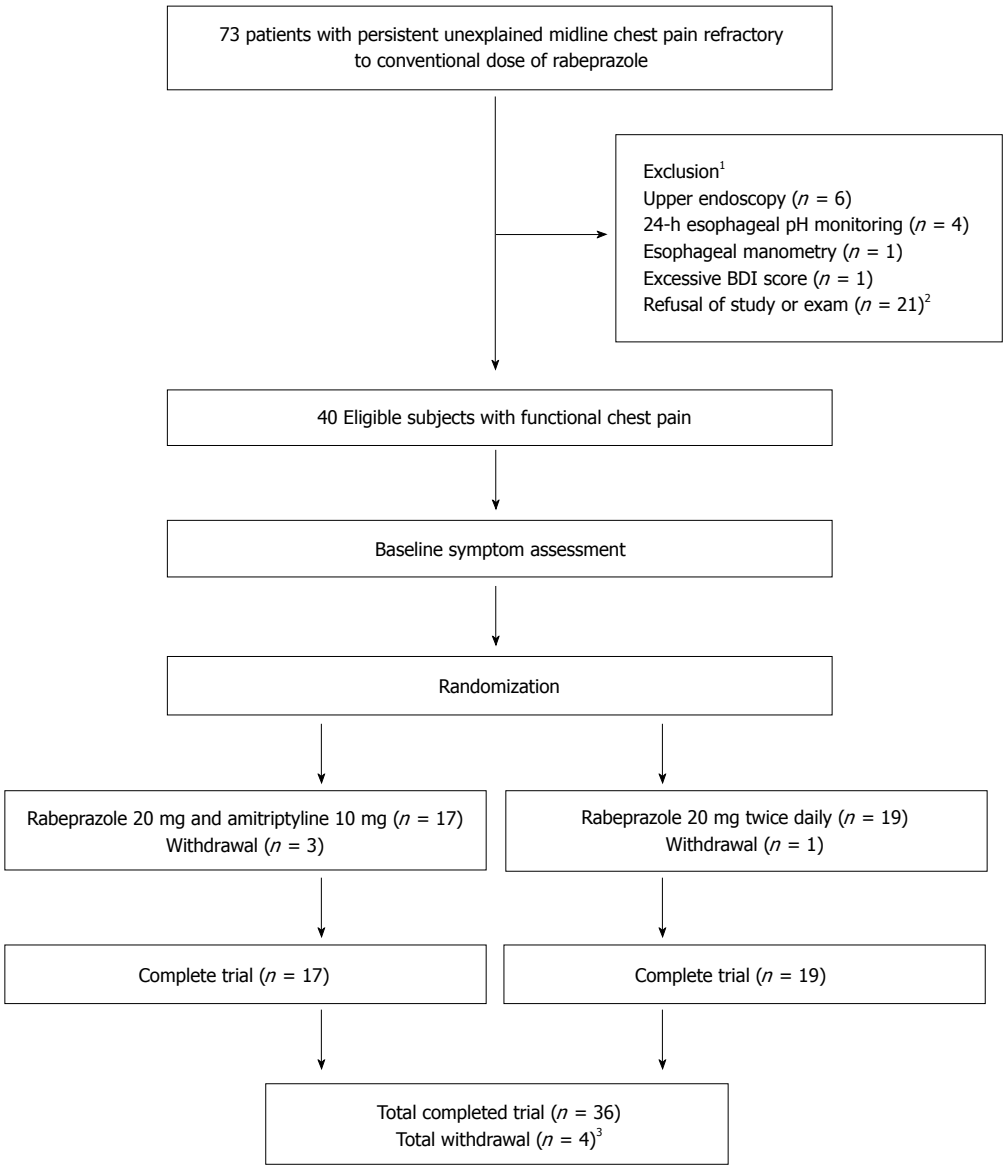

Figure 1 Flow of patients throughout the trial. [1]Upper gastrointestinal endoscopy showed erosive gastroesophageal reflux disease (n = 5), and peptic ulcer disease (n = 1). Pathological acid exposure was found in four patients by ambulatory 24 h esophageal pH monitoring. An esophageal motility disorder was found in one patient by esophageal manometric examination. The Beck Depression Index score of one patient exceeded 19 points; [2]Sixteen patients refused to take part in this study, and five patients refused examination by esophageal manometry or ambulatory 24 h esophageal pH monitoring; [3]Out of 4 patients, three in group A withdrew because of an amitriptyline-associated adverse event. One patient in group B dropped out of the trial because of loss to follow-up. BDI: Beck Depression Inventory.

female predominance in both groups: 55% in group A and 60% in group B. Of those in groups A and B respectively, 15% *vs* 20% used alcohol and 20% *vs* 45% smoked cigarettes. The mean BMI was 21.56 ± 1.74 for subjects in group A and 21.79 ± 2.2 in group B. There were no significant differences between group A and group B regarding symptom index, acid exposure time, and baseline BDI, indicating adequate randomization. Furthermore, subjects in both treatment groups of the ITT population showed generally similar values for most laboratory test results.

Global symptom score assessment

The global symptom scores over time associated with each treatment are shown in Figure 2. The overall mean difference between the two groups was not significantly different (3.75 ± 0.31 *vs* 4.35 ± 0.29, P = 0.172). However, we found that the time effect and time × group interaction effect were significant (P < 0.001 and P = 0.006, respectively). For instance, the global symptom scores significantly declined in group A. For group B, however, the global symptom scores decreased until week 2 and then somewhat increased after week 5 until the end of study follow-up (Table 2). Consequently, the between-group difference in global symptom scores was statistically significant at the last week (P < 0.001). Figure 3A shows the response rates in patients with FCP treated with amitriptyline and rabeprazole *vs* the double-dose of rabeprazole on the PP analysis. We found that 70.6% of amitriptyline and rabeprazole-treated patients showed im-

Table 1 Baseline characteristics of the intention to treat population

Variables	Group		*P* value
	A (*n* = 20)	B (*n* = 20)	
Age (yr)	51.1 ± 8.5	49.7 ± 9.59	0.628
Gender (female)	11 (55)	12 (60)	0.749
Alcohol	3 (15)	4 (20)	0.677
Smoking	4 (20)	9 (45)	0.091
BMI (kg/m^2)	21.56 ± 1.74	21.79 ± 2.2	0.716
Region (rural)	9 (52.94)	7 (36.84)	0.332
Symptom index[1]	5 (25)	5 (25)	-
Acid exposure time	0.52% ± 0.68%	0.44% ± 0.8%	0.719
Baseline BDI	6.9 ± 2.22	7.35 ± 1.93	0.498

Data are expressed as absolute numbers (percentage) or mean ± SD. [1]Symptom index indicated number of patients who has score of symptom index by > 50% on 24-h esophageal pH monitoring. BMI: Body mass index; BDI: Beck Depression Inventory.

Table 2 Global symptom scores by follow-up time

Time	Group A	Group B	*P* value[1]
Week 0	5.85 (5.09-6.61)	6.10 (5.34-6.86)	0.642
Week 1	4.90 (4.14-5.66)	5.10 (4.34-5.86)	0.709
Week 2	3.94 (3.08-4.80)	3.90 (3.06-4.74)	0.948
Week 3	3.76 (2.86-4.66)	3.95 (3.10-4.80)	0.755
Week 4	3.82 (2.96-4.68)	3.90 (3.10-4.70)	0.889
Week 5	3.18 (2.38-3.98)	3.80 (3.05-4.55)	0.259
Week 6	3.00 (2.21-3.79)	3.95 (3.21-4.69)	0.084
Week 7	2.89 (2.12-3.65)	4.05 (3.33-4.77)	0.031
Week 8	2.47 (1.68-3.27)	4.37 (3.63-5.11)	0.001

Data are least square means (95%CI). Group A: rabeprazole + amitriptyline group *vs* Group B: double dose of proton pump inhibitor group. [1]*P* value < 0.006 is considered statistically significant after adjusting the significance level of 0.05 using Bonferroni correction method for multiple comparisons. The significant difference between two groups was found at week 8 only (*P* < 0.006).

provement by > 50%, and that 29.4% failed to respond, given a response of < 50%. On the contrary, only 26.3% of patients showed a response to a double-dose of rabeprazole. This difference was significant (χ^2 = 7.06, *df* = 1, *P* = 0.008).

Health-related QOL assessment

Table 3 shows the health-related QOL as assessed by the SF-36 before and after treatment. There were no statistically significant differences between the two patient groups at baseline in any of the eight SF-36 domains. Moreover, the treatment effect at the end of the study was not significantly different between most domains of the SF-36, except for the body pain and general health perception factors. Patients who received amitriptyline and rabeprazole treatment had a significantly greater improvement in the domains of body pain and general health perception than those who received a double-dose of rabeprazole treatment (*P* = 0.031 and 0.01, respectively). The majority of the other domains of the SF-36 did not reach statistical significance.

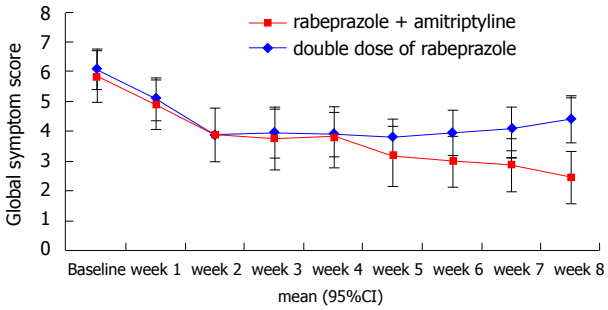

Figure 2 Efficacy of both treatment groups on the global symptom score. The global symptom scores over time was associated with each treatment. The overall mean difference between the two groups was not significantly different (*P* = 0.172). However, we found that the time effect and time × group interaction effect were significant (*P* < 0.001 and *P* = 0.006, respectively). Consequently, the between-group difference in global symptom scores was statistically significant at the last week (*P* < 0.001).

Depression

The overall mean difference of BDI at baseline and after the 8-wk treatment period was not significantly different between group A and group B (6.9 ± 2.22 *vs* 7.35 ± 1.93, *P* = 0.498, and 6.71 ± 1.99 *vs* 7.16 ± 1.89, *P* = 0.49, respectively). There was no significant difference in the depression score from baseline to the end of treatment in the double dose of PPI treatment group (*P* = 0.3). In the group receiving rabeprazole and amitriptyline, this result was marginally significant (*P* = 0.06). The change in value of the BDI scores associated with treatment is shown in Figure 3B.

Tolerability and safety assessment

Three patients withdrew from the study because of non life-threatening adverse events while receiving the combination of amitriptyline and rabeprazole (excessive sleeping, dizziness and general weakness).

DISCUSSION

The primary aim of this study was to determine the efficacy of low-dose amitriptyline with a conventional dose of PPI for the treatment of FCP with refractory symptoms to a conventional dose of PPI. We found favorable evidence for the efficacy of antidepressants in improving global symptom scores and health related QOL in patients with FCP refractory to conventional doses of PPIs. In other words, we found that adding low-dose amitriptyline to a conventional dose of PPI resulted in significantly decreased symptoms compared with a double-dose of PPI, with minimal side effects. Interestingly, this outcome is very similar to the open-label response to antidepressants seen with irritable bowel syndrome[21].

Anti-reflux therapy with PPIs plays an important role in the diagnosis and treatment of patients with NCCP because the major cause of NCCP is GERD, and since the management of NCCP is largely empirical[22,23]. However, in patients with non GERD-related NCCP (espe-

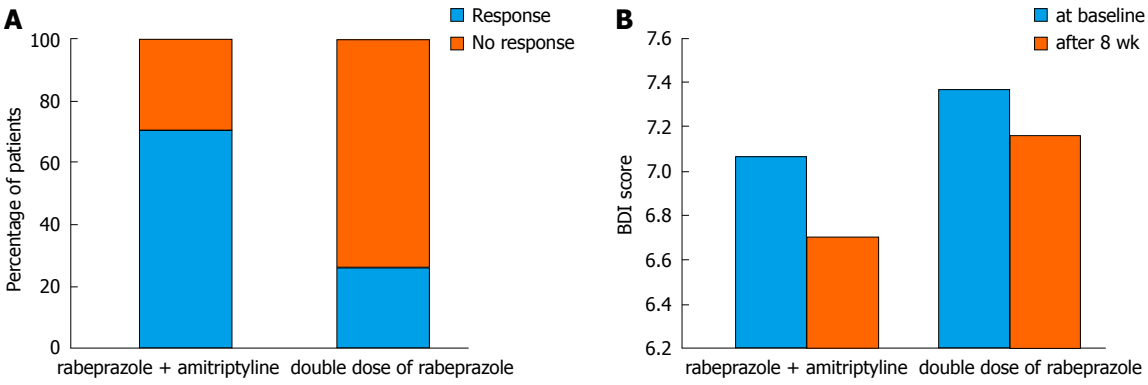

Figure 3 Symptom response rate (A) and Beck Depression Inventory scores (B) after treatment. A: The response rates in patients after treatment with rabeprazole + amitriptyline *vs* double-dose of rabeprazole on the per-protocol analysis are demonstrated. In this analysis, the difference of the response rate between both groups was statistically significant; B: The Beck Depression Inventory (BDI) scores after treatment with amitriptyline and rabeprazole *vs* double-dose of rabeprazole are represented. The overall mean difference of BDI scores after 8 wk of treatment was not significantly different between group A and group B (6.71 ± 1.99 *vs* 7.16 ± 1.89, respectively; $P = 0.49$).

Table 3 Treatment effect on the health-related quality of life assessed with Short-Form 36

SF-36 score	Group		*P* value
	Group A (n = 20)	Group B (n = 20)	
Physical functioning			
Baseline	42.01 ± 13.47	32.83 ± 17.06	0.211
End of treatment	37.28 ± 12.76	38.42 ± 7.83	0.753
Role -physical			
Baseline	31.51 ± 14.9	29.45 ± 15.51	0.670
End of treatment	37.88 ± 10.16	39.02 ± 12.49	0.768
Role-emotional			
Baseline	32.01 ± 12.12	38.39 ± 12.68	0.112
End of treatment	36.99 ± 12.62	30.14 ± 16.95	0.182
Social functioning			
Baseline	37.95 ± 14.06	33.82 ± 13.90	0.356
End of treatment	36.41 ± 13.03	30.07 ± 12.83	0.151
Body pain			
Baseline	34.94 ± 14.39	30.13 ± 12.09	0.260
End of treatment	52.37 ± 17.00	41.32 ± 12.34	0.031
General health perceptions			
Baseline	38.63 ± 11.66	31.82 ± 12.94	0.088
End of treatment	47.95 ± 18.58	31.84 ± 16.84	0.010
Mental health			
Baseline	38.82 ± 14.72	39.20 ± 10.67	0.927
End of treatment	44.69 ± 10.79	38.88 ± 10.50	0.111
Energy/vitality			
Baseline	38.08 ± 12.50	34.39 ± 12.64	0.360
End of treatment	42.95 ± 15.32	35.14 ± 10.87	0.084

All results are expressed as mean ± SD. Group A received amitriptyline and rabeprazole and group B received double dose of rabeprazole. *P* values are for the comparison of amitriptyline and rabeprazole *vs* double dose of rabeprazole at baseline and end of the treatment. Patients who received amitriptyline and rabeprazole treatment had a significantly greater improvement in the domains of body pain and general health perception than those who received a double dose of rabeprazole treatment ($P = 0.031$ and $P = 0.01$, respectively). The majority of the other domains of the Short-Form 36 (SF-36) did not reach statistical significance.

cially those refractory to conventional doses of PPI), treatment should be targeted to alternative drugs, such as pain modulating agents[24]. Indeed, therapeutic gains with PPI treatment have been obtained in only 9%-39% of non GERD-related NCCP patients[6,22,25]. Recent studies have focused on modulating nociception and visceral pain sensation pathways for decreasing chest pain using antidepressants[11,26]. For these reasons, it would be reasonable to assume a beneficial effect of antidepressant drugs, such as TCAs, on the symptoms of FCP. In fact, to investigate the efficacy of antidepressant treatments for FCP, one meta-analysis of seven studies and 319 participants indicated that there was strong evidence for an association of antidepressants with a reduction in pain and psychological symptoms. However, the drugs assessed in this analysis were varied and included a TCA, selective serotonin reuptake inhibitors, and a serotonin-norepinephine reuptake inhibitor[14]. Therefore, the possible effect of amitriptyline for FCP is unclear. Moreover, until now there has been no randomized controlled study to investigate the effects of combining TCAs with PPIs for the treatment of FCP refractory to conventional therapy.

The results of our clinical study suggest that FCP may respond favorably to low-dose amitriptyline in combination with a PPI. Eventually the symptomatic overlap with functional gastrointestinal disorders, the recognized association of functional dyspepsia with visceral hypersensitivity, and the response of several other functional gut disorders to TCAs may all hold clues to the seeming success in our patients. In our study, we used doses of amitriptyline far below those necessary for an antidepressant benefit. This likely explains the positive association of amitriptyline for reducing pain in the absence of a benefit for depressive symptoms. The synergistic effects of amitriptyline in combination with a conventional dose of PPI could have an important role in improving QOL of those with FCP. Because the therapeutic effects of amitriptyline are usually achieved within 4-6 wk, the duration of our trial was considered adequate for evaluating the efficacy of this drug. Our results show that an 8-wk treatment regimen with amitriptyline and rabeprazole significantly improved the global symptom score given the response rate of 70.6%, which was in comparison to the

response rate for a double-dose of rabeprazole of only 26.3%. However, analysis of the SF-36 as a QOL measurement showed satisfactory efficacy in only two domains. This might have been caused by the small sample size, which may have been inadequate for detecting differences in secondary outcomes, although it was adequate for detecting the required difference in the primary outcome variable.

Our study has a few limitations. The open-label nature of this study could lead to some biases with generalization of the results. The sample size was also relatively small and further investigation based on a larger number of patients is necessary to corroborate our data. Finally, our study duration was relatively short. Indeed, the short duration of most studies and the lack of follow-up after treatment cessation leave the question unanswered whether antidepressants have long-term beneficial effects on FCP symptoms, as well as the optimal treatment duration. Nevertheless, this study is of value because it is the first study examining the efficacy of amitriptyline on patients with FCP with symptoms refractory to a conventional dose of PPI. We did not encounter major or unexpected side effects related to amitriptyline.

In conclusion, the combination of low-dose amitriptyline with a standard PPI regimen was more effective than a double-dose of PPIs in patients with FCP refractory to conventional PPI therapy, without serious adverse events. The safety profile and efficacy in the subjects using low-dose amitriptyline as well as the significant improvement in global symptom scores may justify the addition of amitriptyline for the treatment of refractory FCP.

COMMENTS

Background

Despite extensive evidence indicating that the causes of functional chest pain (FCP) are visceral hypersensitivity and psychiatric pathology, the underlying mechanism for FCP is largely unknown. This problem makes the treatment of FCP quite difficult.

Research frontiers

The principal treatment of FCP has moved towards hypersensitivity modulation and antidepressant agents on the basis that the underlying mechanisms were increased pain perception or visceral hyperalgesia in addition to psychologic causes. In this study, the authors demonstrate that amitriptyline, as a tricyclic antidepressant, would appear to have a beneficial effect on the symptoms of FCP.

Innovations and breakthroughs

Recent reports have highlighted the psychological treatment of FCP with tricyclic antidepressants, selective serotonin reuptake inhibitors, and serotonin-norepinephine reuptake inhibitors. This is the first study to report that adding low-dose amitriptyline to a conventional dose of proton pump inhibitor (PPI) is more effective than a double-dose of PPI in patients with FCP resistant to a conventional dose of PPI treatment.

Applications

The authors' result demonstrates that the safety profile and efficacy in the subjects using low-dose amitriptyline as well as the significant improvement in global symptom scores may justify the addition of amitriptyline for the treatment of refractory FCP.

Terminology

FCP was defined as recurrent angina-like retrosternal chest pain with normal coronary anatomy and no detectable gastroenterological and respiratory causes after an adequate evaluation by Rome Ⅲ criteria. The SF-36 as health-related quality of life contains 36 items that, when scored, yield eight domains. The BDI instrument as assessment for anxiety and depression is a self-administered 21-item self-reported scale measuring supposed manifestations of depression.

Peer review

The authors have focused on modulating nociception and visceral pain sensation pathways for decreasing chest pain using antidepressants. It revealed that the combination of low-dose amitriptyline with a standard PPI regimen was more effective than a double-dose of PPIs in patients with FCP refractory to conventional PPI therapy, without serious adverse events.

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BRIEF ARTICLE

Residual common bile duct stones on direct peroral cholangioscopy using ultraslim endoscope

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Author contributions: Huang SW wrote the manuscript; Tsou YK conceptualized and designed the study, performed direct peroral cholangioscopy and analyzed and interpreted the data; Lee MS and Sung KF collected data; Lin CH performed direct peroral cholangioscopy, revised the manuscript and gave final approval of the version to be published.

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Abstract

AIM: To detect and manage residual common bile duct (CBD) stones using ultraslim endoscopic peroral cholangioscopy (POC) after a negative balloon-occluded cholangiography.

METHODS: From March 2011 to December 2011, a cohort of 22 patients with CBD stones who underwent both endoscopic retrograde cholangiography (ERC) and direct POC were prospectively enrolled in this study. Those patients who were younger than 20 years of age, pregnant, critically ill, or unable to provide informed consent for direct POC, as well as those with concomitant gallbladder stones or CBD with diameters less than 10 mm were excluded. Direct POC using an ultraslim endoscope with an overtube balloon-assisted technique was carried out immediately after a negative balloon-occluded cholangiography was obtained.

RESULTS: The ultraslim endoscope was able to be advanced to the hepatic hilum or the intrahepatic bile duct (IHD) in 8 patients (36.4%), to the extrahepatic bile duct where the hilum could be visualized in 10 patients (45.5%), and to the distal CBD where the hilum could not be visualized in 4 patients (18.2%). The procedure time of the diagnostic POC was 8.2 ± 2.9 min (range, 5-18 min). Residual CBD stones were found in 5 (22.7%) of the patients. There was one residual stone each in 3 of the patients, three in 1 patient, and more than five in 1 patient. The diameter of the residual stones ranged from 2-5 mm. In 2 of the patients, the residual stones were successfully extracted using either a retrieval balloon catheter (n = 1) or a basket catheter (n = 1) under direct endoscopic control. In the remaining 3 patients, the residual stones were removed using an irrigation and suction method under direct endoscopic visualization. There were no serious procedure-related complications, such as bleeding, pancreatitis, biliary tract infection, or perforation, in this study.

CONCLUSION: Direct POC using an ultraslim endoscope appears to be a useful tool for both detecting and treating residual CBD stones after conventional ERC.

Key words: Balloon-occluded cholangiography; Common bile duct stones; Endoscopic retrograde cholangiography; Peroral cholangioscopy; Residual stones

Core tip: Balloon-occluded cholangiography is generally performed to confirm bile duct clearance after performing endoscopic retrograde cholangiography (ERC) for stone retrieval. However, balloon-occluded cholangiography may be an imperfect tool for this diagnostic purpose. In this case series, we demonstrated that 22.7%

of patients still had residual stones detected on peroral cholangioscopy after a negative balloon-occluded cholangiography was obtained. All of the residual stones were retracted on the cholangioscopy. Our results reveal that peroral cholangioscopy appears to be a useful tool for both detecting and treating residual common bile duct stones after conventional ERC.

Huang SW, Lin CH, Lee MS, Tsou YK, Sung KF. Residual common bile duct stones on direct peroral cholangioscopy using ultraslim endoscope. *World J Gastroenterol* 2013; 19(30): 4966-4972 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4966.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4966

INTRODUCTION

Endoscopic sphincterotomy (ES) has become the cornerstone of therapeutic endoscopic retrograde cholangiography (ERC). It is most commonly performed to remove common bile duct (CBD) stones[1-3]. Endoscopic papillary balloon dilatation (EPBD) has been used as an alternative approach to ES[4,5]. After ES/EPBD for stone retrieval, balloon-occluded cholangiography is generally performed to confirm bile duct clearance. However, small stones may be left undetected by the balloon-occluded cholangiography[6-8]. These small stone fragments run the risk of acting as nidi for future stone formation, leading to the recurrence of CBD stones[9-11]. Therefore, it is crucial to achieve a level of stone clearance that is as complete as possible to prevent stone recurrence. Intraductal ultrasound (IDUS) has been applied to confirm the clearance of CBD stones after stone retrieval by ES[6,9,12]. Tsuchiya *et al*[9] reported that performing IDUS after stone extraction decreased the recurrence rate of CBD stones to 3.4% from the 13.2% recurrence rate of the control group. However, IDUS poses problems, such as probe fragility and a high cost. In addition, it is a highly operator-dependent technology. Poor images and the consequent oversight of residual stones are possible, especially in patients with extensive pneumobilia[9]. Ohashi *et al*[6] reported that IDUS examination failed to detect residual stones after bile duct clearance in 14.6% (6/41) of the patients.

Cholangioscopy offers a crucial advantage over IDUS in that it permits direct visualization of the bile duct and further management of any bile duct stones[13-15]. Direct peroral cholangioscopy (POC) may play a role in the detection of bile duct stones, but there is a lack of studies on this subject[16]. Although conventional POC using a mother-baby endoscopic system has been available for more than three decades, its role remains limited because of its many disadvantages[17,18]. Recently, direct POC using an ultraslim endoscope has been reported to be feasible and superior to the conventional mother-baby endoscopic system because it provides superior endoscopic images and a larger working channel[19-22]. Furthermore, it can be performed by a single endoscopist. The aim of this study is to evaluate the utility of ultraslim endoscopic POC in the diagnosis and management of residual CBD stones after performing bile duct clearance and confirming the procedure using balloon-occluded cholangiography.

MATERIALS AND METHODS

Patients

From March 2011 to December 2011, the patients who underwent ERC performed by the two endoscopists (Tsou YK and Lin CH) in Chang Gung Memorial Hospital who met the following criteria were prospectively enrolled in this study: CBD stones were diagnosed based on imaging studies, such as abdominal ultrasonography, computed tomography scans, and/or magnetic resonance cholangiopancreatography before the index ERC (n = 92); The exclusion criteria were as follows: (1) patients who were younger than 20 years of age, pregnant, or critically ill (n = 5); (2) patients with concomitant gallbladder stones (n = 42); (3) patients with CBD diameters of less than 10 mm (n = 9); and (4) patients who were unable to provide informed consent for POC (n = 14).

ERC

ERC was performed using a duodenoscope (JF or TJF 260-V, Olympus, Tokyo, Japan) under conscious sedation with the patients in a prone position. ES was performed using a standard pull-type sphincterotome (Ultratome; Boston Scientific Co., Spencer, IN, United States). EPBD was carried out using a controlled radial expansion (CRE) balloon. During the index ERC, EPBD was carried out in 13 patients (including the 8 patients who had previously undergone ES or EPBD); ES then EPBD were performed in 6 patients (including 1 patient who had received a previous ES); and extended ES was performed in 1 patient to facilitate the stone extraction and/or the performance of the POC. Two other patients did not undergo ES or EPBD during the index ERC because the papillary orifice created by EPBD during the previous procedure(s) was large enough to permit stone extraction and performing the POC.

A contrast medium at a 1:1 dilution was used for the cholangiography. The instruments used to extract the CBD stones included a retrieval balloon catheter alone (n = 16) and a combination of a balloon and a basket catheter (n = 6). Two patients underwent lithotripsy because the CBD stones were difficult to extract using a balloon catheter. After the stone extraction, balloon-occluded cholangiography was performed to confirm the complete clearance of the CBD stones. If any residual stones were observed during the balloon-occluded cholangiography, additional endoscopic treatments were performed until the balloon-occluded cholangiography was negative (Figure 1).

Direct peroral cholangioscopy

An ultraslim endoscope (GIF-N260, Olympus) and an

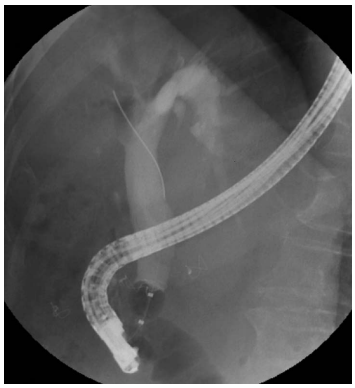

Figure 1 Balloon-occluded cholangiography failed to reveal any filling defects of stones in the biliary tree after endoscopic retrograde cholangiography with bile duct clearance.

overtube (ST-SB1, Olympus) were used for the POC procedures. All of the POC procedures were performed by two endoscopists (Tsou YK and Lin CH) who are experienced in this endoscopy and were carried out immediately after a negative balloon-occluded cholangiography was obtained during a single endoscopic session. The details of the POC procedures are described in our previous study[20]. Briefly, the overtube containing the endoscope is advanced into the distal gastric antrum (or into the afferent loop for patients with a post-operative stomach); the overtube balloon is then inflated to anchor the overtube. The endoscope is further advanced into the orifice of the major papilla either directly or after performing a J-turn of the endoscopic tip. Then, the endoscope is advanced into the bile duct as far as possible. The POC time is defined as the interval between the ultraslim endoscope entering the mouth of the patient and reaching the farthest site of the biliary tree.

In the text and tables, the continuous variables are expressed in the form mean ± SD. The study protocol was approved by the ethical committee at Chang Gung Memorial Hospital (IRB No: 99-2585C).

RESULTS

A cohort of 22 patients with CBD stones undergoing both ERC and direct POC were prospectively enrolled in this study (Table 1). The patient age was 73.4 ± 11.8 years (range, 40-89 years), and 15 (68%) of the patients were men. The patients were categorized into the following gallbladder status groups: intact without stones (n = 3), post laparoscopic cholecystectomy (n = 2), and post open cholecystectomy (n = 17). Eight patients (36.4%) had juxtapapillary diverticulum. Three patients (13.6%) had a medical history of subtotal gastrectomy with Billroth-Ⅱ anastomosis (n = 2) or total gastrectomy with Roux-en-Y anastomosis (n = 1).

