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EDITORIAL

Use of second generation contrast-enhanced ultrasound in the assessment of focal liver lesions

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Abstract

Ultrasound (US) is often the first imaging modality employed in patients with suspected focal liver lesions. The role of US in the characterisation of focal liver lesions has been transformed with the introduction of specific contrast media and the development of specialized imaging techniques. Ultrasound now can fully characterise the enhancement pattern of hepatic lesions, similar to that achieved with contrast enhanced multiphasic computed tomography (CT) and magnetic resonance imaging (MRI). US contrast agents are safe, well-tolerated and have very few contraindications. Furthermore, real-time evaluation of the vascularity of focal liver lesions has become possible with the use of the newer microbubble contrast agents. This article reviews the enhancement pattern of the most frequent liver lesions seen, using the second generation US contrast media. The common pitfalls for each type of lesion are discussed. The recent developments in US contrast media and specific imaging techniques have been a major advance and this technique, in view of the intrinsic advantages of US, will undoubtedly gain popularity in the years to come.

Key words: Microbubble contrast agents; Ultrasound; Focal liver lesions

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INTRODUCTION

The non-invasive detection and characterisation of focal liver lesions is an important component of cross-sectional imaging studies. Depending on their histological nature, different focal liver lesions vary in their blood supply, with the malignant ones generally having a preferential hepatic arterial supply[1]. The enhancement pattern of a lesion is based on the blood supply and constitutes the mainstay of its characterization with contrast-enhanced computed tomography or magnetic resonance imaging[2].

Ultrasound (US) is often the first imaging investigation for patients with liver disease. The sensitivity and specificity of gray-scale US for the characterization of focal lesions is inferior to that of CT or MRI[3,4]. One of the main reasons for this was the absence, until the 1990s, of US contrast media. The advent of microbubble contrast agents has led to improved characterisation and detection of focal liver lesions since the enhancement characteristics can be visualised in real time over a 