The ERC results are listed in Table 2. The maximum diameter of the CBD was 17.9 ± 5.1 mm (range, 10-30 mm). Twelve of the patients (54.5%) had recurrent CBD stones. The number of CBD stones was one each in 10 patients, two in 2 patients, three in 4 patients, and more than three in 6 patients. The maximum stone diameter was 13.4 ± 5.6 mm (range, 5-25.4 mm). Sixteen patients had brown stones, 2 patients had black stones, and 4 patients had mixed brown and black stones. During the index ERC, the CBD stones were removed intact in 11 patients (50%).

Table 1 Patient characteristics n (%)

Characteristics	n = 22
Age (yr)	73.4 ± 11.8 (range, 40-89)
Gender (male)	15 (68)
Prior cholecystectomies[1]	19 (86.4)
Acalculous gallbladder	3 (13.6)
Juxtapapillary diverticulum	8 (36.4)
Subtotal gastrectomy with Billroth-Ⅱ anastomosis	2 (9.1)
Total gastrectomy with Roux-en-Y anastomosis	1 (4.5)
Patients with recurrent CBD stones	12 (54.5)

[1]Including 17 cases of open cholecystectomy and 2 cases of laparoscopic cholecystectomy. CBD: Common bile duct.

Table 2 Results of endoscopic retrograde cholangiography n (%)

Characteristics	n = 22
CBD stones	
No. of stones (one/two/three/more than three)	10/2/4/6
Mean maximum diameter (mm)	13.4 ± 5.6 (range, 5-25.4)
Mean maximum diameter of CBD (mm)	17.9 (range, 10-30)
ES and/or EPBD	
ES	1 (4.5)
EPBD	13 (59.1)
ES + EPBD	6 (27.3)
None[1]	2 (9.1)
Mean maximum inflated diameter during EPBD[2] (mm)	13.6 (range, 12-15)
Stone extraction methods	
Balloon and/or basket	20 (90.9)
Mechanical lithotripter	2 (9.1)
Intact stone extraction	11 (50)

[1]Endoscopic papillary balloon dilatation (EPBD) was performed during the previous endoscopic session, and the papillary orifice was adequate; [2]A total of 15 cases underwent EPBD. CBD: Common bile duct; ES: Endoscopic sphincterotomy.

The results of POC are given in Table 3. The POC time was 8.2 ± 2.9 min (range, 5-18 min). The ultraslim endoscope was able to be advanced to the hepatic hilum or the intrahepatic bile duct (IHD) in 8 patients (36.4%) (Figure 2); to the extrahepatic bile duct where the hilum could be visualized in 10 patients (45.5%); and to the distal CBD where the hilum could not be visualized in 4 patients (18.2%). During POC, residual CBD stones were found in 5 patients (22.7%) (Figure 3). One residual stone was found in each of 3 patients, three in 1 patient, and multiple (more than five) in 1 patient. The diameter of the residual stones ranged from 2-5 mm. In 2 patients, the residual stones were extracted successfully using a retrieval balloon catheter (n = 1) or a basket catheter (n =

Table 3 Results of peroral cholangioscopy *n* (%)

Characteristics	*n* = 22
Mean procedure time (min)	8.2 (range, 5-18)
The endoscope reached	
Hilum or IHD	8 (36.4)
CBD and the hilum was seen	10 (45.5)
Distal CBD and the hilum was not seen	4 (18.2)
Residual stones on the POC	
No. of patients	5 (22.7)
No. of residual stones (one/three/multiple)	3/1/1
Maximum diameter of stones (range, mm)	2-5

IHD: Intrahepatic duct; CBD: Common bile duct; POC: Peroral cholangioscopy

Table 4 Clinical features between the patients with and without residual bile duct stones *n* (%)

	With residual stones (*n* = 5)	Without residual stones (*n* = 17)
Age (yr)	69 ± 18.6	74.7 ± 9.5
Sex (male)	4 (80)	11 (64.7)
Recurrent CBD stones	4 (80)	7 (41.2)
Prior choledocholithotomy	3 (60)	6 (35.3)
Mean maximum CBD diameter (mm)	19 ± 7.6	17.6 ± 4.5
Stones number (single)	2 (40)	8 (47)
Mean maximum stone diameter (mm)	14.1 ± 7.2	13.2 ± 5.2
Lithotripsy	0 (0)	2 (11.8)
Intact stone extraction	1 (20)	10 (58.9)
Diameter of EPBD[1] (mm)	13.5 ± 1.7 (*n* = 4)	13.5 ± 1.4

[1]Mean maximum inflated diameter of the balloon during endoscopic papillary balloon dilatation (EPBD). CBD: Common bile duct.

1) under direct endoscopic visualization (Figure 4). In the remaining 3 patients, the residual stones were removed using direct endoscopic suction after normal saline irrigation. For the patient with multiple residual stones, a balloon catheter was inserted proximally to the stones through the endoscope and the endoscopic tip was placed distally to the stones. Using synchronic normal saline irrigation *via* the balloon catheter and endoscopic suction, the stones and the endoscope were slowly pulled down to the distal bile duct and finally to the duodenum. No serious procedure-related complications, such as bleeding, pancreatitis, biliary tract infection, or perforation, were observed in this study. The ultraslim endoscope used as the cholangioscope did not sustain any obvious damage during the study period.

The clinical data on the patients with (*n* = 5) and without (*n* = 17) residual stones on POC are listed in Table 4. Because the patient sample size was small, we did not perform any statistical analysis. However, recurrent CBD stones and prior choledocholithotomy were more frequently observed in the patients with residual stones than in the patients without (80% *vs* 41.2% and 60% *vs* 35.3%, respectively). Intact stone extraction during the index ERC was less common in patients with residual stones than in patients without (20% *vs* 58.9%, respectively). These patients were followed up for 17.5 ± 4.9 mo (range, 12-24 mo) after the POC. Four patients (18.2%) had recurrent CBD stones documented on ERC during a follow-up; two of these four had residual stones on POC.

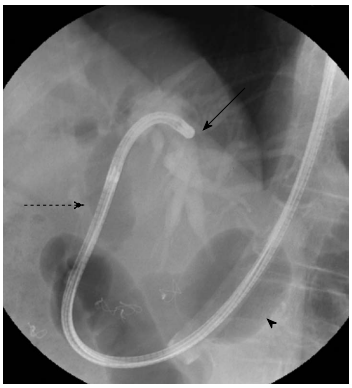

Figure 2 Ultraslim endoscope was advanced to the common bile duct (dotted arrow) and up to the left intrahepatic duct (arrow) using an over-tube balloon (arrow head)-assisted technique.

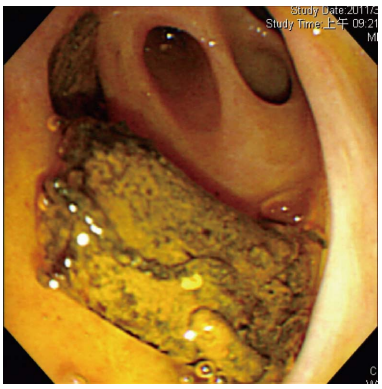

Figure 3 Residual stones in the bile duct visualized using direct peroral cholangioscopy.

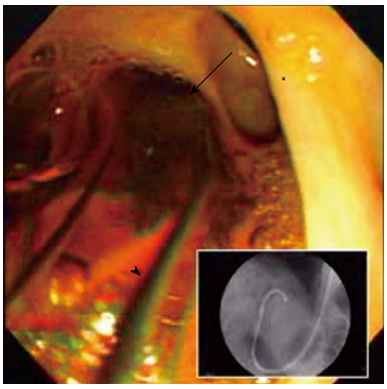

Figure 4 A basket catheter (arrow head) was used to retrieve the residual bile duct stones (arrow) under direct endoscopic control.

DISCUSSION

Balloon-occluded cholangiography is an imperfect tool to confirm complete bile duct clearance after ES/EPBD for

stone retrieval. In this study, we observed that balloon-occluded cholangiography failed to detect residual CBD stones in 22.7% of the patients. Tsuchiya *et al*[9] used IDUS and reported that balloon-occluded cholangiography did not detect any residual CBD stones in 23.7% (14/59) of the patients[9]. Itoi *et al*[7] performed POC using a mother-baby system 0-20 d (median, 6.2 d) after ERC with CBD stone retrieval. In all of these patients, the bile duct was confirmed to be free of stones using balloon-occluded cholangiography. It was later found that 24% (26/108) of these patients had residual CBD stones on the POC, although in some of the patients, the CBD stones might have migrated from the gallbladder after the stone retrieval. Our results are consistent with those of other studies, revealing that balloon-occluded cholangiography fails to detect residual CBD stones in nearly one quarter of patients.

The residual CBD stones not detected by balloon-occluded cholangiography in the previous reports using IDUS were usually small[23-25]. The present study confirmed (using POC) that the undetected residual CBD stones are small and no more than 5 mm in diameter. It is unclear whether these small residual stones have clinical significance[26]. Because the orifice of the major papilla after ES/EPBD is large enough, spontaneous passage of the stones is possible. However, Itoi *et al*[7] performed POC an average of 6 d after stone retrieval by ERC and found that 24% of the patients still had residual stones. Their result suggests that residual stones might not be excreted for a long time and may eventually cause stone recurrence. Several studies that have aimed to analyze the risk factors for recurrent CBD stones suggest that residual stones are a possible cause of recurrent CBD stones[9,10,27]. Therefore, it may be important to detect and remove residual CBD stones after ES/EPBD for stone retrieval. Further studies are needed to clarify whether the residual stones have clinical significance and whether the removal of residual stones minimizes the risk of stone recurrence.

The ultraslim endoscopic POC has several advantages over the mother-baby endoscopic system[20,21]. One is that it enables the extraction of residual CBD stones under direct endoscopic visualization, as demonstrated in the present study. In contrast, the residual stones in the 26 patients reported by Itoi *et al*[7] were not directly removable using the mother-baby system. A 5-Fr balloon or basket catheter can pass through the 2-mm working channel of the ultraslim endoscope to grasp the residual stones. However, to remove the grasped stones, the ultraslim endoscope with the balloon/basket must be withdrawn to the duodenum. This maneuver can be complicated when there are multiple residual stones. In this case, the irrigation and suction method described above offers an effective way to remove the stones, especially for small soft stones. Placing the tip of a balloon catheter proximally to the stones can avoid flushing them upstream.

There are two major limitations to this study. One is that the hilum or IHD could be reached by the POC in only 36.4% of the patients. For the purpose of this study, seeing the hilum may be enough to verify bile duct clearance, and in 71.8% of the patients, the hilum could be seen. However, in 18.2% of the patients, the hilum could not be seen by the POC. One possible reason for this limitation was that many (41%) of our patients had undergone choledocholithotomy with T-tube drainage, resulting in a tortuous CBD. Direct POC using an intraductal anchoring balloon method may be able to improve the rate of seeing the hilum, but the anchoring device was withdrawn from the market due to an increased risk of air embolism[28]. Therefore, the rate of residual CBD stones may be higher if some of the residual stones were not visualized during the POC in our patients.

The other limitation is that we enrolled only patients without GB stones or without GB to avoid the risk of "migrated GB stones" being confused with the residual stones. As a result, 19 of the 22 patients (86.4%) had a previous cholecystectomy. Therefore, the study results may be only applied to this subgroup of patients. In this study, we found that recurrent CBD stones as an indication for index ERC, prior choledocholithotomy, and fragmented stones during stones retrieval on the index ERC were more frequently observed in patients with residual stones. It might be worthwhile to perform POC for patients with these characteristics.

In conclusion, conventional ERC with balloon-occluded cholangiography is not a reliable method for confirming the complete extraction of CBD stones. Direct POC using an ultraslim endoscope appears to be a useful tool to confirm the clearance of CBD stones and to extract the residual CBD stones in selected patients.

COMMENTS

Background

Balloon-occluded cholangiography is generally performed to confirm bile duct clearance after bile duct stone retrieval. However, small stones may remain undetected on the balloon-occluded cholangiography.

Research frontiers

Cholangiography is a direct image which is at least theoretically better than the indirect image of balloon-occluded cholangiography. Therefore, the authors perform direct peroral cholangiography (POC) to examine if there are residual bile duct stones after obtaining a negative balloon-occluded cholangiography. The method of POC is using an ultraslim endoscope with overtube balloon-assisted technique.

Innovations and breakthroughs

The authors demonstrate that 22.7% of the patients still have residual stones detected on the direct POC after a negative balloon-occluded cholangiography is obtained. All of the residual stones are small (range 2-5 mm) and extracted successfully during the POC.

Applications

The results indicate that direct POC using an ultraslim endoscope appears to be a useful tool for both detecting and treating residual bile duct stones after conventional endoscopic retrograde cholangiography.

Terminology

Direct POC is to insert an endoscope perorally into the bile ducts. Direct POC using a mother-baby endoscope system is not widely used because of its many disadvantages. Recently, direct POC using an ultraslim endoscope has been

reported to be feasible and superior to the conventional mother-baby endoscopic system because it provides superior endoscopic images and a larger working channel. Furthermore, it can be performed by a single endoscopist.

Peer review

In this study, the value of peroral cholangioscopy for detecting remaining bile duct stones after balloon-occluded cholangiography was evaluated in 22 patients. Despite a negative balloon-occluded cholangiography, additional bile duct stones were detected in 5 patients (23%). The stone diameter was generally small (2-5 mm). Unfortunately the authors did not perform intraductal ultrasound in their 22 patients simultaneously, that would have allowed comparison of the two methods.

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BRIEF ARTICLE

A systematic analysis of pneumatosis cystoids intestinalis

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Author contributions: Wu LL and Yang YS contributed substantially to the conception and design of the data, drafted the paper and approved the version to be published; Wu LL, Dou Y and Liu QS contributed substantially to the analysis and interpretation of the data and approved the version to be published.
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Abstract

AIM: To increase the understanding, diagnosis and treatment of pneumatosis cystoides intestinalis (PCI) and to find the characteristics and potential cause of the disease in China.

METHODS: We report here one case of PCI in a 70-year-old male patient who received a variety of treatment methods. Then, we systematically searched the PCI eligible literature published from an available Chinese database from May 2002 to May 2012, including CBM, CBMDisc, CMCC, VIP, Wanfang, and CNKI. The key words were pneumatosis cystoides intestinalis, pneumatosis, pneumatosis intestinalis, pneumatosis coli and mucosal gas. The patients' information, histories, therapies, courses, and outcomes were reviewed.

RESULTS: The study group consisted of 239 PCI cases (male:female = 2.4:1) from 77 reported incidents. The mean age was 45.3 ± 15.6 years, and the median illness course was 6 mo. One hundred and sixty patients (66.9%) were in high altitude areas. In addition, 43.5% (104/239) of the patients had potential PCI-related disease, and 16.3% had complications with intestinal obstruction and perforation. The most common symptom was abdominal pain (53.9%), followed by diarrhea (53.0%), distention (42.4%), nausea and vomiting (14.3%), bloody stool (12.9%), mucous stool (12.0%) and constipation (7.8%). Most multiple pneumocysts developed in the submucosa of the colon (69.9%). The efficacy of the treatments by combined modalities, surgery, endoscopic treatment, conservative approach, oxygen, and antibiotics were 100%, 100%, 100%, 93.3%, 68.3% and 26.3%, respectively.

CONCLUSION: PCI can be safely managed by conservative treatments, presents more frequently in males, in the large bowel and submucosa, than in females, in the small intestine and subserosa. High altitude residence maybe associated with the PCI etiology.

Key words: Pneumatosis cystoides intestinalis; Pneumatosis; Cyst; Intestinal; Colon

Core tip: Pneumatosis cystoids intestinalis (PCI) is a rare disease characterized by the presence of multiple gas-filled cysts in the submucosa and/or subserosa of the intestinal wall. PCI is still a poorly understood entity, and nearly all of the studies for PCI are case reports. In this work, we systematically evaluated and demonstrated for the first time the characteristics of PCI patients in China.

Wu LL, Yang YS, Dou Y, Liu QS. A systematic analysis of pneumatosis cystoids intestinalis. *World J Gastroenterol* 2013; 19(30): 4973-4978 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4973.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4973

INTRODUCTION

Pneumatosis cystoids intestinalis (PCI) is an uncommon disease with an unknown etiology, characterized by the

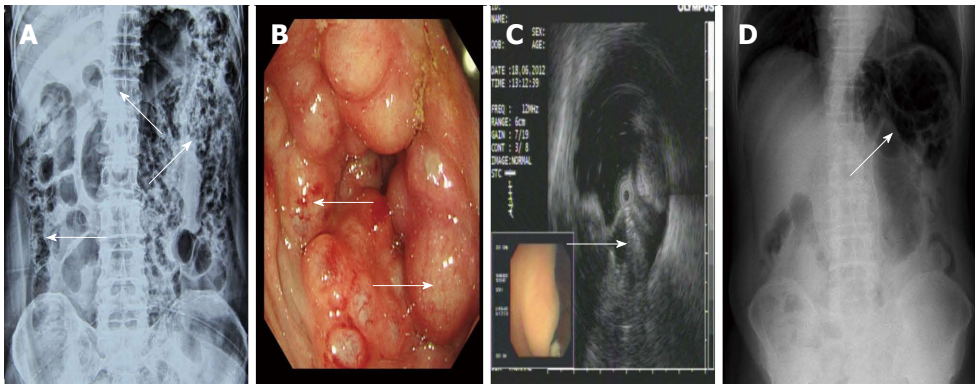

Figure 1 Imaging features of pneumatosis cystoides intestinalis. A: Barium enema study revealing multiple polypoid lesions with air shadows (arrow) and grape-like intramural gas in the whole colon; B: Colonoscopy revealing multiple round and smooth-surfaced elevated lesions (arrow) similar to submucosal tumors in the colon; C: Endoscopic ultrasonography revealing hyperechoic lesions and acoustic shadows in the submucosal layer (arrow); D: Plain radiography of the left upper quadrant abdomen revealing dilatation of the intestine and small linear, round radiolucent areas (arrow) on the clusters in the wall of the colon.

presence of gas within the submucosa or subserosa of the intestine[1,2]. Since it was first described by Du Vernoy[1] in autopsy specimens in 1730 and subsequently named by Mayer as PCI in 1825, it has been reported in various publications. However, after reviewing the literature, we found no epidemiologic studies, no randomized clinical trials, very few case series, and a large number of case reports. Many of the patients underwent misdiagnosis, mistreatment or even surgical exploration[3-5].

Our case report and systematic analysis are based on the Chinese publications to increase the understanding, diagnosis and treatment of PCI and to find the potential cause of the disease in China.

MATERIALS AND METHODS

Materials

A 70-year-old male was admitted for intermittent diarrhea accompanied by abdominal pain and bloody purulent stool for almost 2 years. The abdomen showed no relevant physical findings. Routine biochemical tests, inflammation indices and tumor markers were within normal values. He was diagnosed with hypertension and diabetes 10 years ago. Barium enema (Figure 1A) and colonoscopy (Figure 1B) disclosed multiple submucosal cysts protruding into the lumen of the whole colon. When a cyst was biopsied, it disappeared immediately. Endoscopic ultrasonography revealed gas in the cysts (Figure 1C). PCI was diagnosed. The patient received antibiotics and became asymptomatic with normal bowel movements. However, the diarrhea recurred after 4 mo. The patient then started hyperbaric oxygenation therapy. Unfortunately, he suffered from a hearing disorder and could not tolerate the hyperbaric therapy. Thus, the conservative approach was employed (observation only). Regular follow-up visits half a year later revealed improved clinical and radiological signs of PCI (Figure 1D).

Search strategy

The literature search used the available Chinese databases from May 2002 to May 2012, including CBM, CBMDisc, CMCC, VIP, Wanfang, and CNKI. The key words were pneumatosis cystoides intestinalis, pneumatosis, pneumatosis intestinalis, pneumatosis coli and mucosal gas. The patients' information, histories, therapies, courses, and outcomes were reviewed. Moreover, extended information was collected with regard to the nature and pathophysiology of PCI, and the incomplete reports were removed.

Statistical analysis

All data are presented as the mean ± SE. The demographic characteristics are presented as number (%).

RESULTS

The study group included 77 reports that contained an adequate amount of clinical information on 239 PCI cases (168 male:71 female = 2.4:1). The number of case reports was 62 (80.5%). The mean age was 45.3 ± 15.6 years (range: 2-81 years). The group was nationwide and particularly included high altitude areas and poor areas, including Qinghai, Sinkiang and Gansu (Table 1).

One hundred and four cases (43.5%) had comorbidities that may be related to PCI, with peptic ulcer being the most common concomitant disorder. In addition, 16.3% of the patients had complications including intestinal obstruction and perforation (Table 2).

The illness course from onset to identification ranged from 0 to 20 years, with a median of 6 mo. Overall, 217 cases (90.8%) had symptoms, and the most common symptom was abdominal pain (n = 117, 53.9%), followed by diarrhea (n = 115, 53.0%), distention (n = 92, 42.4%), nausea and vomiting (n = 31, 14.3%), bloody stool (n = 28, 12.9%), mucous stool (n = 26, 12.0%) and constipation (n = 17, 7.8%) (Figure 2A). PCI was most frequently diagnosed by colonoscopy (51.9%, 124/239), followed by surgery (40.6%, 97/239) and X-ray (10.9%, 26/239) (Figure 2B). The primary involved site was the colon, followed by the small intestine, especially the descending

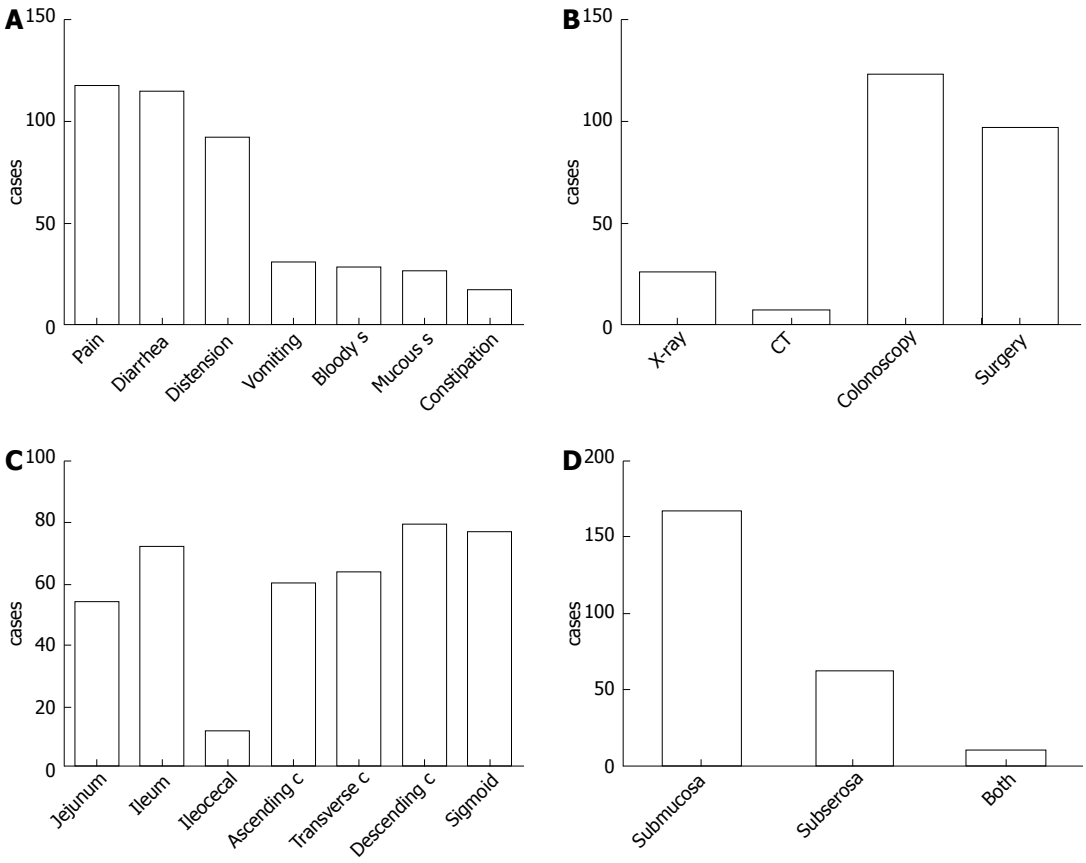

Figure 2 Clinical information of all 217 cases. A: The chief complaints; B: The methods of diagnosis; C: The primary involved site; D: The localization of gas in the intestinal wall. S: Stool; C: Colon; CT: Computed tomography.

colon (*n* = 79, 33.1%), sigmoid (*n* = 77, 32.2%) and ileum (*n* = 72, 30.1%) (Figure 2C). The majority of the cysts were found in the submucosa (69.9%, 167/239). Only 11 (4.6%) patients had both submucosa and subserosa involvement (Figure 2D).

The management of PCI included antibiotics, oxygen therapy, endoscopic therapy, surgery and the conservative approach. The efficiency of the conservative treatment reached up to 93.3% (Table 3). During the follow-up, which ranged from 1 mo to 20 years (median, 1 year), no symptoms recurred.

DISCUSSION

PCI is a rare disease and is still poorly understood. In a retrospective review of PCI, Koss[6] found a 3.5:1 male-to-female ratio of the occurrence of PCI in an age group of 30-50 years, and Jamart[7] found a 3:1 male-to-female ratio (aged from 41-50 years old) of the occurrence of PCI. However, both of these old reports contained few patients. A prospective study by Knechtle *et al*[8] showed equal incidence among males and females. PCI was previously thought to occur most frequently in the small intestine, but in recent barium enemas and colonoscopies studies, PCI has been reported to more commonly affect the colon. Most older studies showed PCI to occur more commonly in the small intestine. However, Horiuchi *et al*[9] showed that PCI appeared more commonly in the colon (61.8%) of females (mean age 55.4 years), followed by the small intestine (15.4%). Recently, Morris *et al*[10] showed the incidence of PCI was 46% in the colon; 27% in the small intestine, only 7% in the colon and small intestine combined. In contrast to previous reports using different ethnic cohorts, PCI in the patients in this study (Chinese cohort) showed a male-to-female ratio of 2.4:1 and a mean age of 45.3 ± 15.6 years. The most frequent location of PCI was the colon instead of the small bowel (rate of 1.3:1), with only 2.9% (7/239) of the cases being combined colon and small intestine. The most common localization of gas was in the submucosa (69.9%) (Figure 1).

PCI has been associated with a wide variety of underlining etiologies to explain the abnormal accumulation of gas[11-23]. There are five major theories: (1) The mechanical theory: Intestinal obstruction, inflammatory bowel disease, ischemic bowel disease, gastroenteric tumor, anorectal surgery, bowel preparation or colonoscopy resulting in intestinal wall injury or increased intraluminal pressure serve as the driving force in PCI that causes the intramural gas[14,15]. However, this theory cannot explain how the cysts are maintained once they have formed; (2) The pulmonary theory: Pulmonary diseases, such as chronic obstructive pulmonary disease, asthma, and interstitial pneumonia, may result in pulmonary alveolar rupture

Table 1 Geographical distribution of pneumatosis cystoides intestinalis in China *n* (%)

Province	Mean altitude (m)	Cases
Qinghai	4000	92 (38.5)
Sinkiang	2000	28 (11.7)
Beijing	50	21 (8.8)
Gansu	3000	17 (7.1)
Shanghai	4	10 (4.2)
Yunnan	1500	8 (3.3)
Shanxi	1000	8 (3.3)
Henan	1500	7 (2.9)
Tianjin	5	7 (2.9)
Sichuan	500	7 (2.9)
Tibet	4000	7 (2.9)
Shandong	1500	4 (1.7)
Shaanxi	1000	4 (1.7)
Zhejiang	50	3 (1.3)
Jiangxi	50	2 (0.8)
Guangdong	100	2 (0.8)
Kiangsu	50	2 (0.8)
Hainan	200	2 (0.8)
Heilongjiang	200	2 (0.8)
Jilin	800	2 (0.8)
Liaoning	500	2 (0.8)
Inner mongolia	1000	1 (0.4)
Hebei	400	1 (0.4)

Table 2 Pneumatosis cystoids intestinalis concomitant diseases and pneumatosis cystoids intestinalis complications *n* (%)

Total	*n* = 239
PCI concomitant diseases	104 (43.5)
Pyloric obstruction	31 (29.8)
Duodenal ulcer	18 (17.3)
Gastric ulcer	17 (16.3)
Pulmonary diseases	15 (14.4)
Intestinal diseases	17 (16.3)
Abdominal external injury or surgery	10 (9.6)
Malnutrition	27 (26.0)
Connective tissue disease	3 (2.9)
Diabetes mellitus	5 (4.8)
Hypertension	3 (2.9)
PCI complications	39 (16.3)
Intestinal obstruction	20 (51.3)
Intestinal perforation	14 (35.9)
Atypical hyperplasia and canceration	4 (10.2)
Intussusception and intestinal necrosis	1 (2.6)

PCI: Pneumatosis cystoids intestinalis.

and then produce a pneumomediastinum that dissects along the aorta and then along the mesenteric vessels to the bowel wall. However, this theory alone also fails to account for the finding that hydrogen, a gas never produced by mammalian cells, may comprise up to 50% of the gas content of the cysts[16]; (3) The bacterial theory: The gas is produced by gas-forming bacteria that enter the mucosal barrier through mucosal rents or increased mucosal permeability and produce the gas within the bowel wall. Indirect support for this theory was obtained by the successful treatment of PCI with antibiotics. However, the presence of aerogenic bacteria in the cysts has not yet been proven. Although Yale *et al*[17] reported that pneumatosis has been produced in germ-free rats by the injection of Clostridium species into the wall of the intestine, the isolation and cultivation of these organisms is rarely possible. Conversely, many of the patients who have pneumoperitoneum resulting from the rupture of cysts show no signs of peritonitis, prompting the theory that in this population, the gas is not caused by bacteria; (4) The chemical theory or nutritional deficiency theory: Malnutrition can prevent the digestion of carbohydrates and increased bacterial fermentation in the intestine, producing large volumes of gas leading to distention and ischemia and subsequently the submucosal dissection of gas. Recently, the development of PCI during the treatment with α-glucosidase inhibitors (α-GI) has been reported. The cessation of α-GI therapy is the key to the successful treatment of PCI[18,19], which supports the fourth theory; and (5) There have been some recent reports on PCI associated with chemotherapy, hormonal therapy and connective tissue disease that are not generally accepted[20-23].

Although many theories exist to explain the etiology and pathogenesis of PCI, no theory can explain the entire pathologic processes. Our experiments showed that many patients accompanied with pyloric obstruction, peptic ulcer, malnutrition and pulmonary diseases may support the theories of mechanical, pulmonary and nutritional deficiency (Table 2). Moreover, we also found that many of the patients came from highland areas, such as Qinghai, Sinkiang and Gansu (Table 1). The passage of intraluminal gas into the submucosa requires damage to the mucosa, which might be possible in these geographic areas. Further studies should be performed in the highland areas.

Although there are many symptoms of PCI, including abdominal pain, abdominal distention, diarrhea, mucous stool and bloody stool, none is disease specific (Figure 2A). The cysts may cause obstruction by internal or external compression of the bowel lumen when the cysts become larger. Complications associated with PCI occur in approximately 16.3% of cases and include intestinal obstruction or intestinal perforation (Table 2).

Approximately 85% of cases fall under secondary PCI[6], which results from other diseases. In these cases, the main symptoms and also the main treatments are related to the primary disease. Thus, some scholars do not think PCI is a disease by itself but rather a secondary manifestation of the reaction of the body to a variety of conditions; therefore, they believe that it does not have a single etiology[24].

In general, the diagnosis of PCI is based on endoscopy or plain radiography of the abdomen and is usually not difficult because the typical radiolucency appears as grape-like clusters or honeycomb-shaped shadows along the wall of the intestine. After the identification of PCI, a prompt further evaluation, including concomitant radiographic findings, of the patient should be conducted. Although only a few patients were diagnosed through an abdominal CT scan in our study, CT is a useful method for diagnosing PCI and is important because it provides

Table 3 Pneumatosis cystoids intestinalis therapies and their efficiency *n* (%)

Methods	*n*	Efficiency
Antibiotics	19	5 (26.3)
Oxygen	41	28 (68.3)
Endoscopy	12	12 (100)
Surgery	97	97 (100)
Conservative	15	14 (93.3)
Antibiotics + Oxygen	1	1 (100)
Oxygen + Endoscopic	53	53 (100)
Oxygen + Surgery	6	6 (100)

data on other abdominal pathologies[25-27]. Radiographic signs of bowel perforation or peritonitis and endoscopic signs of a tumor often indicate the need for emergency surgery[28]. Using two different preoperative imaging modalities to make a precise diagnosis is necessary.

The appropriate therapy is related to the underlying cause of PCI. The majority of patients (93.3%, Table 3) without pronounced symptoms were cured without any treatments. If the symptoms are pronounced, a conservative approach to treatment is allowed, such as gastrointestinal decompression, intestinal "rest", parenteral nutrition, and fluid and electrolyte supplementation. However, in contrast to the case reports, we found the efficiency of treatment by antibiotics was only 26.3%. The most efficient treatment was therapeutic alliance. Although oxygen was first used by Forgacs *et al*[16] in 1973, the optimal concentration, duration and effect of oxygen have not been established. The application of 200-300 mmHg PO_2 pressure for 1.5-2.5 h/d for 2-14 d or 55%-75% oxygen inhalation for 1-3 h/d for 2-5 d was suggested to lead to gas absorption within the cysts. Surgery is reserved either for cases of suspected inconvertible intestinal obstruction or perforation or cases with precancerous conditions[29]. The extremely high rate of surgical resection in China (40.6%) is associated with the lack of realization and the misdiagnosis of PCI as many cases can recover with non-operative management. Therefore, diagnosing PCI as early as possible and providing fast and adequate therapy to treat PCI are extremely important[30,31].

In conclusion, after recognizing the disease for almost three centuries, PCI is still a poorly understood phenomenon. Several theories explaining PCI and the variety of treatments reflect the lack of knowledge regarding the underlying pathophysiology. A long-term follow-up study is suggested to evaluate the long-term outcome of these therapies.

COMMENTS

Background

Pneumatosis cystoides intestinalis (PCI) is a rare disease characterized by the presence of multiple gas-filled cysts in the submucosa and/or subserosa of the intestinal wall. PCI is still a poorly understood entity, and nearly all of the studies for PCI are case reports.

Research frontiers

PCI is a rare disease that has not been unequivocally addressed. In this study, the authors systematically evaluated and demonstrated for the first time the characteristics of PCI patients in China.

Innovations and breakthroughs

For the first time, the authors determined the prevalence and the characteristics of PCI in Chinese people.

Applications

The study results suggest that PCI in Chinese patients presents in the large bowel more often than in the small intestine and more frequently in middle-aged males than in females. The majority of the cysts are found in the submucosa rather than the subserosa. High altitude residence may be associated with the PCI etiology. The majority of PCI can be safely managed by conservative treatment.

Peer review

The authors review PCI in the published literature from China and report on 239 cases. Stats on geography and altitude is hare to interpret - as population density may be more important - would incidence per 100000 population at high or low altitude be more helpful.

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BRIEF ARTICLE

Appropriate treatment of acute sigmoid volvulus in the emergency setting

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Abstract

AIM: To investigate an appropriate strategy for the treatment of patients with acute sigmoid volvulus in the emergency setting.

METHODS: A retrospective review of 28 patients with acute sigmoid volvulus treated in the Department of Colorectal Surgery, Changhai Hospital, Shanghai from January 2001 to July 2012 was performed. Following the diagnosis of acute sigmoid volvulus, an initial colonoscopic approach was adopted if there was no evidence of diffuse peritonitis.

RESULTS: Of the 28 patients with acute sigmoid volvulus, 19 (67.9%) were male and 9 (32.1%) were female. Their mean age was 63.1 ± 22.9 years (range, 21-93 years). Six (21.4%) patients had a history of abdominal surgery, and 17 (60.7%) patients had a history of constipation. Abdominal radiography or computed tomography was performed in all patients. Colonoscopic detorsion was performed in all 28 patients with a success rate of 92.8% (26/28). Emergency surgery was required in the other two patients. Of the 26 successfully treated patients, seven (26.9%) had recurrent volvulus.

CONCLUSION: Colonoscopy is the primary emergency treatment of choice in uncomplicated acute sigmoid volvulus. Emergency surgery is only for patients in whom nonoperative treatment is unsuccessful, or in those with peritonitis.

Key words: Sigmoid colon; Volvulus; Emergency; Colonoscopy

Core tip: Early and correct diagnosis of acute sigmoid volvulus is essential for appropriate treatment aimed at correcting abnormal pathophysiological changes and restoring intestinal transit caused by the volvulus. There is still much debate as to the ideal management of sigmoid volvulus. The results of this study suggest that colonoscopic decompression and derotation is the primary emergency treatment of choice in uncomplicated acute sigmoid volvulus and is a safe treatment modality for recurrent sigmoid volvulus. Emergency surgery is required for patients in whom nonoperative treatment is unsuccessful, or in those with peritonitis, bowel gangrene or perforation.

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INTRODUCTION

Acute sigmoid volvulus is defined as torsion of the sigmoid colon around its mesenteric axis, which leads to

acute large intestine obstruction, which, if left untreated, often results in life-threatening complications, such as bowel ischemia, gangrene, and perforation[1,2]. Early and correct diagnosis of this disease is essential for appropriate treatment aimed at correcting abnormal pathophysiological changes and restoring intestinal transit caused by the volvulus.

Despite significant progress in the treatment of this disease, no consensus has been reached[3-8]. Emergency surgery is the appropriate treatment for those who present with diffuse peritonitis, intestinal perforation or ischemic necrosis[9,10]. Nonoperative treatment is adopted if there is no evidence of these conditions. Barium enema, rectal tubes, rigid and flexible sigmoidoscopy as therapeutic methods have been adopted by clinicians[11,12]. Colonoscopy, besides being a therapeutic measure, allows the evaluation of colonic mucosa and therefore the presence or absence of signs of ischemia[13], and is effective in more than 70% of patients[14]. The initial management of clinically stable patients in good general condition with sigmoid volvulus is colonoscopic decompression as a first therapeutic option in the emergency setting in our hospital. The objectives of this study were to review our experience and the benefits of colonoscopy in the treatment of patients with acute sigmoid volvulus in the emergency setting.

MATERIALS AND METHODS

We performed a retrospective clinical data review of patients with acute sigmoid volvulus treated in the Department of Colorectal Surgery, Changhai Hospital, Shanghai, China, from January 2001 to July 2012. We included 28 patients who were diagnosed with acute sigmoid volvulus in the emergency department and then admitted to our department for treatment. Patient data included demographics, comorbidities, clinical manifestations, radiological investigations, colonoscopic findings and interventions, and clinical outcome.

Following the diagnosis of acute sigmoid volvulus, an initial colonoscopic approach was adopted if there was no evidence of diffuse peritonitis. If patients failed in the colonoscopic derotation, surgical intervention would be performed. Figure 1 illustrates the flowchart of patients who were admitted to our hospital with acute sigmoid volvulus.

RESULTS

Of the 28 patients with acute sigmoid volvulus, 19 (67.9%) were male and 9 (32.1%) were female. The mean age was 63.1 ± 22.9 years (range, 21-93 years). Six patients (21.4%) had a history of abdominal surgery. Seventeen patients (60.7%) had a history of constipation.

All patients presented with acute large intestine obstruction. The interval between the development of symptoms and hospitalization ranged from 15 h to 7 d (mean 37.3 ± 28.2 h). Clinical manifestations included abdominal pain in all patients (100%) and vomiting in 8 patients (28.6%). Abdominal examination revealed marked abdominal distension in 20 patients (71.4%). A visible intestinal loop was noted in 8 patients (28.6%). Following rectal examination, blood on the examining finger was absent in all patients (0%).

During the diagnostic period, all 28 patients underwent plain abdominal X-rays (100%), followed by computed tomography (CT) scan of the abdomen in 20 (71.4%). Blood chemistry and hematological profile were routinely studied. Abdominal radiographs showed multiple air/fluid levels and a dilated sigmoid colon in all patients (Figure 2). All patients were found to have positive sigmoid volvulus findings by CT scanning, such as a dilated sigmoid colon and a whirl pattern in the mesentery (Figure 3).

After early and effective resuscitation, colonoscopy was performed in all patients in order to complete decompression and derotation, and was successful in 26 (92.9%) patients. Emergency surgery was performed in a 41-year-old woman in whom colonoscopic derotation was unsuccessful. A 90-year-old man who did not receive derotation underwent immediate surgery due to a perforation with inlaid adipose tissue 50 cm from the edge of the anus (Figure 4).

There was no mortality or morbidity in the colonoscopic treatment group. One of two patients in the surgical treatment group died. Exploration revealed a 3-cm perforation in the sigmoid colon with omentum adhesion, and a dilated, dusky segment of the descending and transverse colon in this patient. Left hemicolectomy and end colostomy were performed. Postoperatively, he was treated in the intensive care unit but died 1 day later due to sepsis with progressive multiple organ failure.

The overall recurrence rate was 26.9% (7/26) after colonoscopic derotation. These patients underwent repeat colonoscopic detorsion without mortality or morbidity.

DISCUSSION

Acute sigmoid volvulus is the third most common cause of large bowel obstruction[15]. It has a wide geographic variation and it differs significantly between high-incidence countries and low-incidence countries[16]. This variation may be associated with differences in anatomy[17]. Acute sigmoid volvulus usually occurs in adult men. The mean age was found to be between 56 and 77 years and nearly one-third of all colonic emergencies in elderly patients are due to sigmoid volvulus[18]. In our patient group, age ranged from 21 to 93 years with a mean of 63.1 ± 22.9 years which showed that the Chinese population with acute sigmoid volvulus also included many younger patients. A preponderance in males compared with females was found and the ratio in our group was 2.1:1 (19 *vs* 9). This indicated that male preponderance in acute sigmoid volvulus is pronounced in the Chinese population.

The presence of a redundant and mobile sigmoid co-

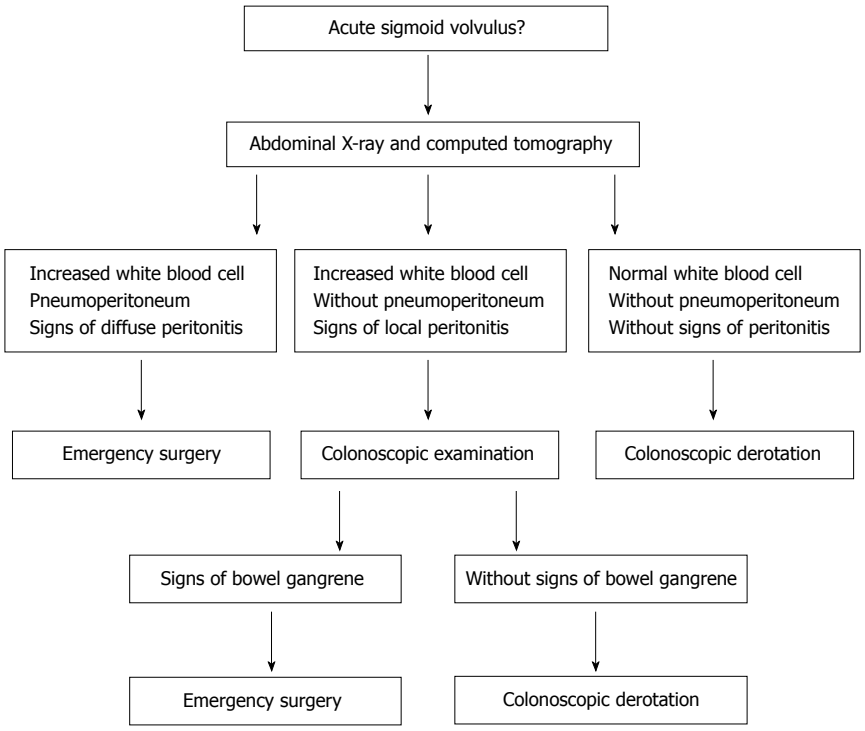

Figure 1 Flowchart of emergency therapeutic strategy for acute sigmoid volvulus.

lon, with a narrow base at the mesenteric root, is one of the major predisposing factors for sigmoid volvulus. Other predisposing factors, such as a high-fiber diet, constipation, previous abdominal surgery, pregnancy, diabetes, or neurological and psychiatric diseases such as dementia or schizophrenia have been described in the literature[19]. In our group, 60.7% of the patients had a history of chronic constipation, and over 15% suffered from diabetes or neurological diseases, and 21.4% of patients had a history of previous abdominal surgery.

The diagnosis of acute sigmoid volvulus is established by clinical and radiological findings. In the majority of patients, a thorough physical examination and abdominal radiographs are adequate to achieve the diagnosis. Typical symptoms include sudden abdominal pain and distension followed by constipation. The most common signs are abdominal tenderness and asymmetrical abdominal distention. Other findings include abnormal bowel sounds, abdominal tympany, a palpable abdominal mass, empty rectum, and dehydration[18]. Plain radiographs are diagnostic in 57%-90% of patients[20,21]. The classical sign of acute sigmoid volvulus is the coffee bean sign. Abdominal CT usually reveals a dilated colon with an air/fluid level and the "whirl sign", which represents twisted colon and mesentery[22].

Raveenthiran *et al*[23] recently provided more insight into the pathophysiology of acute sigmoid volvulus. Increasing intraluminal pressure impairs capillary perfusion following the occurrence of acute sigmoid volvulus. Mechanical obstruction due to twisting of mesenteric vessels and thrombosis of mesosigmoid veins contribute to ischemia. Ischemic injury in the mucosa occurs earlier than in other colonic layers and facilitates bacterial translocation and toxemia. A competent ileo-cecal valve converts the proximal colon into a second "closed loop". Increased intra-abdominal pressure results in the "abdominal compartment syndrome". Prompt and optimal correction of these pathophysiological features is vital to improve the prognosis of acute sigmoid volvulus.

The treatment of colonic volvulus remains controversial, and depends on the elected procedure and the most appropriate therapeutic approach in terms of the clinical status of the patient, the location of the problem, the suspicion or presence of peritonitis, bowel viability and the experience of the surgical team[19].

Emergency surgery is associated with significant mortality and morbidity. Kassi *et al*[24] reported that the mortality rate was 12% (n = 3) for Hartmann's procedure. Surgical site infections (42.86%) were the most common complications. Eleven (50%) of 22 patients had intestinal continuity restored. Bhatnagar *et al*[25] reported that the risk factors for mortality were: (1) age over 60 years; (2) presence of shock on admission; and (3) positive history of a previous episode of volvulus. With regard to the former two risk factors, special efforts are necessary by intensive care staff to monitor homeostatic disturbance and reduce mortality in older patients (> 60 years) and those presenting with shock at the time of admission. One of our patients died due to sepsis with progressive multiple organ failure on postoperative day 1.

Nonoperative detorsion is advocated as the primary treatment choice in uncomplicated acute sigmoid volvulus. Although rectal tubes, barium enemas or rigid sigmoidoscopy have been widely used, flexible sigmoid-

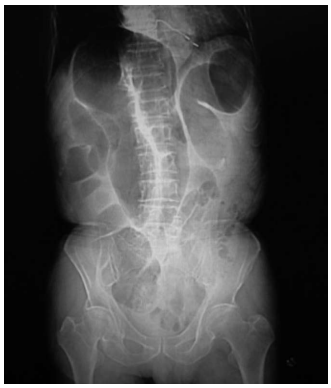

Figure 2 Plain abdominal X-ray film obtained in the supine position reveals gross dilatation of the colon.

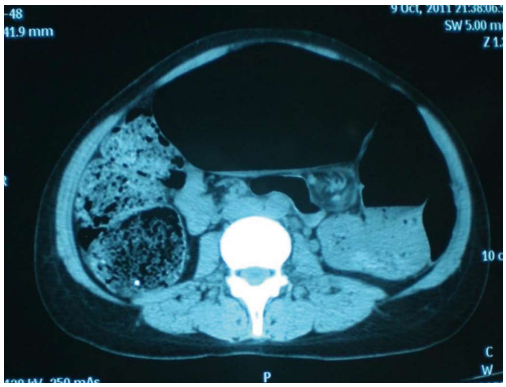

Figure 3 Computed tomography reveals dilated colon with an air/fluid level, as well as the "whirl sign" composed of mesentery and twisted colon.

oscopy is now the preferred nonoperative procedure. Nonoperative treatment is successful in 70%-91% of cases, with reported complication rates of 2%-4.7% in geriatric patients[26,27]. However, in our patients, the success rate of colonoscopic derotation was 92.9%. Compared with sigmoidoscopy, colonoscopy is readily used in our hospital for endoscopic derotation for several reasons. In addition to a superior success rate and safety profile, it is of adequate length to reach beyond the second constricting point. It also allows better visualization of the colonic mucosa and can guide the decompression and derotation procedure. Colonoscopic suctioning of the proximal colon facilitates quick recovery by removing bacterial toxins. Furthermore, the authors adopt modified double-operating colonoscopy which can help minimize the risk of perforation. Colonoscopic derotation simply converts an emergency into an elective procedure, which facilitates treatment of comorbidity and allows preparation of the bowel prior to definitive surgery. The results from the present study suggest that colonoscopy is a safe and effective treatment modality for acute sigmoid volvulus.

Following derotation, ischemia-reperfusion injury aggravates intestinal dysfunction, and even intestinal ulcer and perforation. Peritoneal exudate, high intestinal fluid accumulation, electrolyte disturbances, and hypoproteinemia lead to serious adverse consequences. Consequently,

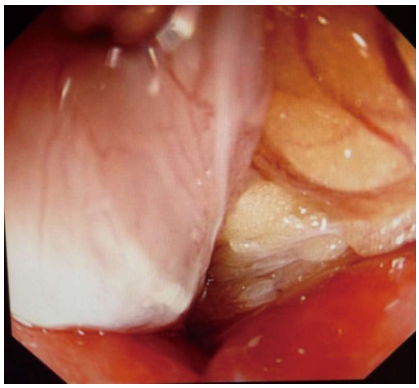

Figure 4 Colonoscopic examination shows a perforation with inlaid adipose tissue.

effective treatment following colonoscopic derotation is very important. Fluid resuscitation should be performed immediately. Vasodilator therapy should be continued, as the use of these agents can ameliorate intestinal tissue microcirculation. Broad-spectrum antibiotics are indicated to control bacterial translocation across the ischemic intestinal wall.

Colonoscopic derotation was followed by recurrence in 26.9% of our patients. These patients underwent successful repeat colonoscopic detorsion without mortality or morbidity. These results suggest that colonoscopy is the primary choice in the treatment of recurrent sigmoid volvulus, particularly in elderly patients who refuse elective surgery.

In conclusion, colonoscopic decompression and derotation is the primary emergency treatment of choice in uncomplicated acute sigmoid volvulus and is a safe treatment modality for recurrent sigmoid volvulus. Emergency surgery is reserved for gangrene and failed decompression, and in patients with a high recurrence rate it may be prudent to consider interval semi-elective resection and primary anastomosis several days after successful decompression.

COMMENTS

Background

Acute sigmoid volvulus is common worldwide, and leads to acute large intestine obstruction, which, if left untreated, often results in life-threatening complications, such as bowel ischemia, gangrene, and perforation. Despite significant progress in the treatment of this disease, no consensus has been reached.

Research frontiers

As the authors described, there is still considerable debate on the ideal management of acute sigmoid volvulus. In this study, the authors demonstrated that colonoscopy is the primary emergency treatment of choice in uncomplicated acute sigmoid volvulus. Surgery is reserved for patients in whom nonoperative treatment is unsuccessful, or in those with peritonitis.

Innovations and breakthroughs

Barium enema, rectal tubes, rigid and flexible sigmoidoscopy have been adopted by clinicians as therapeutic methods. Colonoscopy, besides being a therapeutic measure, allows evaluation of the colonic mucosa and therefore the presence or absence of signs of ischemia, and is effective in more than 70% of patients. The initial management of clinically stable patients in good general condition with sigmoid volvulus is colonoscopic decompression as a first therapeutic option with satisfactory results in the emergency setting.

Applications

By understanding abnormal pathophysiological changes associated with acute sigmoid volvulus, this study may represent a future strategy for therapeutic intervention in the treatment of this condition.

Terminology

Acute sigmoid volvulus is defined as torsion of the sigmoid colon around its mesenteric axis, which leads to acute large intestine obstruction, which, if left untreated, often results in life-threatening complications, such as bowel ischemia, gangrene, and perforation.

Peer review

The authors present a well-written case series describing their experience with sigmoid volvulus. There is still much debate as to the ideal management for this condition. Emergency surgery is reserved for gangrene and failed decompression, and due to a high recurrence rate, it may be prudent to consider interval semi-elective resection and primary anastomosis several days after successful decompression.

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BRIEF ARTICLE

Prevalence of minimal hepatic encephalopathy and quality of life evaluations in hospitalized cirrhotic patients in China

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Abstract

AIM: To investigate the prevalence of minimal hepatic encephalopathy (MHE) and to assess corresponding health-related quality of life (HRQoL) in hospitalized cirrhotic patients in China.

METHODS: This multi-center cross-sectional study included 16 teaching hospitals, which were members of "Hepatobiliary Cooperation Group, Society of Gastroenterology, Chinese Medical Association", from different areas of China carried out between June and October in 2011. All the eligible hospitalized cirrhotic patients (n = 538) were required to complete triplicate number connection tests combined with one digit symbol test

for diagnosing MHE. Patients' clinical examination data were complemented by a modified questionnaire assessing HRQoL. Written informed consent was obtained from each patient.

RESULTS: Male was predominant (68.6%) in 519 patients who met the criteria of the study, with a mean age of 49.17 ± 11.02 years. The most common cause of liver cirrhosis was chronic hepatitis B (55.9%). The prevalence of MHE was 39.9% and varied by Child-Pugh-Classification score (CPC-A: 24.8%, CPC-B: 39.4% and CPC-C: 56.1%, $P < 0.01$). MHE ($P < 0.01$) and higher CPC scores ($P < 0.01$) were associated with a high HRQoL scores (reflecting poorer quality of life). The prevalence of MHE was proportionate to CPC ($P = 0.01$) and high quality of life scores ($P = 0.01$).

CONCLUSION: Hospitalized cirrhotic patients have a high prevalence of MHE that is proportionate to the degree of liver function and HRQoL impairment.

Key words: Minimal hepatic encephalopathy; Health-related quality of life; China; Child-Pugh Classification; Liver cirrhosis

Core tip: This study showed that 39.9% of hospitalized patients with liver cirrhosis had minimal hepatic encephalopathy (MHE), and patients with Child-Pugh Classification-C had a high prevalence of MHE (56.1%) and increased health-related quality of life scores that reflected poorer life status. Increasing awareness of its adverse impact on life should be emphasized. Recommendations to screen for MHE may be applicable for evaluating the risks of driving and work accidents in patients with cirrhosis.

Wang JY, Zhang NP, Chi BR, Mi YQ, Meng LN, Liu YD, Wang JB, Jiang HX, Yang JH, Xu Y, Li X, Xu JM, Zhang G, Zhou XM, Zhuge YZ, Tian DA, Ye J, Liu YL. Prevalence of minimal hepatic encephalopathy and quality of life evaluations in hospitalized cirrhotic patients in China. *World J Gastroenterol* 2013; 19(30): 4984-4991 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/4984.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.4984

INTRODUCTION

Hepatic encephalopathy (HE) is a serious complication of liver cirrhosis that represents a continuous spectrum of neurologic and neuropsychiatric abnormalities[1,2]. Minimal HE (MHE), the mildest form of HE[1,2], is defined as patients with normal mental and neurological examinations but with a number of neuropsychiatric and neuro-physiological defects identified by psychometric tests[3]. Patients with MHE have various subtle abnormalities in the cognitive functioning that detrimentally affects their fitness to drive[4] and handle complex mechanical machines[5]. In a study by Prasad *et al*[6], significant impairment was observed in HE patients' social interactions, alertness, emotional behavior, sleep, work, household management, recreation, and pastimes.

There are several methods of diagnosing MHE, including comprehensive neuropsychological examinations, standard psychometric batteries, neuro-physiological testing, and computerized testing[3]. However, there are no current guidelines for the standardized diagnosis of MHE. The Working Group on HE recommended that at least two of the following neuropsychologic tests should be used for diagnosing MHE: number connection test-A (NCT-A), NCT-B, block-design test (BDT), and the digit-symbol test (DST)[7]. The current definition of MHE is based on psychometric test results that are two SD more than normal on at least two psychometric tests. As there is no gold standard for diagnosis of MHE, the prevalence of MHE in patients with cirrhosis ranges from 30% to 80%[8-11]. Recently, the estimated prevalence of MHE varied from 29.2% to 57.1% in China[12-14]. Some studies only employed one neuropsychologic tests (NCT) for MHE diagnosis. Therefore, the exact prevalence of MHE is unknown in China.

MHE is associated with potential progression to HE, diminished quality of life, driving impairment that increases the risk of traffic accidents, and negative health-related quality of life (HRQoL)[15-17]. Quality of life (QoL) is a multidimensional index that comprehensively addresses all aspects of human well-being, including physical and cognitive capabilities, functional behavior, emotional status, and psychosocial adjustment. As compared with generic measures of impairment, disease-specific measures are more likely to be sensitive to small, yet clinically meaningful, differences in HRQoL. Our group[18] developed and verified a reliable and valid HRQoL instrument that measures the functional and health status of patients with MHE. That study also demonstrated that HRQoL in patients with MHE deteriorates as the disease becomes more severe, although the study had a small sample size and a limited regional scope.

This study investigated the prevalence of MHE in hospitalized cirrhotic patients from different areas of China, and HRQoL evaluations among them.

MATERIALS AND METHODS

Study population

A multi-center cross-sectional study was initiated by the Hepatobiliary Cooperation Group of Society of Gastroenterology, the Chinese Medical Association. The study was conducted in 16 teaching hospitals representing different areas of China (4 in the East, 3 in the West, 1 in the South, 4 in the North and 4 in the central region). All consecutive cirrhotic hospitalized patients aged between 18 and 70 years and without overt HE (OHE) were screened for MHE between June and October 2011. Cirrhosis was diagnosed based on available clinical data,

including laboratory tests, endoscopy, diagnostic imaging, or liver histology. Exclusion criteria included the presence or a history of OHE, a history of taking lactulose or any antibiotics, alcohol intake, gastrointestinal hemorrhage, or spontaneous bacterial peritonitis during the previous 6 wk, significant concurrent diseases such as heart, respiratory, or renal failure, and neurologic abnormalities such as Alzheimer's disease, Parkinson's disease, non-hepatic metabolic encephalopathy, electrolyte disorders, inability to perform psychometric tests or complete the questionnaire (caused by either insufficient knowledge of the Chinese language or poor vision). All patients provided written informed consent. Study protocols were approved by the ethics committees of the participating hospitals in accordance with the Principles of Declaration of Helsinki.

Physical examination, laboratory testing, and medical history documentation

Qualified physicians documented routine physical examinations and laboratory assessments that included biochemical tests (alanine aminotransferase, aspartate aminotransferase, bilirubin, albumin, creatinine, prothrombin time, serum potassium, serum sodium, and serum chloride), virological tests [hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), anti-HBe, anti-hepatitis C virus (HCV), hepatitis B virus (HBV) DNA levels, and HCV RNA levels], and diagnostic imaging [ultrasonography, computer tomography (CT), or magnetic resonance imaging], etiology of cirrhosis, a history of medication use and other medical histories. The Child-Pugh-Classification (CPC) scoring system was used to assess the severity of liver disease.

Psychometric testing

All patients underwent a series of psychometric tests, including triplicate NCT-A and one DST. The NCT-A measures cognitive motor abilities by having patients connect numbers, from 1 to 25 on printed paper, as quickly as possible. DST: Subjects are asked to insert symbols in the blank squares below the numbers using the key provided. The exercise is timed and the number correctly completed in 90 seconds recorded. Bao *et al*[19] established age-based normal parameters of psychometric measures for NCT-A and DST in China in 2006. Normative values for NCT-A and DST were based on those from healthy volunteers with the same geographical background as liver cirrhosis patients.

According to the normative parameters for NCT-A and DST established by Bao *et al*, diagnostic criteria for MHE were as follows: time greater than two SD from the mean for the NCT, and score less than two SD from the mean for the DST. For the NCT-A, diagnostic criteria were: > 34.3 s in patients aged < 35 years; > 45.7 s in patients aged 35-44 years; > 52.8 s in patients aged 45-54 years and > 61.9 s in patients aged > 55 years. Diagnostic criteria for the DST were: < 40.5 in patients < 35 years; < 35 in patients aged 35-44 years; < 28.5 in patients aged 45-54 years and < 26 in patients aged > 55 years. Patients with abnormal results from both psychometric tests were diagnosed as having MHE.

Assessment of HRQoL

A modified Chinese QoL questionnaire with 30 questions verified in Chinese populations in 2009 was used to assess all patients' HRQoL index[18] The domains included physical functioning (8 questions), psychological well-being (7 questions), symptoms/side effects (7 questions), social functioning (4 questions), and self-evaluation about general health (4 questions). Impact scores for each question ranged from 1 to 5. These scores increase as the QoL declines. The total QoL scores were obtained from the sum of each question's score.

Statistical analysis

Continuous variables were expressed as mean ± SD or median (range), where appropriate. Categorical variables were described as the number and proportion of each category. In order to determine relevant risk factors for MHE occurrence, characteristics such as age, gender, pre-existing ascites, variceal bleeding, occupation, driving, alcohol drinking, hepatitis B antigen status, and antiviral therapy for HBV-related cirrhosis were included in the univariate analysis. The χ^2 or Fisher's exact test was used for categorical variables, and the Mann-Whitney *U* test or analysis of variance (ANOVA), was performed as appropriate to determine associations for continuous data. All tested variables with *P* values < 0.5 were entered into logistic regression. All statistical testing was two-tailed at the 5% level. Software used for analysis was Statistical Package for Social Science (SPSS, version 14.0, SPSS Inc. Chicago, IL, United States) and Science Analysis Software (SAS, version 9.13; SAS Institute Inc., Cary, NC, United States).

RESULTS

Study patients

Of the patients screened (n = 538), 519 patients met the study's criteria for inclusion. Excluded patients included those who were older than 70 years (n = 7) and who did not complete all psychometric tests (n = 12). Liver cirrhosis was diagnosed based on diagnostic imaging (ultrasonography, CT and/or magnetic resonance imaging), histopathology, endoscopy, or clinical and laboratory data. Most patients (n = 356, 68.6%) were male with a mean age of 49.17 ± 11.02 years, and 100 (20.3%) of them had college degrees or higher levels of education. Included patients were diagnosed with liver cirrhosis for 2.54 ± 3.16 years. The most common causes of liver cirrhosis were chronic hepatitis B (CHB) (55.7%), followed by CHB accompanied by alcoholic liver disease (ALD) (11.8%), ALD (8.9%), chronic hepatitis C (CHC) (7.3%), and autoimmune hepatitis (AIH) (7.5%). According to the CPC scoring system, 161 (31.0%) patients were classified as CPC-A, 203 (39.1%) were as CPC-B, and 155 (29.9%) as CPC-C. Of 320 cirrhotic patients with CHB, 41.6% (n = 133) were HBeAg positive, 85.0% (n = 272)

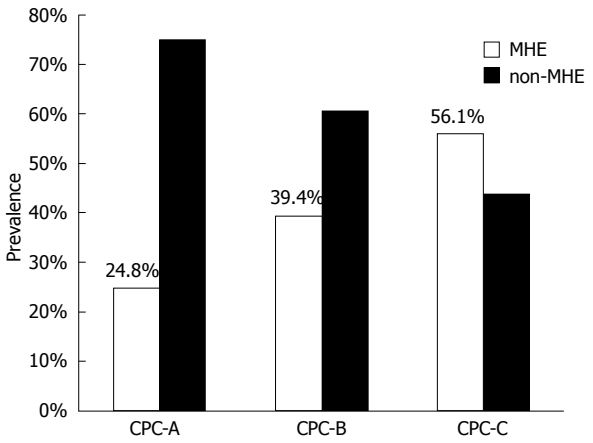

Figure 1 Prevalence of minimal hepatic encephalopathy for various Child-Pugh classes. $P < 0.01$ between minimal hepatic encephalopathy (MHE) and non-MHE. CPC: Child-Pugh classes.

were HBV DNA positive, and 202 (60.3%) had received antiviral treatment.

Prevalence and characteristics of MHE

Cirrhotic patients with concurrent positive NCT and DST results (n = 207, 39.9%) were diagnosed with MHE. The prevalence of MHE differed among CPC-A (24.8%), CPC-B (39.4%) and CPC-C (56.1%) patients (CPC-A *vs* CPC-B, $P < 0.05$; CPC-A *vs* CPC-C, $P < 0.01$; CPC-B *vs* CPC-C, $P < 0.01$) (Figure 1). Older patients and patients with lower levels of education, a history of prior ascites had a higher prevalence of MHE (Table 1). Compared to patients without MHE, those with MHE had lower levels of serum albumin ($P = 0.01$), sodium ($P = 0.01$), potassium ($P = 0.04$), and platelet count ($P = 0.03$); higher levels of serum bilirubin ($P < 0.01$) and blood ammonia ($P = 0.02$); and longer prothrombin times ($P = 0.01$). Many (24.3%) MHE patients were still driving at the time of diagnosis.

There were no statistical differences in HBeAg status ($P = 0.30$) or HBV-DNA levels ($P = 0.19$), duration of HBV infection ($P = 1.00$), antiviral therapy ($P = 0.17$), or duration of antiviral treatment ($P = 0.54$) between patients with and without MHE.

Evaluation of HRQoL

Compared to cirrhotic patients without MHE, patients diagnosed with MHE had higher scores (more dysfunctions) for physical functioning (20.09 ± 6.26 *vs* 18.10 ± 6.02, $P < 0.01$), symptom/side effects (14.98 ± 5.88 *vs* 13.35 ± 5.61, $P < 0.01$), and psychological well-being (15.93 ± 6.62 *vs* 14.80 ± 5.44, $P = 0.04$) (Table 2). Pooled HRQoL scales were higher in MHE patients than in non-MHE ones (69.12 ± 20.40 *vs* 63.89 ± 18.85, $P < 0.01$). Patients with CPC-C had higher HRQoL scores (71.61 ± 21.01) than those with CPC-A (61.13 ± 17.24) and CPC-B (65.50 ± 19.31), $P < 0.01$, which reflect poorer QoL (Table 3).

Table 1 Characteristics of the study population with and without minimal hepatic encephalopathy *n* (%)

Characteristics	MHE	Non-MHE	*P* value
Gender			
Male	140 (67.6)	216 (69.2)	0.77
Female	67 (32.4)	96 (30.8)	
Mean age (yr), mean ± SD	51.56 ± 9.70	47.58 ± 11.55	< 0.01
Level of education			
Grade six or less	93 (44.9)	34 (10.9)	< 0.01
Junior high school	64 (39.9)	76 (24.4)	
Senior high school/vocational school	32 (15.5)	94 (30.1)	
College degree or more	12 (5.8)	88 (28.2)	
Unknown	6 (2.9)	20 (6.4)	
Driving			
Yes	50 (24.2)	89 (28.5)	0.22
No	144 (69.6)	193 (61.9)	
Unknown	13 (6.3)	30 (9.6)	
Primary etiology for chronic liver disease			
Hepatitis B virus	117 (56.5)	172 (55.5)	0.18
Hepatitis B virus and alcohol	21 (10.1)	40 (12.9)	
Alcohol	30 (14.5)	19 (6.1)	
Hepatitis C virus	11 (5.3)	27 (8.7)	
Hepatitis B and C virus	2 (1.0)	1 (0.3)	
Autoimmune hepatitis	12 (5.8)	27 (8.7)	
Other	14 (6.8)	24 (7.7)	
History of prior variceal bleeding			
Yes	43 (20.7)	82 (26.3)	0.21
No	158 (76.3)	226 (72.4)	
Unknown	6 (2.9)	4 (1.3)	
History of prior ascites			
Yes	131 (63.3)	139 (44.6)	0.00
No	66 (31.9)	168 (53.8)	
Unknown	10 (4.8)	5 (1.6)	
Duration of liver cirrhosis (yr), mean ± SD	2.28 ± 3.06	2.73 ± 3.21	0.25

MHE: Minimal hepatic encephalopathy.

Comparison of one single psychometric test and combined psychometric tests

We employed combined NCT and DST tests as "gold standard" in this study. Consistency of diagnosis between one single psychometric test (NCT or DST) and combined psychometric tests was assessed by Kappa statistics (Table 4). Agreement between DST and combined NCT and DST was good, with a Kappa coefficient around 0.98 (95%CI: 0.97-0.99) for diagnosing MHE. Agreement between NCT and combined tests was fair (Kappa value 0.24, 95%CI: 0.19-0.29).

DISCUSSION

This study was the first nationwide investigation of the prevalence of MHE among hospitalized cirrhotic patients in China. The study locations are dispersed in different parts of China, including east, west, north, south and central regions, covering 16 hospitals located in 10 provinces and 3 municipalities under direct administration of the central government. Because each teaching hospital in the capital city of a province provides service

Table 2 Health-related quality of life scales for patients with and without minimal hepatic encephalopathy

	MHE	Non-MHE	*P* value
Physical functioning (8 questions)	20.09 ± 6.26	18.10 ± 6.0	< 0.01
Psychological well-being (7 questions)	15.93 ± 6.62	14.80 ± 5.44	0.04
Symptom/side effects (7 questions)	14.98 ± 5.88	13.35 ± 5.61	< 0.01
Social functioning (4 questions)	9.67 ± 2.73	9.66 ± 2.65	0.95
Self-evaluation regarding general-health (4 questions)	9.74 ± 2.73	9.43 ± 2.57	0.21
Total pooled score (30 questions)	69.12 ± 20.40	63.89 ± 18.85	< 0.01

MHE: Minimal hepatic encephalopathy.

Table 3 Health-related quality of life scales for various Child-Pugh classes

	CPC-A	CPC-B	CPC-C	*P* value
Physical functioning	16.77 ± 5.07	18.94 ± 6.35	20.82 ± 6.33	< 0.01
Psychological well-being	14.43 ± 5.46	15.03 ± 5.93	16.39 ± 6.38	0.02
Symptom/side effects	12.12 ± 5.33	13.56 ± 5.21	16.45 ± 6.05	< 0.01
Social functioning	9.03 ± 2.53	9.60 ± 2.75	10.37 ± 2.58	< 0.01
Self evaluation about general-health	9.20 ± 2.59	9.46 ± 2.74	10.02 ± 2.52	0.02
Total pooled score	61.13 ± 17.24	65.50 ± 19.31	71.61 ± 21.01	< 0.01

CPC: Child-Pugh-Classification.

Table 4 Consistency of diagnosis between one single psychometric test and combined psychometric tests

	NCT and DST		Kappa value (95%CI)	*P* value
	MHE	Non-MHE		
NCT				
MHE	207	223	0.24 (0.19-0.29)	< 0.01
Non- MHE	0	89		
DST				
MHE	207	4	0.98 (0.97-0.99)	0.05
Non- MHE	0	308		

NCT: Number connection test; DST: Digit-symbol test; MHE: Minimal hepatic encephalopathy.

to patients from the entire province, the study population could well represent cirrhotic patients throughout China.

China has the greatest burden of chronic liver disease in the world due to an epidemic of viral B hepatitis. Although the exact nationwide prevalence of liver cirrhosis in China is unknown, a reasonable estimate suggests that up to 1% of the entire population could have histological evidence of cirrhosis[20,21]. The prevalence of MHE in Chinese cirrhotic patients was reported to be 51.3% by Zeng *et al*[22]. However, their study only included local patients and lacked assessment of cognitive impairments and decreased quality of life. Other studies also reported varying and higher than 50% MHE prevalences among cirrhotic patients[10,23-25]. Our study showed that the nationwide prevalence of MHE in hospitalized cirrhotic patients was 39.9%. These discrepancies were due to the different criteria used to diagnose MHE and inter-population variations. The absence of a gold standard for determining MHE is a major challenge for attaining consistency among studies.

Impairments in visuospatial function, attention, response time, and inhibition are specific to MHE in the absence of other neurocognitive disorders; the psychometric HE score (PHES) was specifically designed to detect these impairments. The PHES comprises 5 different tests: the NCT-A, NCT-B, DST, the line-tracing test, and the serial dotting test. NCT-A and NCT-B evaluate concentration, mental tracking, and visuomotor speed. The DST evaluates psychomotor and visuomotor speed with attention on speed and accuracy. According to the consensus of the Working Group on HE[26], if the entire PHES cannot be completed, at least two of the following tests are recommended for the diagnosis of MHE: NCT-A, NCT-B, block design test, and DST.

In this study, we combined two age-based psychometric tests (NCT-A and DST)[19] as "gold standard" to diagnose MHE. Consistency of MHE diagnosis between one single psychometric test (NCT or DST) and combined psychometric tests was assessed by Kappa statistics. Agreement between DST and combined NCT and DST was good, with a Kappa coefficient around 0.98 for diagnosing MHE. This good agreement indicates that DST is equally good as combined NCT and DST[27-29]. However, single NCT, which showed a higher prevalence of MHE, did not have good agreement with combined test. Therefore, in clinical practice, DST can be used as the first test for screening MEH so as to avoid a high rate of false positive diagnosis.

One of the limitations of neuropsychological test is that the results can be influenced by age, educational level, and learning effects. We used three parallel versions of NCT-A to avoid the effects of education. Yet the limitations of our study were that (1) the normality of the NCT-A and DST scores used was not adjusted by educational level; and (2) half of the patients in our study had lower educational levels, which might have influenced the neuropsychological test results.

Furthermore, the prevalence of MHE reported in other studies was higher in cirrhotic patients with CPC-B, CPC-C, advanced age, alcoholism, a previous episode of overt HE, and portosystemic shunts[30]. None of the patients in our study had previous episodes of OHE or histories of portosystemic shunt surgery. Groeneweg *et al*[31] found that cirrhotic patients with normal liver function (CPC-A) had a low prevalence (15%) of MHE, while MHE was present in half of the patients with advanced cirrhosis (CPC-B/C). Our study confirmed that the prevalence of MHE in CPC-C patients was the highest (56.5%). The results demonstrated that cirrhotic patients with MHE had impaired liver function, including reduced hepatic biosynthetic, excretory and/or detoxification capacity, hyponatremia, lower platelet count, and high

blood ammonia.

Patients with MHE had impaired perception, memory, learning, expression (language, constructive abilities, and voluntary motor control), mental activity (attention and mental speed), and executive function[30,32]. There are many aspects of English-published HRQoL instruments which cannot be adapted well to the Chinese due to the differences in cultures and language. In order to define and assess HRQoL appropriately in the Chinese patients, our group developed a modified Chinese questionnaire of HRQoL that was verified in a Chinese population in 2009[18]. The questionnaire was administered to a cohort of patients with varying types and stages of cirrhosis for assessment of its reliability and discriminant validity. As liver disease becomes more severe, the questionnaire documents deterioration in patients HRQoL[18]. Our study confirmed that severity of liver function impairment, based on CPC scoring system, was associated with HRQoL. Patients with CPC-C had higher HRQoL scores and compromised life status compared to patients with CPC-A/B. Patients with MHE had high health-related QoL scores that reflect poorer QoL.

Patients with MHE have higher physical functioning and symptom/side effects scores than patients without MHE. These two domains of the questionnaire also have a higher test-retest reliability (0.94 and 0.96) than other domains[18]. Our study results retested the discriminant validity of the questionnaire for distinguishing among groups with varying CPC classes. However, the validity of this instrument for evaluating the efficacy of MHE treatment needs to be established.

Physicians formerly agreed that it was unnecessary to screen for and treat MHE in cirrhotic patients without a history of OHE[33]. However, given increased knowledge of the impact of MHE, great emphasis on OHE has recently been shifted towards covert HE[34]. Because of psychomotor defects, patients meeting the criteria for MHE have been shown to have reduced driving skills, who are more likely to suffer from falls and develop episodic HE more frequently[4,35,36]. Our study found that 24.2% of MHE patients were driving at the time of diagnosis. Due to the potential risks, there is a need to assess the presence of MHE in cirrhotic patients who drive. Similarly, recommendations for MHE screening of cirrhotic patients may be valuable in reducing the risk of work-related accidents, especially while handling machinery[30,37].

Compared to OHE, there are fewer randomized clinical trials about the treatment of MHE and these trials have smaller case numbers. Some studies show that treatments using lactulose and/or rifaximin can improve the cognitive abilities, QoL[6,38], and driving ability[39] of patients with MHE. Yet the effects of these drugs on MHE patients' ability to work or risk of falling remain unproven. The duration of treatment and choice of medication also remain unclear[40]. Therefore, high quality studies are needed to assess whether patients suffering from liver cirrhosis and MHE require a specific treatment.

In conclusion, our study showed that 39.9% of hospitalized patients with liver cirrhosis had MHE, and this was associated with severe liver function and QoL impairment. Cirrhotic patients with CPC-C had a high prevalence of MHE and increased HRQoL scores that reflected poorer life status. The modified Chinese HRQoL questionnaire performed well in this study. The HRQoL scale results indicated that the questionnaire was suitable for evaluating cirrhotic patients in clinical practice in China. Recommendations to screen for MHE using NCT-A combining DST tests may be applicable for evaluating the risks of driving and work accidents in patients with cirrhosis. In clinical practice, DST can be considered as the first screening test of MHE due to the good agreement between DST and combined psychometric tests.

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COMMENTS

Background

China has the greatest burden of chronic liver disease in the world due to an epidemic of viral B hepatitis. Yet the exact nationwide prevalence of liver cirrhosis in China is unknown. Furthermore, minimal hepatic encephalopathy (MHE) is associated with potential progression to Hepatic encephalopathy (HE), diminished quality of life (QoL), driving impairment that increases the risk of traffic accidents, and negative health-related QoL (HRQoL).

Research frontiers

This is a first multicenter nationwide study to investigate the burden of MHE in hospitalized patients with cirrhosis in China. No such data was reported before.

Innovations and breakthroughs

This study was the first nationwide investigation of the prevalence of MHE among hospitalized cirrhotic patients in China. The study locations are dispersed in different parts of China, including east, west, north, south and central regions, covering 16 hospitals located in 10 provinces and 3 municipalities under direct administration of the central government. Because each teaching hospital in the capital city of a province provides service to patients from the entire province, the study population could well represent cirrhotic patients throughout China.

Applications

The results showed that 39.9% of hospitalized patients with liver cirrhosis had MHE, and patients with Child-Pugh-Classification score-C had a high prevalence of MHE (56.1%) and increased health-related QoL scores that reflected poorer life status. Increasing awareness of its adverse impact on life quality should be emphasized. Recommendations to screen for MHE may be applicable for evaluating the risks of driving and work accidents in patients with cirrhosis.

Terminology

HE is a serious complication of liver cirrhosis that represents a continuous spectrum of neurologic and neuropsychiatric abnormalities. MHE, the mildest form of HE, is defined as patients with normal mental and neurological examinations but with a number of neuropsychiatric and neuro-physiological defects identified by psychometric tests.

Peer review

In this paper, the authors investigated the prevalence of MHE, and assessed corresponding HEQoL in hospitalized cirrhotic patients in China. This is an interesting study regarding the prevalence and clinical features of MHE in China. The article showed a progress in the study of HE in cirrhotic patients. It is an

innovative job of HE study and data is reliable.

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BRIEF ARTICLE

Laparoscopic splenic hilum lymph node dissection for advanced proximal gastric cancer: A modified approach for pancreas- and spleen-preserving total gastrectomy

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Abstract

AIM: To investigate the feasibility and optimal approach for laparoscopic pancreas- and spleen-preserving splenic hilum lymph node dissection in advanced proximal gastric cancer.

METHODS: Between August 2009 and August 2012, 12 patients with advanced proximal gastric cancer treated in Nanfang Hospital, Southern Medical University, Guangzhou, China were enrolled and subsequently underwent laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum lymph node (LN) dissection. The clinicopathological characteristics, surgical outcomes, postoperative course and follow-up data of these patients were retrospectively collected and analyzed in the study.

RESULTS: Based on our anatomical understanding of peripancreatic structures, we combined the characteristics of laparoscopic surgery and developed a modified approach (combined supra- and infra-pancreatic approaches) for laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection. Surgery was completed in all 12 patients laparoscopically without conversion. Only one patient experienced intraoperative bleeding when dissecting LNs along the splenic artery and was handled with laparoscopic hemostasis. The mean operating time was 268.4 min and mean number of retrieved splenic hilum LNs was 4.8. One patient had splenic hilum LN metastasis (8.3%). Neither postoperative morbidity nor mortality was observed. Peritoneal metastasis occurred in one patient and none of the other patients died or experienced recurrent disease during the follow-up period.

CONCLUSION: Laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum LN dissection using the modified approach for advanced proximal gastric cancer could be safely achieved.

Key words: Proximal stomach; Stomach neoplasm; Laparoscopy; Lymph node excision; Splenic hilum

Core tip: Pancreas- and spleen-preserving splenic hilum lymph node dissection in laparoscopic total gastrectomy is challenging. Even though a small number of skilled laparoscopic surgeons have demonstrated the safety and feasibility of this procedure, most surgeons adopt only the suprapancreatic approach. However, exposure and dissection of splenic hilum lymph nodes posterior to the splenic artery, especially its inferior branch is sometimes difficult and unpredicted injury or bleeding is more likely to occur if only through the suprapancreatic approach. We combined the supra- and infra-pancreatic approaches to better expose the posterior

splenic artery lymph nodes at the splenic hilum and dissect more safely.

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INTRODUCTION

The metastatic rate of splenic hilum lymph nodes (LNs) has been reported to range from 8% to 21% in advanced proximal gastric cancer[1-6], and the removal of splenic hilum LNs might bring about potential survival benefit for these patients. Accordingly, splenic hilum LN dissection is recommended in the surgical treatment for advanced proximal gastric cancer[7].

Traditionally, the dissection of splenic hilum LNs and nodes along the splenic artery (SA) is achieved through pancreatosplenectomy or pancreas-preserving splenectomy. However, it has been suggested that the combined resection of pancreas and/or spleen would significantly increase postoperative morbidity and mortality rather than improve prognosis, as well as decrease immunological function[8-12]. As an alternative, pancreas- and spleen-preserving splenic hilum LN dissection might decrease postoperative morbidity without compromising oncological principles[13].

With the rapid development of minimally invasive surgery, the application of laparoscopic surgery for gastric cancer is gradually gaining popularity[14-16]. However, due to the tortuous splenic vessels and possibility of parenchymal injury to the spleen or pancreas, it is still a challenging and technically demanding procedure for conducting laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection. Only a few experienced laparoscopic surgeons have suggested its safety and feasibility[17-19], and most of them adopted the suprapancreatic approach to perform pancreas- and spleen-preserving splenic hilum LN dissection without using the infrapancreatic approach near pancreatic tail, while this method might not facilitate the dissection of LNs posterior to the splenic hilum.

Based on our anatomical understanding of peripancreatic fascia and spaces, we attempted a novel strategy combining supra- and infra-pancreatic approaches to perform laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection in total gastrectomy for treating advanced proximal gastric cancer. Herein, detailed procedure and preliminary results are presented.

MATERIALS AND METHODS

Patients

Between August 2009 and August 2012, 112 patients with endoscopically biopsy-proven proximal gastric cancer underwent laparoscopic total gastrectomy in Nanfang Hospital, Southern Medical University. Among them, twelve consecutive patients underwent laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection with curative intent.

Surgical indications

The indications for this procedure were as follows: (1) tumors were located at the upper- or middle-third of the stomach without distant metastasis; (2) tumors penetrated over the mucosa layer without invading adjacent structures; and (3) no gross involvement of the gastrosplenic ligament or LN number 4sb, at the splenic hilum or along the SA. Preoperative staging was confirmed by endoscopic ultrasound, abdominal high-resolution multidirectional computed tomography (CT), and positron emission computed tomography if necessary.

All surgical procedures were performed by Dr. Li GX, who had experience of over 500 laparoscopic gastrectomies for gastric cancer. All patients were given details about the operative procedure and potential risks before operation and provided written informed consent. This study was approved by the Ethics Committee of Nanfang Hospital.

Surgical procedures

The regional LNs were numbered according to the Japanese Classification of Gastric Carcinoma (JCGC) guidelines and LN dissection was done with laparoscopic ultrasonic shears [laparoscopic coagulation shears (LCSs); Ethicon Endo-Surgery, Cincinnati, OH, United States].

Under general anesthesia, the patient was placed in the supine position with legs set apart in a reverse Trendelenburg position. The surgeon stood on the patient's left side, the assistant surgeon on the patient's right side, and the camera operator stood between the patient's legs. After pneumoperitoneum was established with CO_2 insufflated at a pressure of 12 mmHg, five working ports were introduced (Figure 1)[20]. Exploration of the abdominopelvic cavity was conducted to exclude distant metastasis and carcinomatosis.

The greater omentum was divided along the border of the transverse colon toward the inferior pole of the spleen. By dividing the gastrocolic ligament, the lesser sac was entered. The stomach was then overturned cephalad and the left gastroepiploic vessels were located at the boundary between the gastrocolic ligament and the gastrosplenic ligament, which were then divided at their roots (Figure 2A). By separating the gastrosplenic ligament up to the left side of the esophageal hiatus, the short gastric vessels were divided just adjacent to the spleen and the upper part of the greater curvature was mobilized. LN numbers 4sa and 2 were dissected. The right gastroepiploic vein was identified by tracing proximally along the gastrocolic trunk or dissecting the mesogastrium inferior to the gastric antrum off the transverse mesocolon, which was then ligated and divided at its origin. The right gastroepiploic artery was usually identified

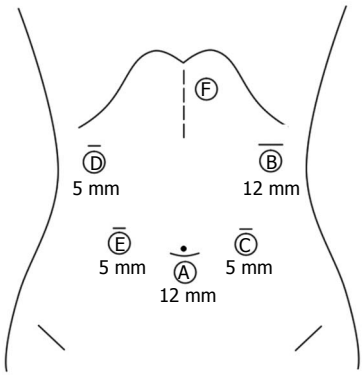

Figure 1 Positions of trocars. The trocars were inserted into the abdomen in the order A-E. Position F stands for the 4-5 cm midline minilaparotomy incision for reconstruction.

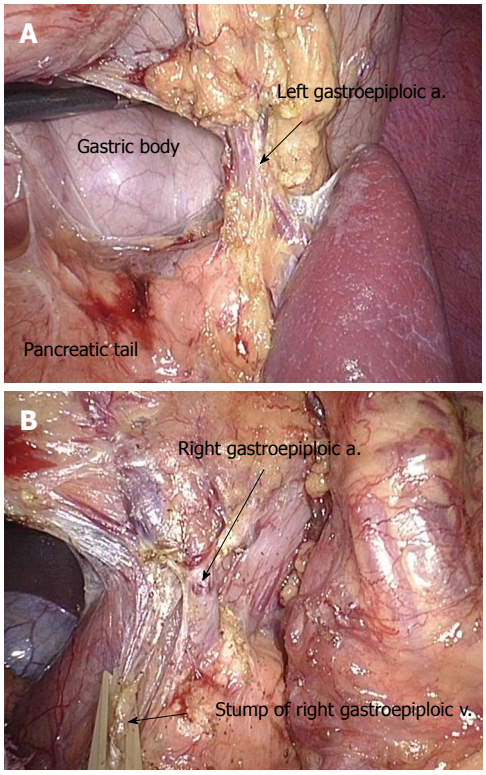

Figure 2 Gastroepiploic artery. A: Dividing left gastroepiploic artery (arrow); B: Dividing right gastroepiploic vessels (arrow). a: Artery; v: Vein.

next to the vein, which was also divided to allow the removal of LN numbers 4d and 6 (Figure 2B). After overturning the gastric antrum cranially, the gastropancreatic fold was exposed. The gastroduodenal artery was usually located in the groove between the duodenum and pancreatic head, which was a clue to trace the celiac trunk and its branches. By following the common hepatic artery, the proper hepatic artery was traced. The right gastric artery was located in the hepatoduodenal ligament as a small branch running from the proper hepatic artery to the supra-pylorus. By ligating the right and left gastric arteries and veins at origin and dissecting the tissues around the proper hepatic artery, common hepatic artery and celiac

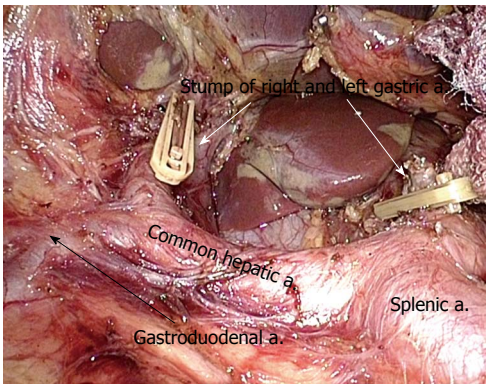

Figure 3 Tracing gastroduodenal artery to locate celiac trunk and its branches (arrows). a: Artery.

trunk, the right side of the suprapancreatic LNs (numbers 5, 7, 8a, 9 and 12a) were removed *en bloc* (Figure 3).

By retracting the pancreas meticulously in the caudal direction, the surgeon could dissect the soft tissue off the superior margin of the pancreatic body and tail in order to enter the retropancreatic space, thus uncovering the proximal SA (Figure 4A). From this step, in order to facilitate this manipulation, the surgeon changed his operating position and stood between the patient's legs. By opening the artery sheath and skeletonizing the SA from the proximal portion towards the distal portion, LN number 11p could be removed. When the bifurcation was reached, two secondary branches of the SA could be seen in most cases. The superior branch coursed towards the superior pole of the spleen and the inferior one coursed directly towards the splenic hilum. The pancreatic tail was mobilized using the infrapancreatic approach to enter the retropancreatic space (Figure 4B). The superior and inferior branches of the SA were then skeletonized until they reached the splenic parenchyma (Figure 5). Meanwhile, the remaining short gastric vessels originating from the SA were further ligated and divided. By skeletonizing the SA, fatty tissues bearing LN numbers 10 and 11d were removed, and all vessels in the splenic hilum area were saved with the preservation of both the pancreas and the spleen.

The duodenum was transected 2 cm distal to the pylorus using an endoscopic linear stapler (Echelon 60 Endopath Stapler; Ethicon Endo-Surgery, Guaynabo, Puerto Rico, United States). Subsequently, the phrenoesophageal and both vagus nerves were divided, along with the removal of LN number 1. The transaction of the esophagus and Roux-en-Y esophagojejunostomy were carried out extracorporeally through a 4-5-cm midline minilaparotomy just below the xiphoid process using a circular stapler. An end-to-side jejunojejunostomy was performed by hand suture.

RESULTS

The clinicopathological characteristics of the patients are shown in Table 1. Surgical outcomes and postoperative

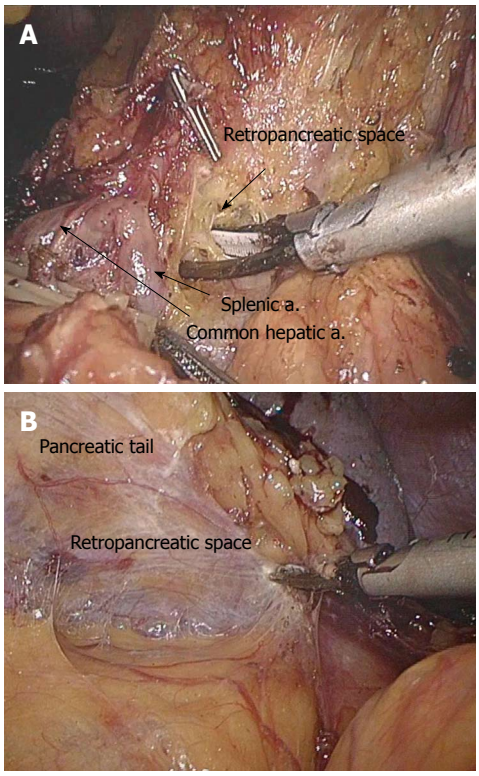

Figure 4 Entering retropancreatic space. A: Near the superior margin of the pancreas (arrows); B: Near the lower margin of the pancreatic tail. a: Artery.

course are summarized in Tables 2 and 3. There were nine male and three female patients, with a mean age of 60.6 years (range, 45-75 years). The mean body mass index was 21.5 kg/m^2 (range, 19.1-25.6 kg/m^2).

Laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum LN dissection was successfully performed in all 12 patients without conversion to open procedure. Only one patient experienced intraoperative bleeding during the skeletonization of the inferior branch of the SA. Pathological findings showed that tumor penetrated into the subserosal layer (T3) in only one patient and into the serosa without invasion to adjacent structures (T4a) in the other 11 patients. In accordance with the American Joint Committee on Cancer (AJCC) cancer staging manual, 7th edition, the TNM stages were distributed as follows: one stage ⅡA, three stage ⅡB, four stage ⅢA, two stage ⅢB, and two stage ⅢC. The mean number of retrieved splenic hilum LNs per patient was 4.8 (range, 2-8) and only one patient had splenic hilum LN metastasis (8.3%). Postoperatively, neither morbidity nor mortality was observed (Table 3).

At a median follow-up of 21 mo (range, 1-37 mo), one patient had peritoneal metastasis after 12 mo of surgery and died 6 mo later. None of the other patients died or experienced recurrent disease during the follow-up period.

DISCUSSION

Splenic hilum LN involvement was reported to range between 8% and 21%[1-6] and was identified as an important prognostic factor for gastric carcinoma in previous studies[4,6,9,21]. Splenic hilum LN involvement rate correlates with the depth of tumor invasion over the mucosal layer[2,3], the tumor is classified as Bormann's type Ⅲ or Ⅳ[3,5,22], the tumor is located at the greater curvature[5], and the tumor size is > 5 cm[4]. Thus, splenic hilum LN dissection should be conducted in patients with advanced proximal gastric cancer, especially those whose tumor has the above mentioned properties.

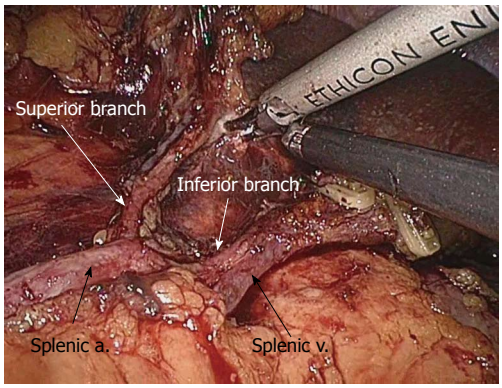

Figure 5 Skeletonizing the branches of the splenic artery (arrows). a: Artery; v: Vein.

For the complete removal of splenic hilum LNs, in traditional open surgery, extended total gastrectomy including pancreatosplenectomy was once recommended as the classic procedure by some surgeons[23,24]. However, combined resection of the distal pancreas is associated with increased postoperative complications, including acute pancreatitis, pancreatic fistula, abdominal abscess, and postoperative diabetes, which may even adversely affect survival. As a result, total gastrectomy with pancreas-preserving splenectomy has been proposed by other surgeons[11,25]. Other studies have demonstrated that splenectomy may result in higher morbidity and mortality, and has no significant survival benefit[1,9,10,22,26]. Accordingly, pancreas- and spleen-preserving total gastrectomy has been attempted in open surgery[13], although it is still controversial.

Laparoscopic gastrectomy, as an alternative to traditional open surgery for early gastric cancer, has been suggested to produce comparable morbidity and mortality, as well as long-term survival as open gastrectomy, while possessing the benefits of minimally invasive approaches[27-32]. With respect to the above reasons, laparoscopic distal gastrectomy has gradually gained popularity for the treatment of early gastric cancer located in the lower portion of the stomach[14-16,32]. However, only a few studies have reported the application of laparoscopic total gastrectomy in advanced proximal gastric cancer[33-35]. With the development of laparoscopic devices and accumulation of experiences, a small number of skilled laparoscopic surgeons in high-volume specialized centers have attempted to extend the indications to advanced proximal gastric cancer using the strategy of splenic hilum LN dis-

Table 1 Clinicopathological characteristics of patients

Patient No.	Gender	Age (yr)	BMI (kg/m^2)	Tumor location	Tumor size (cm)	Tumor depth[1]	TNM stage[1]	No. of retrieved LN[2]	No. of metastatic LN[2]	No. of retrieved splenic hilum LN[2]	No. of metastatic splenic hilum LNc
1	Male	60	19.1	U	5.0	T4a	ⅢC	21	7	3	0
2	Male	73	24.8	U	6.0	T4a	ⅢB	18	5	4	0
3	Male	61	20.6	U	8.5	T4a	ⅢA	34	2	8	0
4	Male	62	20.2	U	7.0	T3	ⅡA	39	0	4	0
5	Male	59	20.8	U	4.5	T4a	ⅡB	16	0	3	0
6	Female	54	20.4	M	5.0	T4a	ⅢA	20	1	5	0
7	Female	57	24.0	U	5.0	T4a	ⅢA	20	1	7	0
8	Male	57	19.1	U	8.0	T4a	ⅢC	28	21	6	3
9	Male	61	25.6	U	3.5	T4a	ⅢA	16	2	4	0
10	Male	63	21.1	M	2.9	T4a	ⅡB	24	0	5	0
11	Female	75	21.8	U	5.5	T4a	ⅢB	19	3	2	0
12	Male	45	20.6	M	4.0	T4a	ⅡB	35	0	6	0

[1]"Tumor depth" and "Tumor node metastasis (TNM) stage" were in accordance with the AJCC cancer staging manual-7th edition; [2]Lymph nodes (LN). BMI: Body mass index.

Table 2 Surgical outcomes, postoperative course and follow-up data of patients

Patient No.	Operating time (min)	Estimated blood loss (mL)	Time to first flatus (POD)	Time to soft diet (POD)	Hospital stay (POD)	Follow-up (mo)	Follow-up outcome
1	230	50	5	10	10	37	No recurrence, alive
2	352	300	6	10	13	37	No recurrence, alive
3	180	100	4	8	11	36	No recurrence, alive
4	314	100	5	7	10	28	No recurrence, alive
5	278	100	4	8	12	24	No recurrence, alive
6	305	100	2	9	11	24	No recurrence, alive
7	298	300	5	8	12	24	No recurrence, alive
8	260	150	2	4	8	18	Peritoneal metastasis, death
9	280	200	3	5	6	6	No recurrence, alive
10	223	50	2	6	7	5	No recurrence, alive
11	221	150	3	4	5	4	No recurrence, alive
12	280	200	3	7	8	1	No recurrence, alive

POD: Postoperative days.

Table 3 Surgical outcomes and postoperative courses of laparoscopic pancreas- and spleen-preserving splenic hilum lymph nodes dissection

Items	mean ± SD (range)
Operating time (min)	268.4 ± 48.0 (180-352)
Estimated blood loss (mL)	150.0 ± 85.3 (50-300)
No. of retrieved LN	24.2 ± 7.9 (16-39)
No. of metastatic LN	3.5 ± 5.9 (0-21)
No. of retrieved splenic hilum LN	4.8 ± 1.8 (2-8)
No. of metastatic splenic hilum LN	0.3 ± 0.9 (0-3)
Time to first flatus (POD)	3.7 ± 1.4 (2-6)
Time to soft diet (POD)	7.2 ± 2.1 (4-10)
Hospital stay (POD)	9.4 ± 2.6 (5-13)
Intraoperative complication	1 (8.3%)
Postoperative complication	0
Mortality	0

LN: Lymph nodes; POD: Postoperative days.

section in pancreas- and spleen-preserving total gastrectomy[17-19,36].

The major difficulties of this laparoscopic procedure lie in the complicated variations of the SA supplying the spleen with its variable branching. The greatest challenges to surgeons are the high probability of injuries to the splenic vessels, unpredicted avulsion of the splenic capsule, skillful manipulation of endoscopic devices in a limited space, and injuries to the splenic hilum during skeletonization of the splenic vessels. Our strategy to deal with these difficulties was based on our thorough understanding of anatomy under laparoscopic view[20] and team cooperation. The SA is located in the retropancreatic space, coursing near the superior margin of the pancreas and usually dividing into two terminal branches near the pancreatic tail[37-39].

The inferior branch of the SA courses directly into the splenic hilum, therefore, the exposure and dissection of the LNs posterior to it are sometimes difficult. From our past experience, if the vascularization and dissection was continued leftward only through the suprapancreatic approach, bleeding and unpredicted injury were more likely to occur due to the exposure limit. Thus, in our clinical practice, the suprapancreatic approach was adopted for the vascularization of the SA trunk, its superior branch, and the upper hemisphere of its inferior branch. Then, the lower margin of the pancreatic tail was mobilized. Since the retropancreatic space was filled with loose

connective tissue near the lower margin of the pancreatic tail[40], exposure of the lower hemisphere could easily be achieved with the assistant turning the pancreatic tail cephalad. The vascularization of the inferior branch was continued until coming across the upper hemisphere, in other words, the inferior branch of the SA was skeletonized both through the supra- and infra-pancreatic approaches. The splenic pedicle was also freed, allowing for the complete removal of the posterior splenic hilum LNs.

Our strategy for laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection is different from that in previous reports in the literature. The hand-assisted technique was adopted by Uyama *et al*[17], taping the SA was applied by Hur *et al*[19], and Hyung stood at the patient's right side and skeletonized the distal portion of the SA as soon as completing division of the gastrosplenic ligament[18]. To our knowledge, all these surgeons adopted the suprapancreatic approach. However, one similarity we noted was that when approaching the splenic hilum, meticulous manipulation was required to avoid injury. In Hyung's report, preoperative assessment of the splenic vascular anatomy was conducted with CT in collaboration with radiologists[18]. In our study, we experienced an episode of major intraoperative bleeding during dissection of the inferior branch of the SA in one of our patients. We applied endoscopic gauze to compress the bleeding area and identified the bleeding point. A hemo-lock was then used to clip onto the artery surface, involving the bleeding point without fully clamping the whole artery. Successfully, the bleeding was finally controlled after some maneuvers. In retrospect, even if this attempt was not successful, splenectomy could be safely conducted because the splenic pedicle was freed. Given these aspects, the average operating time was increased to 268.4 min, and the time was especially longer for the first three cases; nevertheless, our average operating time was still in congruent with that in the previous reports[17-19]. Although the patients in our study suffered from a more advanced stage of gastric carcinoma, the early follow-up results showed satisfactory survival.

In our study, only one out of 12 patients had splenic hilum LN metastasis. Interestingly, this patient had an overall high percentage of positive LNs, experienced peritoneal metastasis at 12 mo after surgery and died 6 mo later. This finding might suggest that splenic hilum LN involvement is always associated with highly advanced proximal gastric cancers, and poorer prognosis in these patients might be predicted. Similarly, Shin *et al*[4] found that splenic hilum LN metastasis had a poor prognosis. However, due to the limitation of our relatively small sample size, the correlation between splenic hilum LN metastasis and oncological outcomes needs to be further confirmed.

Our retrospective study also had several limitations, including patient selection bias and relatively small sample size. However, to the best of our knowledge, this is a modified approach for conducting laparoscopic pancreas- and spleen-preserving splenic hilum LN dissection. The detailed procedure might be useful for surgeons who wish to conduct similar laparoscopic surgery.

In conclusion, using the strategy of combining supra- and infra-pancreatic approach to extend the retropancreatic space in experienced hands, laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum lymph nodes dissection for the treatment of advanced proximal gastric cancer in selected patients could be safe and feasible. However, long-term follow-up and randomized clinical trials to evaluate its surgical safety and oncological efficacy are needed.

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COMMENTS

Background

Laparoscopic gastrectomy, as a minimally invasive alternative treatment to traditional open surgery in treating gastric cancer, is gaining popularity worldwide. For advanced gastric cancer, radical surgery should accomplish adequate lymph node (LN) dissection (D2 lymphadenectomy) according to oncological principles. Splenic hilum LN dissection should be included in the D2 lymphadenectomy when treating advanced proximal gastric cancer.

Research frontiers

Traditional removal of splenic hilum LNs was achieved through combined resection of the pancreas and/or spleen. However, it has been suggested that combined resection would increase postoperative morbidity and mortality and not significantly benefit patient survival. Thus, pancreas- and spleen-preserving total gastrectomy was subsequently attempted in open and laparoscopic surgery.

Innovations and breakthroughs

In laparoscopic total gastrectomy, pancreas- and spleen-preserving splenic hilum LN dissection is challenging because of the tortuous splenic vessels and possibility of parenchymal injury to the spleen or pancreas. To date, only a small number of skilled laparoscopic surgeons in high-volume specialized centers can achieve splenic hilum LN dissection in pancreas- and spleen-preserving total gastrectomy, and most of them only adopt the suprapancreatic approach. This method might not facilitate the dissection of LNs posterior to the splenic hilum and might cause unpredicted injury to splenic vessels. Thus, they modified this strategy by combining both supra- and infra-pancreatic approaches to better expose the posterior splenic artery LNs at the splenic hilum, and dissect more safely.

Applications

Using the strategy of combining supra- and infra-pancreatic approach to extend the retropancreatic space in experienced hands, laparoscopic total gastrectomy with pancreas- and spleen-preserving splenic hilum lymph nodes dissection for the treatment of advanced proximal gastric cancer in selected patients could be safe and feasible. The indications for laparoscopic surgery could be extended to advanced proximal gastric cancer. The detailed procedure described here might be useful for laparoscopic surgeons. However, due to the limited sample size, further long-term follow-up results and randomized controlled trials are needed to ascertain its surgical safety and oncological efficacy.

Terminology

In advanced gastric cancer, the tumor penetrates the mucosal layer of the stomach wall. In advanced proximal gastric cancer, the tumor is located in the upper or middle third of the stomach. Splenic hilum LNs are the LNs located adjacent to the splenic artery distal to the pancreatic tail, those on the roots of the short gastric arteries, and those along the left gastroepiploic artery proximal to its first gastric branch, according to the Japanese Classification of Gastric Carcinoma guidelines.

Peer review

The authors described of clinical impact of laparoscopic splenic hilum lymph node dissection for advanced proximal gastric cancer based on the strategy combining supra- and infra-pancreatic approach for pancreas- and spleen-preserving total gastrectomy. It is well written.

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BRIEF ARTICLE

Stent-grafts for the treatment of TIPS dysfunction: Fluency stent *vs* Wallgraft stent

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Abstract

AIM: To evaluate the clinical efficacy of an expanded polytetrafluoro-ethylene-covered Fluency stent compared with that of a polyethylene terephthalate-covered Wallgraft stent for the management of transjugular intrahepatic portosystemic shunt (TIPS) dysfunction.

METHODS: A retrospective review of patients who underwent TIPS revision with stent-grafts between May 2007 and June 2011 was conducted. The patients were divided into two groups according to the stent-grafts implanted: the Fluency stent (Bard Incorporated, Karlsruhe, Germany) and the Wallgraft stent (Boston Scientific, Galway, Ireland). The primary patency rates were calculated and compared using the Kaplan-Meier method.

RESULTS: A total of 73 patients were evaluated in this study: 33 with Fluency stents and 40 with Wallgraft stents. The primary patency rates at 12 and 24 mo were 91% and 85%, respectively, in the Fluency stent group and 78% and 63%, respectively, in the Wallgraft stent group. The primary shunt patency rates after TIPS revision were significantly better with the Fluency stent than with the Wallgraft stent (P = 0.033).

CONCLUSION: TIPS revision with the Fluency stent has higher medium-term patency rates than that with the Wallgraft stent.

Key words: Expanded polytetrafluoroethylene-covered stent-grafts; Transjugular intrahepatic portosystemic shunt dysfunction; Revision; Fluency

Core tip: There are few data on the clinical use of expanded polytetrafluoroethylene-covered stent-grafts for the management of transjugular intrahepatic portosystemic shunt (TIPS) dysfunction in the literature. The present study was designed to retrospectively evaluate the clinical efficacy of Fluency stent compared with Wallgraft stent in the treatment of TIPS dysfunction. And the results demonstrated that TIPS revision with the Fluency stent has higher medium-term patency rates than that with the Wallgraft stent.

Luo XF, Nie L, Wang Z, Tsauo J, Liu LJ, Yu Y, Zhou B, Tang CW, Li X. Stent-grafts for the treatment of TIPS dysfunction: Fluency stent *vs* Wallgraft stent. *World J Gastroenterol* 2013; 19(30): 5000-5005 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5000.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5000

INTRODUCTION

Transjugular intrahepatic portosystemic shunts (TIPS) have been increasingly used for the management of portal hypertension complications in patients with cirrhosis[1,2]. However, shunt dysfunction is a major drawback of TIPS. The primary patency rate after 24 mo has been reported to be 40%-60% when bare stents were used[1]. Consequently, regular shunt surveillance and reintervention are required to maintain shunt patency.

TIPS dysfunction is the result of acute thrombosis within the stent or of pseudointimal hyperplasia within the TIPS tract in the liver parenchyma or along the outflow hepatic vein[3]. Both phenomena may be associated with a biliary fistula. Several experimental and clinical studies have shown that TIPS creation with an expanded polytetrafluoroethylene (ePTFE)-covered Viatorr stent can remarkably improve the long-term shunt patency[4-6]. Similarly, encouraging results have been obtained when the Viatorr stent was used for the treatment of TIPS dysfunction[7,8].

However, the Viatorr stent is not available in many countries. The Fluency stent, which is a non-dedicated ePTFE-covered stent-graft, has been utilized to establish a transjugular intrahepatic portosystemic shunt[9]. Currently, there is no relevant report on the clinical use of the Fluency stent for the management of TIPS dysfunction in the literature. In this study, we retrospectively evaluated the clinical efficacy of the Fluency stent and of the Wallgraft stent in TIPS revision.

MATERIALS AND METHODS

Patient selection

This retrospective study was approved by the ethics committees of West China Hospital, Sichuan University. Between May 2007 and June 2011, patients who underwent TIPS revision by implantation of stent-grafts were analyzed. Patients were excluded if they already had a previous TIPS revision. Patients who underwent the insertion of a parallel shunt due to failed original shunt revision were also excluded. Thus, a total of 73 patients were evaluated in this study. This study group was further divided into two subgroups according to the stent-grafts received: 33 patients who underwent TIPS revision by implantation of the Fluency stent and 40 patients who underwent TIPS revision by implantation of the Wallgraft stent.

TIPS revision procedure

Written consent was obtained from each patient before the procedure. All procedures were performed by two experienced interventional radiologists. The patients were prepared and draped in the angiographic suite using local anesthesia. After puncturing the right internal jugular vein, a standard 10-F TIPS set (Cook Incorporated, Bloomington, United States) was introduced into the inferior vena cava. Once the previous shunt was accessed through the sheath using an angled hydrophilic guidewire (Terumo Company, Fijinomiya, Japan), a 5-F Cobra catheter (Terumo Company) was advanced into the superior mesenteric or splenic vein. Portography was performed with the 5-F catheter placed in the portal region, and the portosystemic pressure gradient (PPG) was measured. After dilating the stenotic or occluded shunt with a 10 mm angioplasty balloon catheter (Cordis, LJ Roden, the Netherlands), we advanced the 10-F sheath into the main portal vein. The stent-graft delivery set, which was either a Fluency stent or a Wallgraft stent, was then introduced into the sheath. The sheath was withdrawn into the inferior vena cava, and the stent-graft was released to cover the entire length of the shunt up to the junction of the hepatic vein and the inferior vena cava. Shunt venography was performed, and the PPG was measured again. Additional shunt dilation or an additional stent-graft implantation was performed if necessary. Patients with a PPG higher than 12 mmHg despite sufficient dilation of the shunt received prophylactic embolization of the varices with metal coils.

Medication

All patients received a single prophylactic dose of a second-generation cephalosporin 1 h before the procedure. Intravenous heparin (3000 U) was administered immediately after successful TIPS revision, except for those patients with a coagulation disorder. Subsequently, antiplatelet therapy with aspirin was maintained for life.

Follow-up

All patients were evaluated by the same medical team in the gastroenterology clinic according to the follow-up schedule. Doppler duplex ultrasonography (US) was performed 24 h and 1, 3 and 6 mo after the procedure, followed by every 6 mo thereafter or whenever recurrent TIPS dysfunction was suspected clinically. TIPS dysfunction was suspected on the US if the intrastent flow velocity was less than 60 cm/s or higher than 120 cm/s or if there was a change in the direction of the flow in the intrahepatic portal branches compared with previous US findings. TIPS dysfunction was defined as a shunt narrowing of more than 50%, a PPG higher than 12 mmHg or both. Primary patency was defined as the interval of time without an intervention.

Statistical analysis

The results were expressed as the mean ± SD. The 12 and 24 mo primary patency rates were analyzed and compared using the Kaplan-Meier method. The results were compared with the log-rank test. A P value of less than 0.05 was considered statistically significant. All calculations were performed using SPSS version 20.0 software for Windows.

RESULTS

The patient characteristics at the time of the TIPS revision and indications for TIPS revision are documented and summarized in Table 1. Technical success was achieved in all 73 patients, and no severe complications

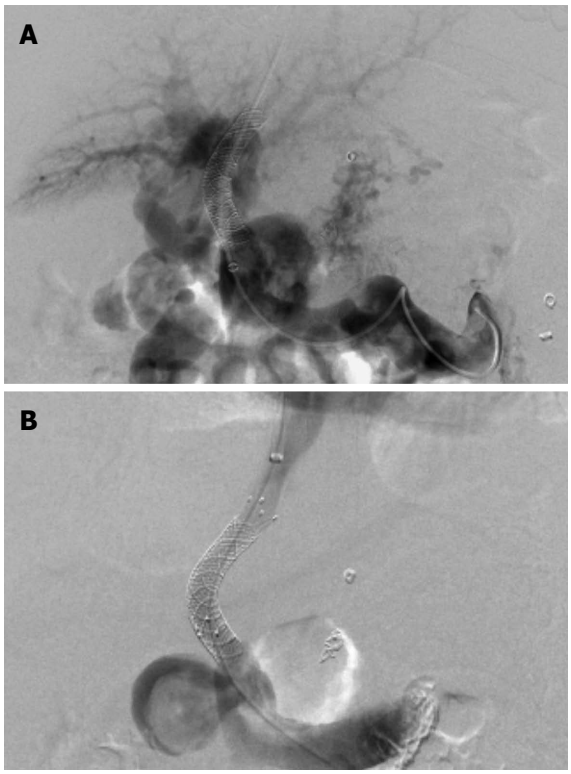

Figure 1 Portal venograms in a 52-year-old woman with hepatitis B cirrhosis who was treated with transjugular intrahepatic portosystemic shunt for recurrent variceal bleeding. A: Portography after crossing the previous shunt shows a patent splenic vein and the occluded main portal vein with cavernous transformation; B: After implantation with a Fluency stent, the portosystemic pressure gradient was reduced from 31 to 11 mmHg.

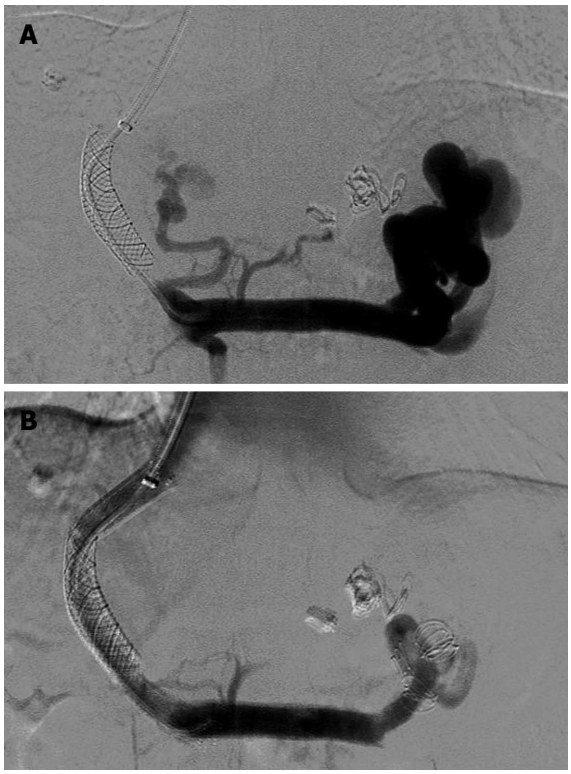

Figure 2 Portal venograms in a 45-year-old man with hepatitis B cirrhosis who had undergone transjugular intrahepatic portosystemic shunt for repeated variceal bleeding. A: The anteroposterior portal venogram obtained 5 mo after the initial transjugular intrahepatic portosystemic shunt procedure shows the complete occlusion of the stent and opacification of a massive spontaneous splenorenal shunt; B: Embolization of the splenorenal shunt was performed to maintain sufficient portal flow to keep the stent open. The portal venogram after Wallgraft stent placement reveals a wide patent shunt. The portosystemic pressure gradient decreased from 37 to 10 mmHg.

occurred (Figures 1 and 2). Four patients had transient discomfort in the upper abdominal area. A total of 15 patients developed new hepatic encephalopathy after the procedure, including nine patients with the Fluency stent and six with the Wallgraft stent. Eleven of these patients were successfully managed by protein restriction and lactulose administration. One patient was treated with shunt reduction by implantation of an additional Fluency stent, which raised the PPG from 7 to 10 mmHg. Hepatic encephalopathy failed to improve in the remaining three patients.

In the 33 TIPS revisions with the Fluency stent, the mean PPG was reduced from 18.5 ± 4.9 mmHg (range: 13-47 mmHg) to 7.4 ± 5.7 mmHg (range: 5-15 mmHg) after the procedure. A single stent-graft was used in 32 cases, and two stent-grafts were used in the other case because the stent was not terminated at the hepatocaval junction. All Fluency stents measured 10 mm in diameter. A PPG value below 12 mmHg after TIPS revision was not achieved in five patients, even though no obvious shunt stenosis was observed. Prophylactic embolization of the varices with metal coils was then performed in these patients.

In the 40 TIPS revisions with the Wallgraft stent, the mean PPG was reduced from 20.8 ± 3.7 mmHg (range: 12-51 mmHg) to 8.6 ± 3.1 mmHg (range: 6-17 mmHg) after the procedure. A single Wallgraft stent was used in 38 patients. Two patients were implanted with an additional stent because the shunt was not extended to the inferior vena cava. All Wallgraft stents measured 10 mm in diameter. The stent-grafts were placed after shunt dilation with 4 mm × 10 mm angioplasty balloons. In three patients, the PPG was not reduced to below 12 mmHg. These three patients received prophylactic embolization of the varices with metal coils. Another patient was treated with embolization of a massive splenorenal shunt to maintain sufficient portal flow in the stent (Figure 2).

The mean follow-up time in the Fluency stent group was 27.7 ± 11.6 mo (range: 3.5-46.5 mo). Of the 33 patients in this group, seven patients developed recurrent TIPS dysfunction during this period. Three patients presented with gastrointestinal hemorrhage, and the other four were diagnosed by US findings (Table 2). Angioplasty and subsequent implantation of a second stent-graft were performed in four of these patients, resulting in the restoration of blood flow within the shunt. One patient underwent orthotopic liver transplantation without revision. The other two patients refused TIPS revision. The primary shunt patency rates after TIPS revision with the Fluency stent were 91% at 12 mo and 85% at 24 mo.

The mean follow-up time in the Wallgraft stent group

Table 1 Patient demographics

Characteristics	Fluency endoprosthesis	Wallgraft endoprosthesis	*P* value
Patients (*n*)	33	40	
Age (yr)	51.4 ± 5.7	50.2 ± 8.5	0.491
Sex (male/female)	25/8	29/11	0.752
Etiology			0.242
Hepatitis B	21	31	
Alcohol	7	4	
Other	5	5	
Child-Pugh classification			0.564
A	6	8	
B	19	25	
C	8	7	
TIPS indication			0.946
Variceal bleeding	25	31	
Ascites	6	5	
Other	2	4	
Indications for TIPS dysfunction			0.762
Abnormal US findings	12	15	
Variceal bleeding	18	23	
Ascites	3	2	

TIPS: Transjugular intrahepatic portosystemic shunt; US: Ultrasonography.

Table 2 Long-term outcome of patients

	Fluency endoprosthesis	Wallgraft endoprosthesis	*P* value
Patients (*n*)	33	40	
Hepatic encephalopathy	9	6	0.196
Variceal rebleeding	7	19	0.020
Shunt dysfunction			
12 mo	3	9	0.722
24 mo	4	15	0.039

was 25.6 ± 13.2 mo (range: 3-48 mo). Of the 40 patients in this group, 19 patients developed recurrent TIPS dysfunction during this period. Eight patients presented with gastrointestinal hemorrhage, three presented with recurrent ascites, and the remaining eight were diagnosed by US findings (Table 2). Angioplasty and subsequent implantation of a second stent-graft were performed in the nine patients who had shunt restenosis or occlusion. Five patients were solely treated with angioplasty because the portography did not show any stenosis or portal vein thrombosis. Three patients were not revised because of overt hepatic encephalopathy. One patient underwent orthotopic liver transplantation. The primary shunt patency rates after TIPS revision with the Wallgraft stent were 78% at 12 mo and 63% at 24 mo. The primary shunt patency rates after TIPS revision were significantly better with the Fluency stent than with the Wallgraft stent (Log-rank test, $P = 0.033$, Figure 3).

DISCUSSION

The causes of TIPS dysfunction include acute thrombosis and pseudointimal hyperplasia in the parenchymal tract of the shunt or in the outflow hepatic vein[3,10]. During TIPS creation with bare stents, the dilatation of the liver parenchymal tract may cause laceration of the bile ducts, resulting in biliary-TIPS fistulas, which have been observed frequently in patients with acute thrombosis or recurrent shunt occlusions. The fibrotic or inflammatory healing response to the trauma of shunt creation induces the fibroblasts from adjacent liver stroma to differentiate into myofibroblasts and to then migrate through the stent mesh into the shunt lumen[3,10]. The overgrowth of pseudointimal hyperplasia could be responsible for stenosis or occlusion within the parenchymal tract.

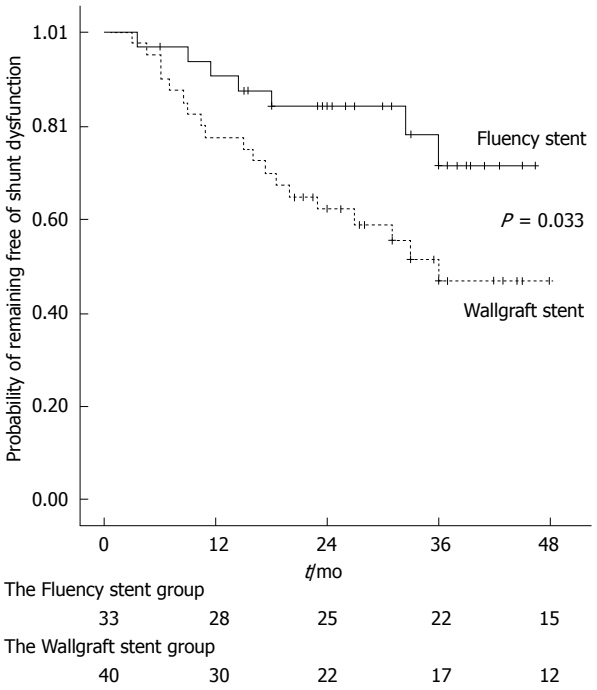

Figure 3 The probability of remaining free of shunt dysfunction.

The preliminary stent position within the outflow hepatic vein plays an important role in TIPS patency. The turbulence and shear stress from increased shunt flow could provoke the acceleration of pseudointimal hyperplasia and predispose the patient to shunt dysfunction. Additionally, late shortening of the self-expanding stent-grafts may occur, leaving the outflow hepatic vein at risk of subsequent intimal hyperplasia or the recoiling of an unsupported outflow section of the parenchymal tract[11]. Clark *et al*[12] demonstrated that the initial stent position within the hepatic venous outflow was predictive of shunt patency, with TIPS extending to the junction of the hepatic vein and the inferior vena cava having longer lifespans than shunts terminating in the hepatic vein. In this study, the primary patency rates at 12 mo were 36% ± 10% among patients with the outflow portion of the stent-grafts terminating in the hepatic vein and 58% ± 8% among patients with the outflow portion of the stent-grafts terminating at the hepatocaval junction[12]. Based on previous studies and our experience, we were careful about bridging the complete tract to the inferior vena cava in the present series.

A few experimental and clinical studies have verified the application of ePTFE-covered stent-grafts in *de novo*

TIPS creation and TIPS revision[4,7,8]. ePTFE was utilized as a cover material for stent-grafts, separating the blood flow within the shunt from the liver parenchyma and from the injured outflow hepatic vein. After TIPS creation with the ePTFE-covered stent-graft, the shunt flow was maintained by inhibiting the overgrowth of pseudointimal hyperplasia in the parenchymal tract or along the outflow hepatic vein. Echenagusia et al described the application of ePTFE-covered stent-grafts in the treatment of TIPS stenosis or occlusion in 12 patients. After TIPS revision, the primary patency rates were 100% at 12 mo and 88.8% at 24 mo[7]. More recently, Jirkovsky et al reported a clinical study in which 121 episodes of dysfunctional TIPS were evaluated retrospectively. The primary patency rates after 12 and 24 mo were 49.7% and 25.3%, respectively, in conventional angioplasty, 74.9% and 64.9%, respectively, with bare stents, 75.2% and 64.5%, respectively, with ePTFE-covered stent-grafts and 88.1% and 80.8%, respectively, with Viatorr stent-grafts. These results showed a tendency favoring ePTFE-covered stent-grafts, especially the Viatorr stent[13]. Unfortunately, the Viatorr stent is not commercially available in mainland China.

Compared with previously published reports, our study has a relatively large number of patients who underwent TIPS revision (n = 73). In our series, the primary shunt patency rates at 12 and 24 mo were 91% and 85%, respectively, with the Fluency stent and 78% and 63%, respectively, with the Wallgraft stent. After TIPS revision by implantation of a second stent-graft, the primary shunt patency rates were significantly better with the Fluency stent than with the Wallgraft stent. The long-term patency of the Fluency stent compared well with the previously published results of ePTFE-covered stent-grafts[13]. To the best of our knowledge, TIPS revision with angioplasty alone may have barely satisfactory short-term patency but results in a high incidence of recurrent shunt dysfunction. Lining the dysfunctional TIPS with a second stent-graft not only enables the restoration of shunt function but may also improve the configuration of the TIPS to prevent future restenosis.

The Fluency stent is a PTFE-encapsulated grid-like cylinder composed of a biocompatible nickel-titanium alloy. The deployment of a Fluency stent is easy because the position of this type of stent-graft is very precise. Based on our data, one patient (3%) with the Fluency stent and two patients (5%) with the Wallgraft stent required additional stent-graft implantation, partly due to previous stent malposition. The Fluency stent is one of the two commercially available stent-grafts in mainland China, the other being the Wallgraft stent[9,14]. Wu *et al*[9] performed a retrospective study on shunt patency in patients treated with TIPS creation using the Fluency stent. The rates of recurrent bleeding, shunt occlusion, hepatic encephalopathy and mortality were 0.03%, 0.0%, 16.7% and 0% after 6.16 ± 3.89 mo of follow-up. Although the follow-up time was not long enough, these results suggested that the Fluency stent was effective in TIPS creation and had a favorable patency rate. The Wallgraft stent is a polyethylene terephthalate-covered stent-graft and has been considered unsuitable for initial TIPS creation according to experimental studies[12,15]. However, the role of the Wallgraft stent in TIPS revision remains unknown.

There are several limitations of the present study that warrant consideration. This study is a single-institution, retrospective study, which increases the likelihood of systematic bias. Ideally, a randomized two-arm clinical trial should be designed to compare the Fluency stent with the Wallgraft stent. Additionally, the decision on the stent-graft selected for revision was based on the operator`s preference.

This study represents one of the largest published case series of patients with dysfunctional TIPS who underwent shunt revision with stent-grafts. Our results suggest that completing TIPS revision with the Fluency stent is safe and effective. Although large prospective studies with longer follow-up periods are needed, our analysis indicates that TIPS revision using the Fluency stent provided better shunt patency than that using the Wallgraft stent in the medium-term. Based on our institutional experience and on results from the literature, the ePTFE-covered Fluency stent could be a valuable solution for TIPS revision.

COMMENTS

Background

Transjugular intrahepatic portosystemic shunts (TIPS) has been widely used in the treatment of complications of portal hypertension. However, shunt dysfunction is a main defect of TIPS. Previously clinical studies have demonstrated that de novo TIPS creation and shunt revision with an expanded polytetrafluoroethylene (ePTFE)-covered stent could improve the long-term shunt patency.

Research frontiers

In this study, the authors demonstrated that TIPS revision with the ePTFE-covered Fluency stent has higher medium-term patency rates than that with the polyethylene terephthalate-covered Wallgraft stent which are commercially available in mainland China.

Innovations and breakthroughs

There are few data on the clinical use of Fluency stent or Wallgraft strent for the management of TIPS dysfunction in the literature. This is believed to be the first study to report that TIPS revision with Fluency stent could provide better second shunt patency.

Applications

Considering TIPS dysfunction would still be an improtant clinical issue in the near future, this study may illustrate a useful management strategy in the treatment of TIPS dysfunction.

Terminology

The Fluency stent is a polytetrafluoroethylene-encapsulated grid-like cylinder composed of a biocompatible nickel-titanium alloy.

Peer review

An interesting publication in which authors show that TIPS revision with the Fluency stent has higher medium-term patency rates than that with the Wallgraft stent. This study will be of interest and the paper is clearly written.

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BRIEF ARTICLE

Predicting a novel pathogenicity island in *Helicobacter pylori* by genomic barcoding

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Abstract

AIM: To apply a new, integrated technique for visualizing bacterial genomes to identify novel pathogenicity islands in *Helicobacter pylori* (*H. pylori*).

METHODS: A genomic barcode imaging method (converting frequency matrices to grey-scale levels) was designed to visually distinguish origin-specific genomic regions in *H. pylori*. The complete genome sequences of the six *H. pylori* strains published in the National Center for Biotechnological Information prokaryotic genome database were scanned, and compared to the genome barcodes of *Escherichia coli* (*E. coli*) O157:H7 strain EDL933 and a random nucleotide sequence. The following criteria were applied to identify potential pathogenicity islands (PAIs): (1) barcode distance distinct from that of the general background; (2) length greater than 10000 continuous base pairs; and (3) containing genes with known virulence-related functions (as determined by PfamScan and Blast2GO).

RESULTS: Comparison of the barcode images generated for the 26695, HPAG1, J99, Shi470, G27 and P12 *H. pylori* genomes with those for the *E. coli* and random sequence controls revealed that *H. pylori* genomes contained fewer anomalous regions. Among the *H. pylori*-specific continuous anomalous regions (longer than 20 kbp in each strain's genome), two fit the criteria for identifying candidate PAIs. The bioinformatic-based functional analyses revealed that one of the two anomalous regions was the known pathogenicity island *cag*-PAI, this finding also served as proof-of-principle for the utility of the genomic barcoding approach for identifying PAIs, and characterized the other as a novel PAI, which was designated as *tfs3*-PAI. Furthermore, the *cag*-PAI and *tfs3*-PAI harbored genes encoding type IV secretion system proteins and were predicted to have potential for functional synergy.

CONCLUSION: Genomic barcode imaging represents an effective bioinformatic-based approach for scanning bacterial genomes, such as *H. pylori*, to identify candidate PAIs.

Key words: *Helicobacter pylori*; Genome analysis; Pathogenicity islands; Genomic bar coding

Core tip: The genomic barcoding technology was recently developed to increase the accuracy of genome analysis, and has facilitated the identification of origin-

specific genomic regions of both eukaryotic and prokaryotic lifeforms. In this study, we applied the genomic barcode imaging approach to screen for pathogenicity islands (PAIs) in *Helicobacter pylori* using the six strains for which the complete genome sequences have been published and performing comparison to a common *Enterobacter* species. Bioinformatic-based functional analysis not only provided proof-of-principle (identifying the known *cag*-PAI) but also identified a novel PAI (designated as *tsf3*-PAI).

Wang GQ, Xu JT, Xu GY, Zhang Y, Li F, Suo J. Predicting a novel pathogenicity island in *Helicobacter pylori* by genomic barcoding. *World J Gastroenterol* 2013; 19(30): 5006-5010 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5006.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5006

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative pathogen that colonizes the stomachs of over half the world's population[1,2]. Despite being one of the most common chronic infections among humans, it often remains undiagnosed until an unknown trigger causes manifestation of gastric diseases (*e.g.*, gastritis[3], ulcers[4], and gastric carcinoma[5]) with varying degrees of symptom severity and outcome. Extensive research efforts have been dedicated to understanding the molecular mechanisms of *H. pylori* pathogenesis, and have identified several (*bona fide* and putative) classes of virulence factors, including adhesins[6,7], cytotoxins[8], and lipopolysaccharide (LPS)[9]. While LPS has received the majority of research attention in the *H. pylori* field, due to its prevalence among pathogenic bacteria and its well-characterized interactions with the Toll-like receptor 4 of the host innate immune system, systematic investigations of the cytotoxins have also elucidated the host-pathogen signaling interactions leading to pathogenic changes in the infected tissues. For example, the vacuolating cytotoxin (VacA) has been shown to induce apoptosis in epithelial cells, and the cytotoxin-associated antigen (CagA) has been shown to counteract the VacA-induced apoptosis to promote survival of infected host cells and facilitate stomach colonization[10].

Recent evidence has suggested that pathogenicity islands (PAIs) in the bacterial genome play an important role in pathogenesis[11,12]. PAIs are defined as large DNA fragments that have been acquired through horizontal transfer and which bear multiple genes encoding bacterial factors with virulence functions[13]. The genes located on each PAI serve as molecular markers for clinical testing to diagnose bacterial pathogens, estimate their pathogenic potential, and predict treatment response (*i.e.*, antibiotic resistance)[14]. Therefore, genomic scanning to determine the PAI profile of *H. pylori* will not only provide insights into the molecular evolution and pathogenic mechanisms of this important human pathogen but also identify putative targets for effective molecular therapies.

The advent of high-throughput sequencing technologies has allowed for the complete genome sequences of a large number of prokaryotes; in conjunction with the rapid accumulation of such minable data in publicly available databases, various *in silico* methods have been developed to detect PAIs[15,16]. Most of these methods depend on finding aberrant G + C content and/or bias in codon usage[17] among various genera and species. Yet, this approach produces a high frequency of false negative results due to post-transfer changes that naturally accumulate in the transferred fragments over the course of evolution in a new environment.

In our previous studies, we addressed the limitations of the *in silico* methods. It was found that when genome scanning was performed using a fixed window size of at least 1000 bp, the frequency of each κ-nucleotide sequence ($2 < \kappa < 7$) was highly stable across a whole genome[18]. As a result, we represented the κ-nucleotide sequence frequency distributions across a whole genome as a 2-D barcode-like image, which was designated as a genomic barcode. By visualizing the barcodes of each genome, we were able to easily identify those sequences of foreign origin, such as horizontally transferred genes[18].

In the current study, we applied the genomic barcode imaging technique to scan the *H. pylori* genome for PAIs. Both known (serving as a proof-of-principle finding) and novel PAIs were detected.

MATERIALS AND METHODS

Genome sequence data

Complete genomes of the 26695, HPAG1, J99, Shi470, G27 and P12 strains of *H. pylori*, as well as those of *Escherichia coli* (*E. coli*) O157:H7 strain EDL933 (serving as a control for comparative analysis), were downloaded from the National Center for Biotechnological Information FTP server (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/) in January 2012. In addition, a random nucleotide sequence was generated by a K-order Markov chain model for use as an additional control.

Generation of genomic barcode images

Each genome was partitioned into non-overlapping fragments of 1000 bp and a 4-nucleotide-based barcode was calculated for each genome. Specifically, the barcode for each genome is a matrix of N (4) columns and genome length/M rows, so that N (4) = 136, with the i^{th} value being the combined frequency of the i^{th} 4-nucleotide and its reverse complement in this fragment. The κ-nucleotide frequencies were then converted to grey-scale levels to visualize the overall barcode image profile for the whole genome. Darker grey levels represent lower frequencies.

Identification of PAIs

The following criteria were applied to identify potential PAIs: (1) barcode distance distinct from that of the general background; (2) length greater than 10000 continu-

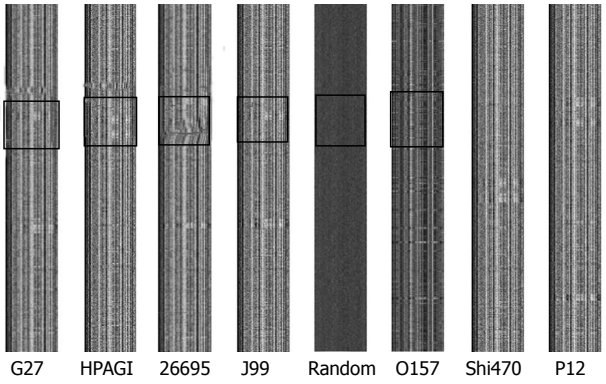

Figure 1 2-D barcode images of genomes of *Helicobacter pylori* strains J99, G27, 26695, HPAG1, P12, and Shi470, *Escherichia coli* O157:H7 strain EDL933, and a random sequence. The y-axis represents the genome axis from top-down, with each pixel representing a fragment of n = 1000 bp; the x-axis represents the 4-nucletide frequencies. The abnormal barcode regions are demarcated by a rectangle.

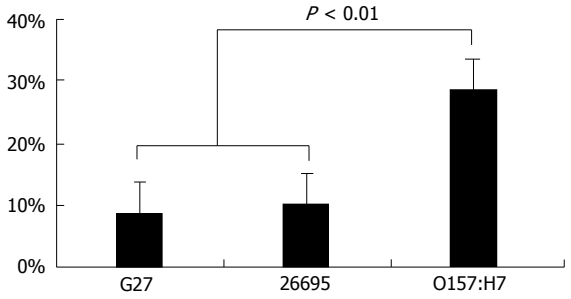

Figure 2 Fraction of anomalous fragments detected by genomic barcode imaging of *Helicobacter pylori* strains G27 and 26695, and *Escherichia coli* O157:H7 strain EDL933.

ous base pairs; and (3) containing genes with known virulence-related functions (as determined by PfamScan[19] and Blast2GO[20]).

Statistical analysis

The distance between two barcodes was calculated as the Euclidean distance between the corresponding 136-dimensional vectors. The distance database was built using Microsoft Excel spreadsheet software, and SPSS 13.0 statistical software was employed for analysis of the data using descriptive methods and the χ^2 test.

RESULTS

Visualization of H. pylori genomes based on genomic barcode images

Each genome was partitioned into a series of non-overlapping fragments of 1000 bp, and the combined frequencies of each 4-nucleotide/reverse complement were calculated. The frequency matrices converted to grey-scale are shown in Figure 1. The unique barcode image for each of these microbial genomes represents the underlying base composition. The 2-D barcode images of the *H. pylori* strains were similar to one another but distinct from that

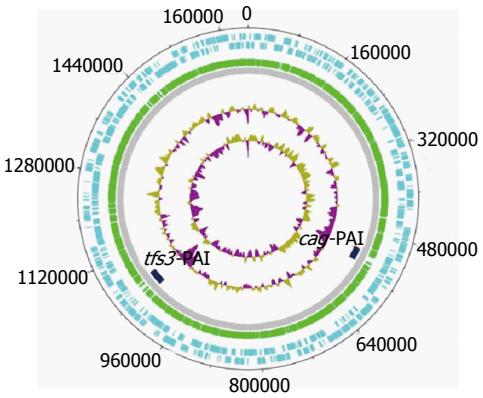

Figure 3 Circular representation of the *Helicobacter pylori* 26695 chromosome. The outermost (first) concentric circle denotes the predicted coding regions on the plus strand. The second concentric circle denotes the predicted coding regions on the minus strand. The third concentric circle denotes the predicted coding regions on both strands. The fourth concentric circle denotes the buffer zone. The fifth concentric circle denotes the predicted pathogenicity island (PAI) candidates. The sixth concentric circle denotes the guanine and cytosine (GC) content. The seventh concentric circle denotes the GC content. The figure was created using GenVision from DNASTAR.

of *E. coli*, demonstrating the close relationship of strains from the *H. pylori* species. It should be noted that no barcode structure was able to be produced for the random nucleotide sequence, indicating that the genomic barcode is an inherent property of the microbial genome.

Identification of H. pylori-specific genomic regions

While the genomes of different *H. pylori* strains possessed the conserved κ-nucleotide frequency producing the visual barcode, some regions appeared to have an abnormal structure. As shown in Figure 1, an abnormal band was apparent across the barcode image of the corresponding genome. In principle, these regions may have been acquired through horizontal gene transfer or derived from phage-mediated gene conversion.

The percentage of the anomalous regions in each genome are shown in Figure 2. As expected, the *H. pylori* strains contain fewer anomalous regions than *E. coli* ($P < 0.01$).

Identification of PAIs in H. pylori

We collected continuous anomalous fragments, longer than 20 kbp in each genome, and kept only those specific for most *H. pylori* genomes. In addition, some anomalous fragments found only in some *H. pylori* genomes, but subdivided into a number of discrete smaller segments in another *H. pylori* genome, were excluded from further analysis since such fragments may have resulted from frequent recombination events[21,22]. As a result of this procedure, two specific genome regions were selected as potential PAI candidates. Figure 3 and Table 1 show the position of these two candidate PAIs in *H. pylori*.

The bioinformatic-based functional analyses revealed that one of the two anomalous regions was the known pathogenicity island *cag*PAI, this finding also served as proof-of-principle for the utility of the genomic barcoding approach for identifying PAIs, and characterized the

Table 1 Pathogenicity island candidates in sequenced *Helicobacter pylori* genomes

	Size	GC	Barcodedistance	ORF
Wholegenome	1.5-1.7 Mbp	38.0% ± 0.2%	114.3 ± 14.9	-
cag-PAI	35 kbp	35.4% ± 0.8%	134.6 ± 20.1	20.0 ± 0.6
tfs3-PAI	30 kbp	33.0% ± 0.8%	138.0 ± 20.0	17.0 ± 3.0

PAI: Pathogenicity island; ORF: Open reading frame; GC: Guanine and cytosine.

other as a novel PAI, which was designated as *tfs3*-PAI and was located at the 3' end of the *Ser*-tRNA gene.

Identification of genes in cag-PAI and tfs3-PAI and prediction of the pathogenic role for each

We verified that the genes located in *cag*-PAI encode components of the type Ⅳ secretion system (T4SS), as characterized by previous studies[22-24].

Compared with *cag*-PAI, *tfs3*-PAI displayed some sequence variability due to rearrangements. The *tfs3*-PAI consisted of three distinct domains separated by mobile genetic elements. The first module contained the largest number of genes and encoded mobile sequence elements including a transposase (IS605), which is an essential element for a PAI. The second module encoded homologous genes of *tfs3* gene clusters, which formed a T4SS. The function of the *tfs3* gene cluster is not yet known, but it may play a role in bacterial conjugation and host cell signaling complementary to that of the *cag*-PAI-encoded system, which indicates a functional synergy. Most genes of the third module encoded hypothetical genes; as these genes have no orthologs in the databases, it is not clear at this point how many of them are in fact pseudogenes. It worth noting that *tfs3*-PAI consists of 17 open reading frames, six of which encode homologous genes of the T4SS. Therefore, this region may be related to pathogenesis in gastroduodenal diseases, and may represent a useful target for new vaccines and antibiotics.

DISCUSSION

The first potential PAI was *cag*-PAI, a well-known pathogenicity island in *H. Pylori*[25]. This approximately 35 kbp cluster of genes was acquired through horizontal transfer from an unknown extraneous source and integrated into the *H. pylori* chromosome. It is known that, compared to Enterobacteriaceae, *H. pylori* has less opportunity to obtain foreign genes by horizontal transfer since only a few bacterial species colonize human stomachs. Indeed, a previous microarray-based study of a larger strain collection suggested that up to 10% of all genes in an individual isolate may be accessory genes[26], which corroborates our finding.

T4SS is one of at least six specialized secretion systems characterized in bacteria. Usually consisting of 12 components, T4SS plays various functions in transporting a wide range of components, from single protein to protein-protein complexes and protein-DNA complexes. Moreover, T4SS facilitates injection of bacteria-encoded effectors into host cells during the infection process. The *cag*-PAI-encoded secretion systems have been implicated in modulation of bacteria-host interactions, interference with host signal-transduction pathways, and promotion of apoptosis, to name a few[22-24].

Pathogenesis of *H. pylori* is a multi-stage process. It is likely that multiple bacterial and host mechanisms are involved; however, a long-standing dogma of infectious biology claims that PAIs of *H. pylori* are stable entities and could be robustly correlated with disease progression or outcome. Screening and functional analysis of PAIs in *H. pylori*, as developed and demonstrated in this study, will aid in the development of more accurate and timely diagnosis and improved control of this common pathogen.

COMMENTS

Background

Recent evidence suggests that pathogenicity islands (PAIs) play an important role in bacterial pathogenesis. Scanning of PAIs in the *Helicobacter pylori* (*H. pylori*) genome will provide insights into the molecular evolution and pathogenic mechanisms of this important human pathogen but also identify putative targets for effective molecular therapies.

Research frontiers

Auhtors have applied the genomic barcode imaging technique to scan PAIs in *H. pylori*. Bioinformatic-based functional analysis not only provided proof-of-principle (identifying the known *cag*PAI) but also identified a novel PAI (designated as *tsf3*-PAI).

Innovations and breakthroughs

A novel PAI, *tsf3*-PAI, was detected in *H. pylori* using the genomic barcode imaging technique. Bioinformatic-based functional analysis revealed that *tsf3*-PAI encodes a type Ⅳ secretion system (T4SS) which may functionally synergize with the T4SS encoded by *cag*-PAI.

Applications

The genomic barcode imaging technique is useful for identifying known and novel PAIs in bacterial genomes. The PAIs identified in this study may be related to the manifestation of *H. pylori*-induced gastroduodenal diseases, and may represent useful targets of new molecular therapies or vaccines.

Terminology

The genomic barcode is generated by measuring the κ-nucleotide sequence frequency distributions across a whole genome using a fixed window size of at least 1000 bp. The 2-D barcode-like image is generated by converting the frequency matrices to grey-scale levels.

Peer review

This manuscript applied genomic barcodes to screen for PAIs in *H. Pylori*, which showed that genomic barcode technique is more usefulness and accuracy tool for genome analysis so far. The proof-of-principle work showed that one known and one novel PAI could be detected using this technique.

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BRIEF ARTICLE

Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection for circumferential mixed hemorrhoids

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Abstract

AIM: To identify a more effective treatment protocol for circumferential mixed hemorrhoids.

METHODS: A total of 192 patients with circumferential mixed hemorrhoids were randomized into the treatment group, where they underwent Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, or the control group, where traditional external dissection and internal ligation were performed. Postoperative recovery and complications were monitored.

RESULTS: The time to wound healing was 12.96 ± 2.25 d in the treatment group shorter than 19.58 ± 2.71 d in the control group. Slight pain rate was 58.3% in the treatment group higher than 22.9% in the control group; moderate pain rate was 33.3% in the treatment group lower than 56.3% in the control group severe pain rate was 8.4% in the treatment group lower than 20.8% in the control group. No edema rate was 70.8% in the treatment group higher than 43.8% in the control group; mild local edema rate was 26% in the treatment group lower than 39.6% in the control group obvious local edema was 3.03% in the treatment group lower than 16.7% in the control group. No stenosis rate was 85.4% in the treatment group higher than 63.5% in the control group; moderate stenosis rate was 14.6% in the treatment group Lower than 27.1% in the control group severe anal stenosis rate was 0% in the treatment group lower than 9.4% in the control group.

CONCLUSION: Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection is the optimal treatment for circumferential mixed hemorrhoids and can be widely applied in clinical settings.

Key words: Milligan-Morgan hemorrhoidectomy; Mixed hemorrhoids; Anal cushion; Internal sphincter

Core tip: We treated 96 patients with circumferential mixed hemorrhoids using Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, and compared their clinical outcomes with those undergoing traditional hemorrhoidectomy. The differences are significant in favor of the modified Milligan-Morgan technique in terms of time to wound healing, anal stenosis, wound pain, edema and other complications. This approach can be widely applied in clinical practice.

Lu M, Shi GY, Wang GQ, Wu Y, Liu Y, Wen H. Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection for circumferential mixed hemorrhoids. *World J Gastroenterol* 2013; 19(30): 5011-5015 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5011.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5011

INTRODUCTION

The treatment of circumferential mixed hemorrhoids is challenging for medical providers. Despite a number of reports on the surgical options at home and abroad, no effective treatment method is currently available[1]. The traditional "external dissection and internal ligation" or Milligan-Morgan technique, is the mainstream treatment for mixed hemorrhoids. However, with larger and more hemorrhoids involved in the circumferential type, surgery becomes more complicated and is inevitably associated with a range of post-operative complications[2]. The most common and serious complication is anal stenosis and incontinence, which results in significant pain in affected patients[3-6]. We treated 96 patients with circumferential mixed hemorrhoids using Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, and compared their clinical outcomes with those undergoing traditional hemorrhoidectomy. The differences were significant in favor of the former group, as shown below.

MATERIALS AND METHODS

General information

A select group of 192 patients with circumferential mixed hemorrhoids treated in our department from August 2010 to November 2012 were randomized into two groups, with 96 patients in each group. The 96 patients in the treatment group underwent Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, while the patients in the control group were treated with the traditional Milligan-Morgan technique. The patients comprised 98 men and 94 women aged 26 to 65 years, with a mean age of 48.5 years. Disease duration ranged between 7 and 48 mo, with an average of 26.5 mo. The two groups were comparable as there were no significant differences in terms of age, gender, and disease duration.

Methods

Treatment group: Each patient was placed in the lateral position after caudal or spinal anesthesia for routine disinfection and draping. Hemorrhoids of higher grades situated in the 3, 7 and 11 o'clock positions were treated in most cases. A V-shape was made from the body of an external hemorrhoid to the anal margin using scissors. With complete exposure after anal dilatation, each internal hemorrhoid was slightly pulled towards the outside with forceps for high suspension and ligation. To improve the effect of suspension, the rectal mucosa 1-2 cm above the internal hemorrhoid was lifted with tissue forceps, clamped with the base of the hemorrhoid that was carried by curved forceps, and ligated with a 10-gauge silk suture at 0.5 cm away from the dentate line, without injuring it. The internal hemorrhoids at the base were individually ligated in the same way, keeping the ligated bodies at different levels of the anal canal with sufficient mucosal bridges between them to prevent postoperative anal stenosis. Each external hemorrhoid was then lifted with forceps and dissected along the V-shaped incision. Subcutaneous varicose veins were stripped off and bleeding was managed appropriately. All external hemorrhoids were treated in the same way. Sufficient flaps were retained between each incision to avoid anal stenosis due to anal skin defects. Finally, the lower edge of the internal sphincter was divided and cut off at the interscalene in the 3 or 9 o'clock position. A second-generation cephalosporin was administered for 2 d after the procedure to prevent infection.

Control group: The traditional external dissection and internal ligation method was used to treat the patients in this group. Postoperative treatment was the same as that in the treatment group.

Outcome evaluation

The therapeutic efficacy was evaluated according to the Diagnosis and Efficacy Standards in Traditional Chinese Medicine issued by the State Administration of Traditional Chinese Medicine of China[7].

The severity of anal stenosis was classified as: (1) no stenosis: index finger can pass smoothly; stool passes easily, without pain or discomfort; mild stenosis: index finger can pass with difficulty; stool passes relatively easily, without obvious pain; (2) moderate stenosis: only the first joint of the index finger can pass; stool passes with difficulty and anal pain; and (3) severe anal stenosis: only the little finger can pass through; stool passes with difficulty and obvious anal pain.

The degree of pain was classified as: grade 0: no pain; grade 1: mild pain; the anal pain is slight and tolerable without disturbing normal life and sleep; no significant changes in mood; grade 2 (moderate): obvious pain; the anal pain is so intense that analgesics are required, normal life and sleep are compromised, and mood changes are present, such as irritability; the condition is still under control with typical analgesics; grade 3 (severe): severe pain; the pain is intolerable and seriously disturbing normal life and sleep, causing autonomic dysfunction; analgesic drugs are necessary. The degree of anal margin edema was classified into: grade 0: no edema; grade 1: mild local edema, without affecting normal activity; and grade 2: obvious local edema, restricting normal activity.

Statistical analysis

The data were processed using SPSS 16.0 for statistical analysis with both the χ^2 test and t test. A P value of less

than 0.05 was considered statistically significant.

RESULTS

Following treatment: the time to wound healing was 12.96 ± 2.25 d in the treatment group shorter than 19.58 ± 2.71 d in the control group, the difference was statistically significant (t = 32.52, P = 0.000). Slight pain rate was 58.3% in the treatment group higher than 22.9% in the control group, the difference was statistically significant (χ^2 = 24.961, P = 0.000); moderate pain rate was 33.3% in the treatment group lower than 56.3% in the control group, the difference was statistically significant (χ^2 = 10.194, P = 0.001); severe pain rate was 8.4% in the treatment group lower than 20.8% in the control group, the difference was statistically significant (χ^2 = 6.021, P = 0.014). No edema rate was 70.8% in the treatment group higher than 43.8% in the control group, the difference was statistically significant (χ^2 = 14.389, P = 0.000); mild local edema rate was 26% in the treatment group lower than 39.6% in the control group, the difference was statistically significant (χ^2 = 3.993, P = 0.046); obvious local edema was 3.03% in the treatment group lower than 16.7% in the control group, the difference was statistically significant (χ^2 = 9.872, P = 0.002). No stenosis rate was 85.4% in the treatment group higher than 63.5% in the control group, the difference was statistically significant (χ^2 = 12.084, P = 0.001); moderate stenosis rate was 14.6% in the treatment group lower than 27.1% in the control group, the difference was statistically significant (χ^2 = 4.547, P = 0.033); severe anal stenosis rate was 0% in the treatment group lower than 9.4% in the control group, the difference was statistically significant (χ^2 = 7.461, P = 0.006).

DISCUSSION

Arising above or beneath the dentate line, the mixed hemorrhoid is a condition where the internal and external hemorrhoidal plexuses of veins merge, presenting the features of both internal hemorrhoid and external hemorrhoid[8]. The circumferential mixed hemorrhoids, as the severe type of the disease, are particularly difficult in surgical treatment. The conventional Milligan-Morgan[9-11] hemorrhoidectomy has been the globally recognized "golden standard" for circumferential mixed hemorrhoids[12]. However, it is limited by severe pain[13,14] after surgery, prolonged wound healing time, and complications such as anal stenosis[15]. In fact, it is an extremely painful procedure for the patients, Anal function and feeling fine and other defects be affected[16]. Based on these results, there were significant differences in favor of the modified Milligan-Morgan technique in terms of time to wound healing, anal stenosis, wound pain, edema and other complications. Anal stenosis is a critical factor that compromises the treatment effect and wound healing, resulting in significant inconvenience to the patient's life and work. Hence, caution should be taken to reduce the risk of this complication. It is proved that reduction of the anal cushions may trigger anal incontinence[17]. However, the conventional Milligan-Morgan hemorrhoidectomy can not achieve the ring-shaped resection of all external hemorrhoids, and anal stenosis can easily occur due to excessive resection. In addition, the massive damage of the dentate line can result in the change of anal function[18]. The modified Milligan-Morgan technique is designed to minimize anal stenosis, anal margin edema, incision pain, and other undesirable factors. During surgery, the anal cushions can be retained by suspending and ligating internal hemorrhoids and mucosa above the dentate line, minimizing injury to the anal cushions[19] and the dentate line. Anal cushions are the normal structures above the dentate line. They have certain immune and endocrine functions, and can effectively cause anal reflex, ensuring normal continence and defecation[20,21]. Bowel movement is induced at the dentate line, which should be preserved during surgery. Destruction of this area may lead to prolapse and incontinence of anal cushions, thus protection of anal cushions and the dentate line is essential for normal anal function after surgery[22-24]. Using the modified operation, only lesions are removed, and normal anal cushions are retained, ensuring normal function of the anus. Sufficient skin bridges and mucosal bridges are required between adjacent incisions to avoid the formation of a mucosal tension band, which may lead to anal stenosis. In addition, as part of the anorectal smooth muscle, the internal sphincter is prone to spasm due to its contractile properties, resulting in spastic pain after surgery and subsequently worsened edema, affecting wound healing. Therefore, the internal sphincter is partially resected during surgery to relieve persistent spasm and reduce the pressure of the sphincter to decrease the anal resting pressure and restore normal blood and lymph circulation. In this way, postoperative anal margin edema can be reduced or avoided, ensuring less pain and better wound healing. Based on our experience, retention of anal cushions and partial resection of the internal sphincter results in considerable benefits in the treatment of mixed hemorrhoids, including: (1) definite efficacy, less invasiveness, faster healing after surgery, and a shorter treatment course; (2) reduced sequelae, less damage to the anal cushions, and maximal retention of the anal canal, anal function and normal structure; (3) fewer complications, effectively reducing the incidence of postoperative hemorrhage, edema, urinary retention and anal stenosis; (4) suspension and high ligation of internal hemorrhoids can elevate the anal cushions that have moved downward in a similar way to the procedure for prolapse and hemorrhoids; this simple and cost-effective operation can be applied in various hospitals of different levels; and (5) with the preserved and elevated anal cushions, external hemorrhoids are also significantly reduced, making it possible for uncompromised, refined postoperative management.

In conclusion, Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphinc-

ter resection is the optimal treatment for circumferential mixed hemorrhoids, as it effectively removes hemorrhoids while retaining the anal canal anatomy and physiology, complying with the physiological function, pathological changes and anorectal dynamics of the anus[25]. In addition, this surgical method overcomes the drawbacks of traditional techniques by eliminating continued spasm of the internal sphincter, and reducing postoperative pain, edema, anal stenosis and other complications. This modified technique is a valuable approach in the treatment of circumferential mixed hemorrhoids resulting in a shorter time to wound healing, improved quality of surgery and can be widely applied in clinical settings.

COMMENTS

Background

The treatment of circumferential mixed hemorrhoids is a challenging procedure for medical providers. Despite a number of reports on the surgical options at home and abroad, no effective treatment method is currently available. The traditional "external dissection and internal ligation" or Milligan-Morgan technique, is a mainstream treatment for mixed hemorrhoids. With larger and more hemorrhoids involved in the circumferential type, however, the surgery becomes more complicated and is inevitably associated with a range of post-operative complications.

Research frontiers

The authors treated 96 patients with circumferential mixed hemorrhoids using Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection, and compared their clinical outcomes with those undergoing traditional hemorrhoidectomy. The differences are significant in favor of the modified Milligan-Morgan technique in terms of time to wound healing, anal stenosis, wound pain, edema and other complications. This approach can be widely applied in clinical practice.

Innovations and breakthroughs

Milligan-Morgan hemorrhoidectomy with anal cushion suspension and partial internal sphincter resection is an optimal treatment of circumferential mixed hemorrhoids, which effectively removes hemorrhoids while retaining the anal canal anatomy and physiology, complying with the physiological function, pathological changes and anorectal dynamics of the anus. Meanwhile, it overcomes the drawbacks of traditional techniques by eliminating continued spasm of the internal sphincter, and reducing postoperative pain, edema, anal stenosis and other complications.

Applications

The modified Milligan-Morgan hemorrhoidectomy provides a valuable approach to the treatment of circumferential mixed hemorrhoids with shortened time to wound healing, and improved the quality of surgery, which can be widely applied in clinical settings.

Terminology

The traditional "external dissection and internal ligation" or Milligan-Morgan technique, is a mainstream treatment for mixed hemorrhoids. PPH stands for "procedure for prolapse and hemorrhoids". With the new PPH or stapled hemorrhoidectomy there is no need to cut out the hemorrhoid tissue at the anus.

Peer review

In this manuscript, the authors made a good study of 96 cases with circumferential mixed hemorrhoids. The paper is well written and interesting for the readers.

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CASE REPORT

Bone metastasis from early gastric cancer following non-curative endoscopic submucosal dissection

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Abstract

A 67-year-old male underwent endoscopic submucosal dissection (ESD) to treat early gastric cancer (EGC) in 2001. The lesion (50 mm × 25 mm diameter) was histologically diagnosed as poorly differentiated adenocarcinoma, with an ulcer finding. Although the tumor was confined to the mucosa with no evidence of lymphovascular involvement, the ESD was regarded as a non-curative resection due to the histological type, tumor size, and existence of an ulcer finding (as indicated by the 2010 Japanese gastric cancer treatment guidelines, ver. 3). Despite strong recommendation for subsequent gastrectomy, the patient refused surgery. An alternative follow-up routine was designed, which included five years of biannual clinical examinations to detect and measure serum tumor markers and perform visual assessment of recurrence by endoscopy and computed tomography scan after which the examinations were performed annually. The patient's condition remained stable for eight years, until a complaint of back pain in 2010 prompted further clinical investigation. Bone scintigraphy indicated increased uptake. Histological examination of biopsy specimens taken from the lumbar spine revealed adenocarcinoma resembling the carcinoma cells from the EGC that had been treated previously by ESD, and which was consistent with immunohistochemical findings of gastrointestinal tract cancer. Thus, the diagnosis of bone metastasis from EGC was made. The reported rates of EGC recurrence in surgically resected cases range 1.4%-3.4%, but among these bone metastasis is very rare. To our knowledge, this is the first reported case of bone metastasis from EGC following a non-curative ESD and occurring after an eight-year disease-free interval.

Key words: Endoscopic submucosal dissection; Early gastric cancer; Non-curative resection; Bone metastasis; Late recurrence

Core tip: This case report provides the first description of an adult male with bone metastasis from early gastric cancer following an eight-year disease-free interval after endoscopic submucosal dissection (ESD). Although the original tumor was confined to the mucosa, with no evidence of lymphovascular involvement, the ESD was regarded as a non-curative resection based upon the tumor's histological type and size, and existence of an ulcer finding. If any patient, who is otherwise fit, initially refuses surgery and requests a contraindicated ESD, efforts should be made to persuade the patient to undergo a gastrectomy with lymph-node dissection.

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gastric cancer following non-curative endoscopic submucosal dissection. *World J Gastroenterol* 2013; 19(30): 5016-5020 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5016.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5016

INTRODUCTION

Endoscopic resection remains the preferred treatment modality for cases of early gastric cancer (EGC) with a negligible risk of lymph-node metastasis[1-3]. The 2010 Japanese gastric cancer treatment guidelines (ver. 3) provide absolute indications and expanded indications for performing endoscopic resections, including endoscopic submucosal dissection (ESD), to treat these cases[4]. In contrast, EGC cases with possible risk of lymph-node metastasis generally require surgical treatment.

Here, we describe our clinical experience with a case of bone metastasis from EGC that developed after an eight-year disease-free interval following non-curative ESD. To our knowledge, this case represents the first of its kind to be reported in the literature. In this particular case, ESD was clinically contraindicated because the EGC consisted of an undifferentiated type adenocarcinoma with an ulcer finding, but was performed in accordance with the patient refusing surgical intervention despite our strong recommendation to the contrary.

CASE REPORT

In 2001, a 58-year-old man was admitted to our hospital upon detection of EGC by screening endoscopy. Physical examination and laboratory data revealed no abnormalities, and the patient had no relevant personal or family history. The endoscopic findings indicated a superficial depressed type (0-Ⅱc) EGC confined to the mucosa (in terms of invasion depth), along with an ulcer (50 mm) located in the gastric angle (Figure 1). Histological examination of the biopsied specimens indicated poorly- to moderately-differentiated adenocarcinoma. Ultrasound and computed tomography (CT) examinations revealed no metastasis.

Based on the 2010 Japanese gastric cancer treatment guidelines[4], endoscopic resection was not indicated for this lesion. The patient was informed of the possible risk of lymph-node metastasis and the need for a surgical resection; however, the patient refused surgery and the ESD was performed in October 2001 (Figure 2). Histological examination of the resected tissues revealed the tumor (50 mm × 25 mm diameter) to be primary composed of poorly-differentiated adenocarcinoma and confirmed the proximal ulcer (Figure 3). The tumor was confined to the mucosa, with no evidence of lymphovascular involvement. Both the vertical and horizontal margins were free of tumor cells. However, a slight tear was observed inside the lesion and was considered to have occurred incidentally during the ESD.

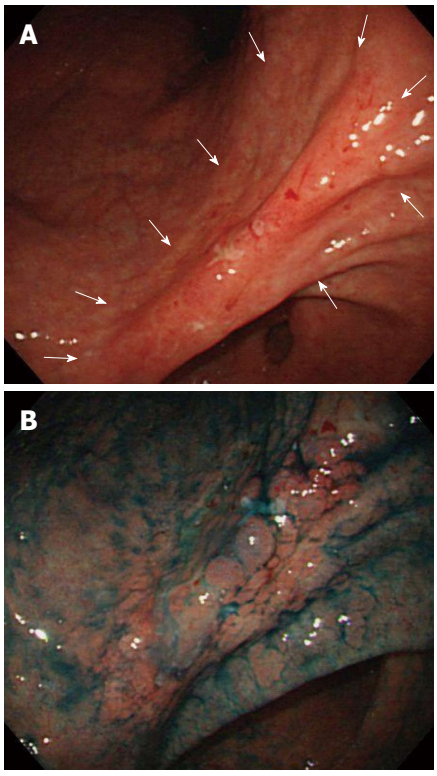

Figure 1 Endoscopic findings upon initial admission for early gastric cancer. A: Conventional endoscopy showing a superficial depressed type lesion, 50 mm in size with an ulcer scar located in the gastric angle (arrows); B: Chromoendoscopy with indigo-carmine dye (blue) showing the lesion margin.

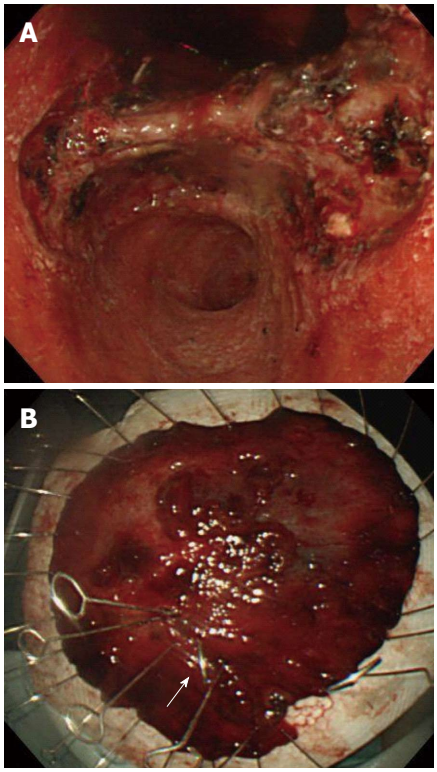

Figure 2 *In situ* locale of the lesion and gross appearance of the resected tumor. A: Intraoperative image taken during the endoscopic submucosal dissection (ESD) procedure. ESD was performed in October 2001; B: A tear (arrow) was present inside the lesion and likely was incident to the ESD procedure.

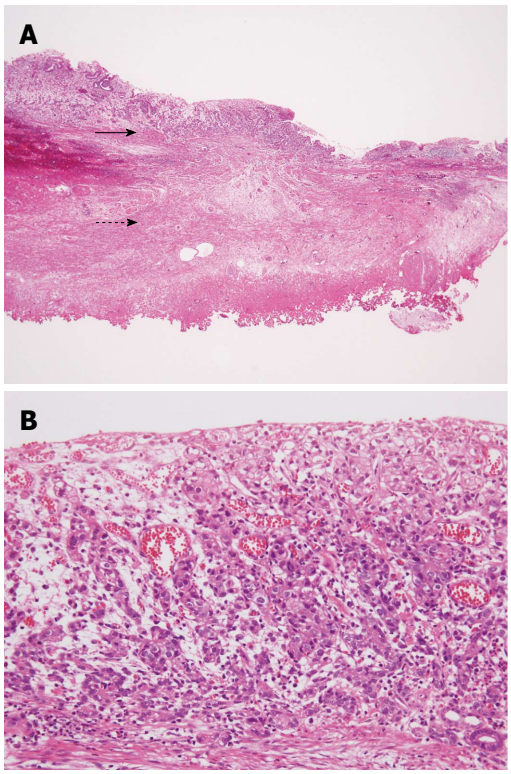

Figure 3 Histological findings of the endoscopic submucosal dissection-resected lesion tissues. A: The tumor is confined to the mucosa (solid arrow) and an ulcer scar (dashed arrow) (magnification: × 20); B: The tumor is composed of poorly differentiated adenocarcinoma with signet ring cells (magnification: × 100).

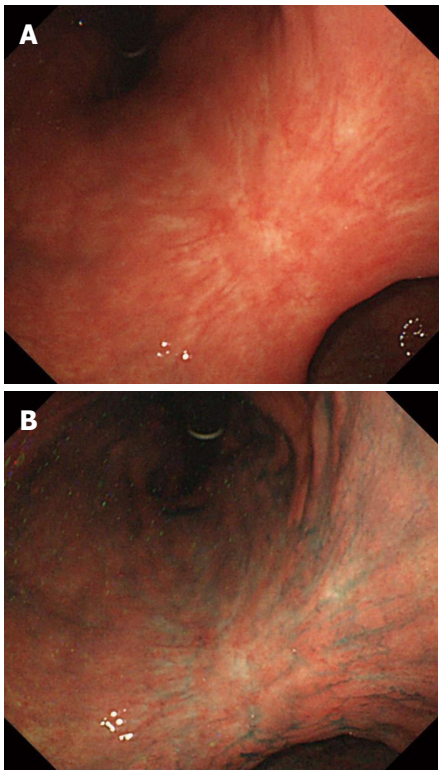

Figure 4 No endoscopic evidence of recurrence was found at the endoscopic submucosal dissection site eight years after endoscopic submucosal dissection. A: Conventional endoscopy; B: Chromoendoscopy with indigocarmine dye. Endoscopic examination provided no indications of recurrence at the endoscopic submucosal dissection site.

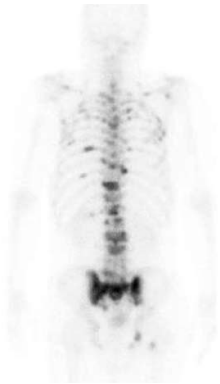

Figure 5 Bone scintigraphy results from the eight-year follow-up showing increased uptake in the spine and pelvic bone.

Since the ESD was regarded as a non-curative resection, we repeated our strong recommendations for gastrectomy, but the patient continued to refuse. As a result, we designed a monitoring strategy in which the patient would present to clinic biannually for testing of serum tumor markers and endoscopic and CT examinations; after five years with no remarkable findings, the clinical follow-ups were decreased to once a year. The patient's condition remained stable, with no evidence of recurrence, for eight years after the ESD.

In April 2010, however, the patient presented with a complaint of back pain. Serum testing revealed a remarkably enhanced level of alkaline phosphatase (1172 IU/L, normal range: 120-340 IU/L), but endoscopic examination provided no indications of recurrence at the ESD site (Figure 4). Bone scintigraphy indicated increased uptake in the spine and pelvic regions (Figure 5). Histological examination of biopsied specimens taken from the lumbar spine revealed a poorly-differentiated adenocarcinoma that resembled the carcinoma cells in the original ESD-treated EGC (Figure 6A). Immunohistochemical examination showed the tumor cells were positive for caudal-type homeobox transcription factor 2, which is consistent with gastrointestinal tract cancer (Figure 6B). There were no findings of malignancy in any other organs. Thus, the diagnosis was made of bone metastasis from the EGC following a non-curative ESD after an eight-year disease-free interval.

The patient received chemotherapy (methotrexate + fluorouracil), but developed disseminated intravascular coagulopathy and died in August 2011.

DISCUSSION

This is the first report of bone metastasis from EGC after an eight-year disease-free interval following non-curative ESD. This case presents several aspects for discussion, including contraindicated ESD, metastasis limited to bone, and late recurrence.

A recent report of EGC indicated a lower survival

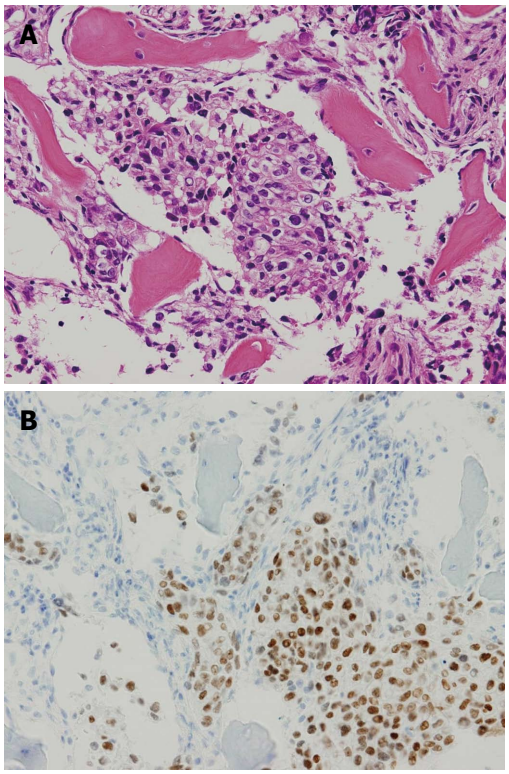

Figure 6 Histological findings of the biopsy specimen taken from the lumbar spine. A: The biopsy specimen taken from the lumbar spine revealed a poorly differentiated adenocarcinoma histologically resembling the carcinoma cells in the early gastric cancer treated previously by endoscopic submucosal dissection; B: Using immunohistochemical staining, the tumor cells were positive for caudal-type homeobox transcription factor 2.

rate after contraindicated ESD because of clinically diagnosed submucosal invasion and concluded that ESD was ineffective in such cases[5]. Although the case described herein did not involve clinically diagnosed submucosal invasion, ESD was contraindicated because of a possible risk of lymph-node metastasis due to the undifferentiated type adenocarcinoma with an ulcer finding. The risk of lymph-node metastasis has been reported as 13.4% for undifferentiated type EGC with ulcer finding, and as 5.9% in undifferentiated intramucosal cancer[6]. Bone metastasis is a hematogenous metastasis, so it may not have been avoided in the present case even if the patient had undergone a gastrectomy with lymph-node dissection. Nonetheless, we believe that gastrectomy would likely have provided some benefit to the patient, who was fit for surgery.

Bone metastasis is relatively common in patients with advanced breast, lung and prostate cancer, but not in gastric cancer patients (occurring in only 0.99%-2.1% of advanced cases)[7,8]. Recurrence of EGC has been reported to be in the range of 1.4%-3.4% for surgically resected cases, but among these bone metastasis is very rare[9]. In a previous investigation of 1452 EGC patients treated with curative resection, Lee *et al*[10] found that 21 patients (1.4%) experienced recurrence, including 4 (19.0%) with local lymph-node recurrence, 2 (9.5%) with peritoneal dissemination, 9 (42.9%) with distant metastasis, and 6 (28.6%) with mixed type recurrence. Similarly, Kobayashi *et al*[9] reviewed Japanese case reports to determine the characteristics of bone metastasis in EGC for this patient population. Among the tumors, 51.4% had infiltrated the mucosa and 48.6% had infiltrated the submucosa, 65.0% of the metastases were metachronous and 35.0% were synchronous, 18.8% of the primary tumors were differentiated and 81.2% were undifferentiated, and lymph-node metastasis was present in 55.2% of the patients.

In our case, the metastasis that was diagnosed after a lengthy interval (eight years) following non-curative ESD was only found in bone. One possible explanation of such a late recurrence involves the concept of tumor dormancy, a state in which cancer cells exist with no to low-level activity concomitant to normal, healthy tissues until the dormancy is disturbed by an instigating event, such as infection or immunosuppression[11]. In such a case, the activated cancer cells will have a negative impact on the prognosis of the cancer patient, regardless of the duration of the disease-free interval. Recurrence of gastric cancer is generally believed to occur within five years of the primary surgery, but there are some reports of very late recurrence, including bone metastasis after surgery, and particularly involving EGC cases[9,12-14]. It may be necessary, therefore, to follow-up EGC patients for more than five years, especially those who were treated by ESD in lieu of surgical resection or those with expanded indications.

The number of patients who undergo ESD instead of surgery has increased in recent years, in part because the indications have been expanded[3,5,15,16]; however, it is important to apply the indications properly. If any patient, who is otherwise fit, initially refuses surgery and requests a contraindicated ESD, all efforts should be made to persuade the patient to undergo a gastrectomy with lymph-node resection in order to optimize the patient prognosis.

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CASE REPORT

Mucocele of the appendix due to endometriosis: A rare case report

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Abstract

Mucocele of the appendix due to endometriosis is extremely rare, and there are only 10 previously reported cases in the English literature. We report a case of mucocele of the appendix due to endometriosis and provide the first review of the literature. A 43-year-old woman was admitted to the hospital because of recurrent right lower abdominal pain during her menstrual periods. Colonoscopy revealed submucosal tumor-like elevations of the appendiceal orifice. Computed tomography and magnetic resonance imaging of the abdomen suggested cystic lesions near the appendix. Consequently, mucocele of the appendix was suspected preoperatively. An open ileocecal resection was performed. Multiple cystic lesions were observed around the appendix. The cystic lesions contained mucus. Histopathological examination was consistent with a mucocele of the appendix due to endometriosis. The postoperative course was uneventful. We present the first review of the literature to clarify the clinical features.

Key words: Mucocele; Appendix; Endometriosis

Core tip: We report an extremely rare case of mucocele of the appendix due to endometriosis. Although it is uncommon, preoperative diagnosis of the mucocele is important; however, diagnosis is difficult using imaging modalities. We report a more accurate diagnostic possibility using preoperative imaging modalities, such as colonoscopy, ultrasonography, computed tomography, and magnetic resonance imaging. Furthermore, this report is important because it is the first review of the literature for mucocele of the appendix due to endometriosis.

Tsuda M, Yamashita Y, Azuma S, Akamatsu T, Seta T, Urai S, Uenoyama Y, Deguchi Y, Ono K, Chiba T. Mucocele of the appendix due to endometriosis: A rare case report. *World J Gastroenterol* 2013; 19(30): 5021-5024 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i30/5021.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i30.5021

INTRODUCTION

Mucocele of the appendix is an uncommon disease. It is observed in 0.2%-0.3% of appendectomies and 8%-10% of appendiceal tumors[1]. According to the modern classification[2,3], mucocele of the appendix includes four histological groups: simple mucocele, mucosal hyperplasia, mu-

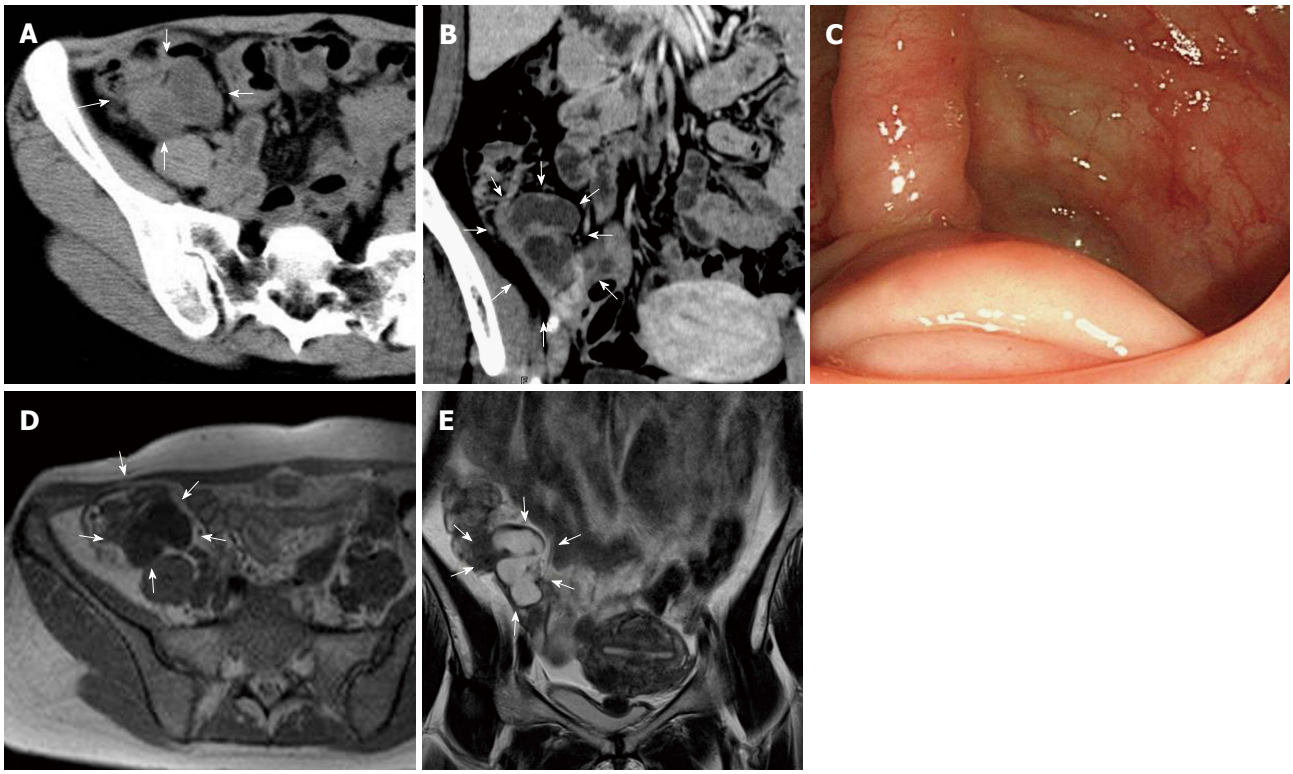

Figure 1 Preoperative image of the mucocele. A: Computed tomography (CT) (axial image); a tumor was detected (arrows); B: CT (coronal image); a tumor was detected (arrows); C: Colonoscopy; submucosal tumor-like elevations of the appendiceal orifice; D: Magnetic resonance imaging (MRI) (T1-WI, axial image); E: MRI (T2-WI, coronal image).

cinous cystadenoma, and mucinous cystadenocarcinoma. Simple mucocele is caused by mucus distention secondary to an obstruction of the appendix due to fecaliths, post-inflammatory scarring, or rarely, endometriosis. There are only 10 previously reported cases of mucocele of the appendix due to endometriosis in the English literature[4-12]. We present a rare case of mucocele of the appendix due to endometriosis and a review of the literature.

CASE REPORT

A 43-year-old woman was admitted to the hospital because of recurrent right lower abdominal pain during her menstrual periods. On physical examination, she presented with mild right lower abdominal tenderness without rigidity. Her blood and urine tests were normal. Abdominal computed tomography (CT) revealed low-density lesions near the appendix. Colonoscopy revealed submucosal tumor-like elevations of the appendiceal orifice. A biopsy was performed and result was negative. Subsequent magnetic resonance imaging (MRI) revealed hyperintensity on T2-weighted imaging (WI) (Figure 1). Consequently, mucocele of the appendix was diagnosed preoperatively. Her recurrent right lower abdominal pain during menstrual periods suggested the involvement of endometriosis. Our patient was offered open surgical resection because malignancy could not be ruled out. During surgery, several cystic lesions were observed around the appendix. Features of endometriosis were not observed in the pelvis, the uterus, or the rest of the abdominal cavity. An open ileocecal resection was performed. Multiple cystic lesions were observed around the appendix. The contents of the cystic lesions consisted of mucus (Figure 2). Histopathological examination indicated that the cysts were a simple type of mucocele and that endometriosis and smooth muscle hypertrophy were present in the muscle layer of the appendix around the mucocele (Figure 3). Consequently, we reached a diagnosis that the mucocele of the appendix was due to endometriosis. The postoperative course was uneventful.

DISCUSSION

A mucocele of the appendix is a rare lesion. It occurs in 0.2%-0.3% of all appendectomies performed and 8%-10% of all resected appendiceal tumors[1]. Endometriosis of the appendix is also a rare lesion, and is observed in 0.054%-0.8% of all appendectomies performed[13-15]. Finally, mucocele of the appendix due to endometriosis is extremely rare.

According to the modern classification[2,3], mucocele of the appendix includes four histological groups: simple mucocele, mucosal hyperplasia, mucinous cystadenoma, and mucinous cystadenocarcinoma. A simple mucocele is characterized by degenerative epithelial changes due to obstruction and distention of the appendix. This type represents 20%-30% of cases. A simple mucocele is caused by mucus distention secondary to an obstruction of the appendix due to fecaliths, post-inflammatory scarring, or rarely, endometriosis. Mucosal hyperplasia is similar to a hyperplastic colon polyp histologically. This type represents

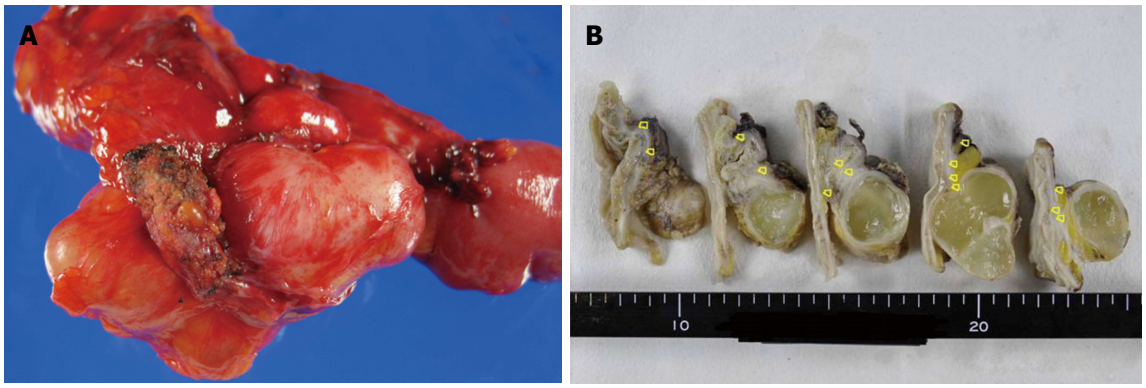

Figure 2 Macroscopic appearance of the resected specimen. A: Resected specimen contained thin-walled cystic masses; B: Cystic masses contained yellow mucin. Endometriosis was observed around the cyst (circle).

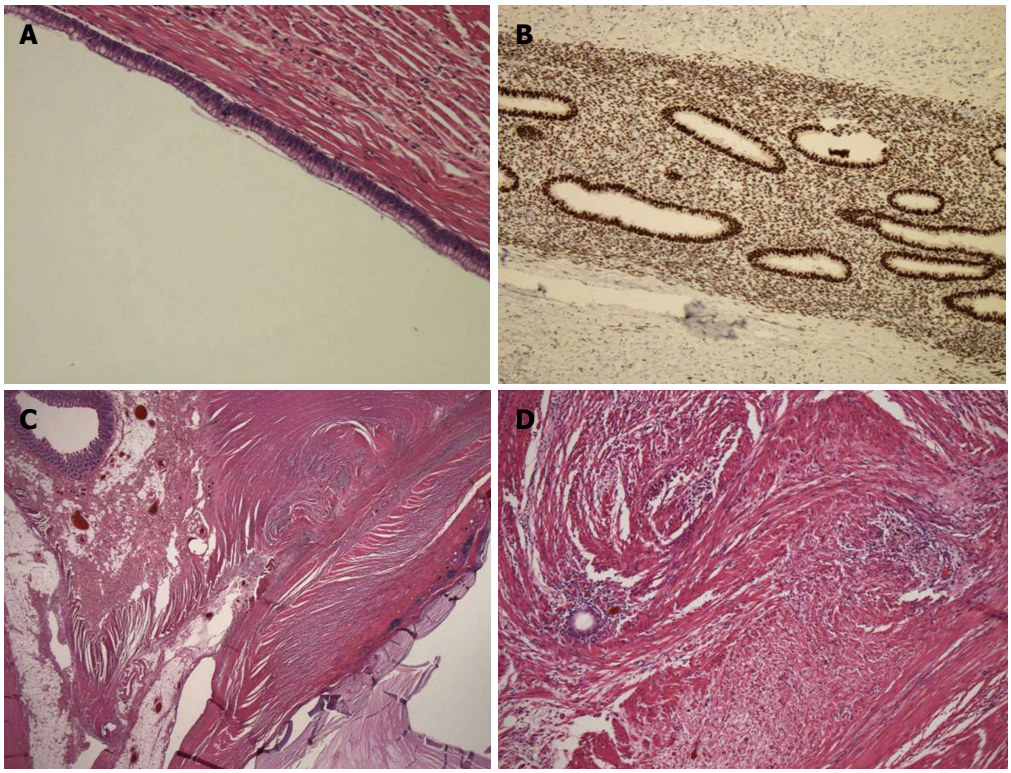

Figure 3 Pathological findings and immunohistochemical staining. A: Appendiceal mucosa was observed on the cyst wall; B: Estrogen receptor; C, D: Obstructive lesion of the appendiceal lumen. Endometriosis and smooth muscle hypertrophy were observed in the muscle layer (hematoxylin/eosin staining, C: × 20; D: × 100).

20%-30% of cases. Mucinous cystadenoma is a neoplasm that presents with a tubular or papillary pattern, with mucus production and adenomatous epithelium. This type represents 30%-50% of cases. Mucinous cystadenocarcinoma differs from cystadenoma because glandular and stromal invasion is involved. Previously, it was believed that only mucinous cystadenocarcinoma could cause pseudomyxoma peritonei (PMP), but it has recently been reported that other types of mucocele can cause PMP. Ruiz-Tovar *et al*[3] presented a case of simple mucocele that was apparently not perforated and developed PMP.

Consequently, the preoperative diagnosis of mucocele of the appendix is crucial. Colonoscopy, ultrasonography (USG), CT and MRI are used for diagnosis. On colonoscopy, the appearance of the appendiceal orifice at the center of the mound has been called the "volcano sign". USG can be used to differentiate between acute appendicitis and mucocele. Dilatation of the appendiceal lumen to ⩾ 15 mm suggests mucocele with 83% sensitivity and 92% specificity. CT offers better specificity in establishing a diagnosis of mucocele. The typical features are cystic masses that are well circumscribed with low attenuation. Wall calcifications are observed in 50% of cases, and they strongly suggest mucocele. In addition, enhancing nodules in the mucocele wall suggest cystadenocarcinoma. MRI is also useful in establishing a diagnosis of mucocele. On MRI, mucocele appears as a cystic mass with low to intermediate signal intensity on T1-WI and high

signal intensity on T2-WI. These findings can be attributed to the high protein content of a mucocele[16]. In our case, the CT, MRI and colonoscopy imaging results were compatible with these findings.

When mucocele of the appendix is diagnosed preoperatively, open surgery is favored over laparoscopy to prevent rupture of the mucocele, which may induce PMP. If mucocele is detected during a laparoscopic procedure, the patient must undergo conversion to open surgery.

Appendiceal endometriosis is diagnosed pathologically. Glandular tissue, endometrial stroma, and hemorrhage are typically assessed in patients who present with endometriosis[17]. Approximately half of the cases of endometriosis of the appendix involve the body and half involve the tip of the appendix. Muscular and seromuscular involvement occurs in two-thirds of patients, and the serosal surface is involved in one-third of patients. The mucosa is not involved and the submucosa is involved in one-third of patients. There was no relationship between the location of the endometriotic foci and the symptoms of our patient, who had endometriosis with muscular involvement.

Hapke *et al*[5] noted that the progression of mucocele of the appendix due to endometriosis consists of the following steps. Endometriosis results in smooth muscle hypertrophy of the appendix, including the muscularis mucosa, with obstruction of some of the gland crypts. These obstructions lead to local increased mucin production from multiple small cysts. Ultimately, several of these small cysts coalesce, resulting in a single layer cyst that can be dissected through the submucosa proximally. In our case, multiple small cysts were observed surrounding the appendix. These findings confirm the proposal of Hapke *et al*[5].

There are only 10 previously reported cases of a similar condition in the English literature[4-12]. We reviewed these reported cases and the present case. The mean age at presentation was 34 years (22-56 years). The patients presented various symptoms: four had recurrent abdominal pain during menstrual periods; three had chronic pelvic pain; two had acute abdominal pain with vomiting; one had increasing menorrhagia (she had complicated uterine myomas); and another had no symptoms. Preoperative diagnosis of mucocele was made in five cases; diagnostic laparoscopy was performed in five cases; and in one case, it was found by chance during surgery. Open surgery was performed in six cases, and two experienced rupture during surgery. The mean tumor size was 2.5 cm (1.3-5.5 cm). Nine cases had a single cyst, and two had multiple cysts. One case was complicated with intussusception and another with ureteric obstruction.

In summary, we report a rare case of mucocele of the appendix due to endometriosis and provide the first review of the literature.

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CASE REPORT

Anticoagulation and delayed bowel resection in the management of mesenteric venous thrombosis

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Author contributions: Kim HK, Chun JM and Huh S performed surgical operation; Kim HK organized the report; Kim HK and Chun JM wrote paper.

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Abstract

Acute mesenteric venous thrombosis is potentially lethal because it can result in mesenteric ischemia and, ultimately, bowel infarction requiring surgical intervention. Systemic anticoagulation for the prevention of thrombus propagation is a well-recognized treatment modality and the current mainstay therapy for patients with acute mesenteric venous thrombosis. However, the decision between prompt surgical exploration *vs* conservative treatment with anticoagulation is somewhat difficult in patients with suspected bowel ischemia. Here we describe a patient with acute mesenteric venous thrombosis who presented with bowel ischemia and was treated with anticoagulation and delayed short-segment bowel resection.

Key words: Thrombosis; Mesenteric vein; Anticoagulation; Small intestine; Resection

Core tip: Recently, we experienced a patient with acute mesenteric venous thrombosis who presented with bowel ischemia and was treated with anticoagulation and delayed short-segment bowel resection. The decision between prompt surgical exploration or conservative treatment with anticoagulation in patients with suspected bowel ischemia is difficult and one of the main purpose is the preservation of bowel. So, in equivocal patients, anticoagulation for potentially reversible bowel ischemia and delayed bowel resection for stricture if developed could be an appropriate management technique to prevent or limit future bowel resection.

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INTRODUCTION

Acute mesenteric venous thrombosis (MVT) accounts for 5%-10% of acute mesenteric ischemia, although it is rare in the absence of abdominal malignancy or liver cirrhosis[1,2]. Acute MVT is potentially lethal because it can result in intestinal ischemia and, ultimately, intestinal infarction requiring surgical intervention. Although intestinal gangrene resulting from mesenteric venous occlusion was first reported by Elliot[3], it was only after the detailed publication of Warren *et al*[4] in 1935 that MVT became known as a distinct clinical entity related to mesenteric ischemia.

Recent advances and widespread use of diagnostic modalities, in particular computed tomography (CT), have facilitated the early detection of MVT before laparotomy; systemic anticoagulation as early treatment for MVT prevents thrombus propagation and has resulted in a decrease in the reported mortality rate[5,6]. Nevertheless, the decision between laparotomy *vs* conservative treatment with anticoagulation can be difficult in patients with suspected bowel ischemia. Complicating matters, the

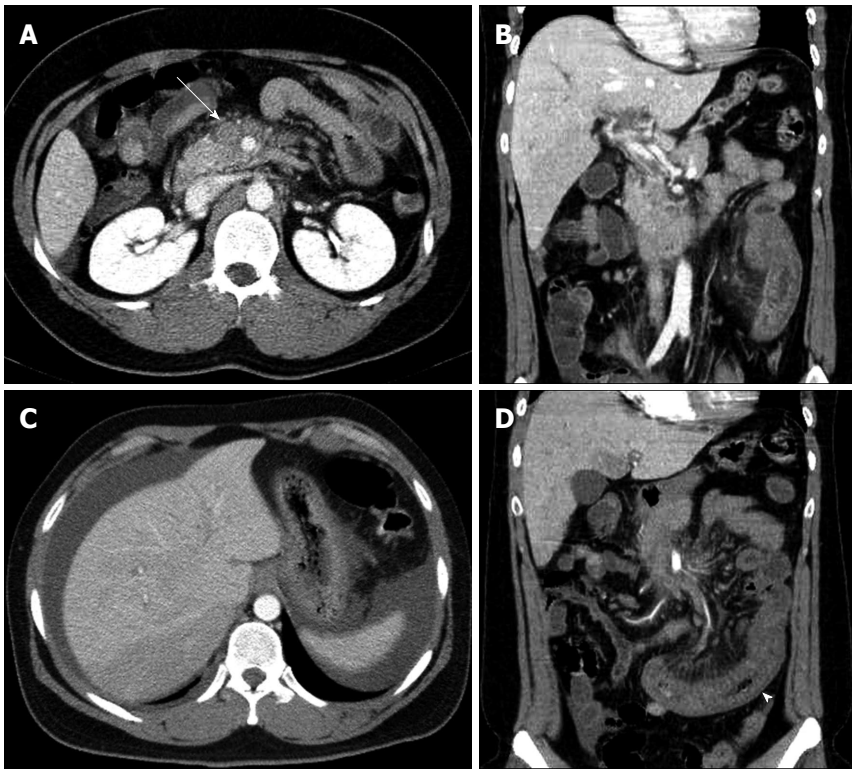

Figure 1 Abdominal computed tomography demonstrates an acute mesenteric venous thrombosis at the time of initial presentation. A: A thrombus (arrow) and perivenous infiltration at the proximal superior mesenteric vein; B: extension into the portal vein; C: An abnormal fluid collection around the liver and spleen; D: The affected small bowel (arrow head) with long-segment wall thickening and decreased enhancement.

border between ischemic bowel and viable bowel is often diffuse when exploratory laparotomy is performed in the acute stage. Because the viability of bowel is difficult to determine, overly aggressive bowel resection may result with consequent short bowel syndrome. We describe herein a patient with acute MVT and bowel ischemia who was treated with prompt anticoagulation and delayed short-segment bowel resection.

CASE REPORT

A 25-year-old male was referred to our emergency department from an outside hospital after experiencing 3 d of abdominal pain. The pain was gradually increasing in intensity and was squeezing and continuous in nature; he also noted blood-tinged stools. On abdominal examination, the patient complained of epigastric and left lower-quadrant tenderness to palpation, and the bowel sounds were decreased. Laboratory evaluation showed leukocytosis with a left shift (white blood cell count, 14100/mm^3; segmented neutrophils, 94.5%). The erythrocyte sedimentation rate was 29.0 mm/h, the C-reactive protein level was 18.61 mg/dL, the platelet count was 258000/mm^3, the hemoglobin level was 14.8 g/dL, the international normalized ratio (INR) was 1.22, the activated partial thromboplastin time was 39.6 s, and the D-dimer level was increased to 1344 μg/dL (normal range, < 340 μg/dL). Hypercoagulability testing, including protein S, protein C, and antithrombin-Ⅲ, was within normal limits. None of the following were detected: factor V Leiden mutation, prothrombin G20210A mutation, activated protein C resistance, anticardiolipin antibodies, antiphospholipid antibodies, or lupus anticoagulant. CT demonstrated complete thrombosis of the superior mesenteric vein (SMV) and partial thrombosis of the portal vein. An abnormal intraperitoneal fluid collection was noted around the liver and the pouch of Douglas, and the affected small bowel had an edematous and thickened wall with decreased enhancement, suggesting bowel ischemia (Figure 1).

The initial treatment included intravenous fluid administration, prophylactic antibiotics, bowel rest, and nasogastric-tube bowel decompression, with close monitoring for signs of bowel necrosis. Anticoagulation was started at the time of diagnosis with low molecular weight heparin (Enoxaparin, 1 mg/kg subcutaneous injection twice daily). The patient's abdominal pain gradually decreased over 1 wk of conservative management. With continuing parenteral nutrition and anticoagulation, his clinical condition stabilized 18 d after the initiation of treatment and he started oral intake with sips of water. He was able to progress to some oral food intake, however intermittent abdominal discomfort and fullness occurred when he tried to increase his oral intake further. At this point, we were concerned about the possibility of bowel stricture and discussed with him the likely ultimate need for surgical exploration. The patient strongly desired a longer period of conservative management, so after 5 wk in the hospital he was discharged on oral anticoagulation.

During the follow-up period, his INR was regulated at the outpatient clinic within the optimal range of 2.0 to

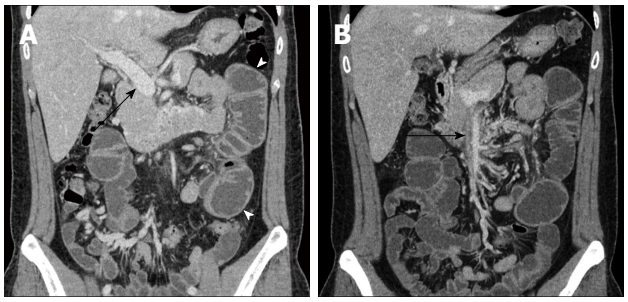

Figure 2 Abdominal computed tomography at second admission. A: Dilated proximal jejunal loop (arrow heads) and resolution of thrombus in the main portal vein (arrow); B: Remnant thrombus in superior mesenteric vein (arrow).

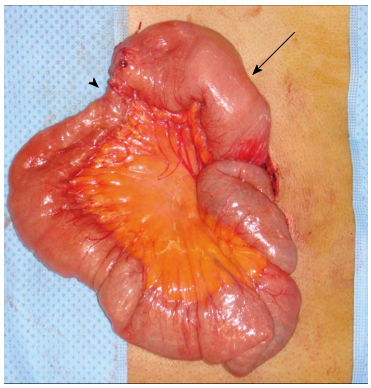

Figure 3 Intraoperative findings: sequelae of the mesenteric venous thrombosis. A dilated proximal jejunum (arrow) and short-segment stricture (arrow head) are noted.

3.0. Unfortunately, he returned to our emergency department 5 wk after discharge with abdominal pain, distension, and vomiting. A small bowel ileus was detected on plain abdominal radiography, and CT showed dilation of the proximal jejunal loop. The thrombus of the main portal vein had resolved, and the SMV demonstrated a remnant thrombus with obliteration; mesenteric congestion with development of collateral circulation was also present (Figure 2). We suspected bowel stricture due to the previous MVT with bowel ischemia and his complaint of abdominal distension, along with a weight loss of 8 kg over 2 mo. He received bowel decompression through a nasogastric tube and underwent scheduled laparotomy 2 d after the second admission. At surgery, a short-segment stricture of the distal upper jejunum with proximal dilatation was noted; the bowel color was normal (Figure 3). We performed segmental resection of 6 cm of the small bowel and a functional side-to-side anastomosis with staplers. The patient's postoperative course was uneventful and he was discharged 1 wk after surgery. He receives regular follow-up at the outpatient clinic and has been taking oral anticoagulation, without a recurrence, for 2 years.

DISCUSSION

Currently, MVT is recognized as a multifactorial disorder predisposed by some genetic and acquired risk factors. Several genetic and acquired risk factors such as factor V Leiden mutation, prothrombin G20210A mutation, protein S deficiency, protein C deficiency, antithrombin-Ⅲ deficiency, activated protein C resistance, and antiphospholipid syndrome have been reported to be associated with MVT. In our case, we tried to find out risk factors mentioned above, but we could not find any risk factors including a local inflammatory disease. The JAK-2 V617F mutation, which is associated with myeloproliferative disorder, has been recently reported to be associated with splanchnic vein thrombosis in a number of series[7,8]. Therefore, determination of this mutation may contribute to the search for genetic determinant of MVT, and further research will define the role and clinical significance of this mutation.

The management of acute MVT has changed over recent decades, however there is no consensus on its optimal management, especially in patients with bowel ischemia. Currently, systemic anticoagulation for the prevention of thrombus propagation is a well-recognized treatment modality and the mainstay of treatment in patients with acute MVT. Abdu *et al*[5], in their literature review involving 372 patients, reported that the addition of anticoagulation to previous treatment modalities improves survival rates and reduces recurrence rates in patients with MVT. However, they still recommend prompt surgical intervention. Since the 1990s, several studies have reported the feasibility of non-operative management for acute MVT[2,9-11]. Brunaud *et al*[2] determined that the morbidity, mortality, and survival rates are similar in surgical and non-surgical groups, with a shorter length of hospital stay in patients who avoid surgery. They also reported non-transmural infarction in 83% of resected specimens in the surgical group and concluded that the non-operative approach, when indicated, could avoid the resection of small bowel that is macroscopically infarcted but potentially curable with anticoagulation.

There are 2 potential difficulties in the management of patients with acute-stage MVT. The first is the decision between prompt surgical exploration or conservative treatment with anticoagulation, and the second is the difficulty in confirming bowel viability if surgical exploration is conducted. Most of the literature on the subject considers surgical exploration to be indicated if there are signs of peritoneal irritation at presentation. However, Brunaud *et al*[2] discussed the finding that peritoneal signs may not strictly correlate with the severity of bowel ischemia and suggested that new criteria, such as bowel-wall thickness and bowel-wall enhancement on the arterial phase of CT, need further evaluation. Our patient complained of abdominal pain and had tenderness to palpation, with the CT findings of an abnormal fluid collection and edematous bowel with wall thickening. However, conservative management with bowel rest and anticoagulation did not lead to transmural infarction of the affected bowel (bowel gangrene or perforation). We agree that further research into alternate criteria for surgical exploration, perhaps specific CT findings, is necessary

to improve management in these patients.

Limited areas of infarcted bowel can be surgically resected and anastomosed without significant morbidity, however when extensive ischemia is present, surgeons should try to conserve as much bowel as possible. Unfortunately, the border between ischemic bowel and viable bowel is often diffuse, making viability difficult to determine. Bowel viability is typically assessed using Doppler examination and the clinician's judgment; when the diagnosis is in doubt, a second-look operation is usually planned and performed[12,13]. Currently, numerous other techniques for assessing intestinal viability are available[14], however there is no consensus regarding their clinical usefulness. Further studies are needed to determine the value of various methods in the diagnosis of MVT and to standardize these methods.

After patients recover from the acute stage of MVT, the development of a small bowel stricture is among the possible complications during the chronic stage[15,16]. Arguably, conservative management with anticoagulation requires a more prolonged treatment period and increases patient discomfort due to the diet restrictions and need for hospitalization. Therefore, in patients with limited acute-stage bowel involvement on CT, early surgical exploration could be a more appropriate treatment, eliminating the above-mentioned shortcomings of conservative treatment. Nevertheless, the more important consideration in these patients is the possibility of recurrence. Though the reported MVT recurrence rate seems to be low while patients are receiving anticoagulation[17], recurrence is still possible, and its surgical treatment may lead to catastrophic sequelae such as short bowel syndrome.

In summary, surgical exploration in acute MVT is appropriate to be limited to the patients with definite signs of bowel infarction; in equivocal patients, anticoagulation for potentially reversible bowel ischemia could be an appropriate management technique to prevent or limit future bowel resection. Of course, patients undergoing conservative management need to be closely observed for evidence of clinical deterioration.

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In press

3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

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10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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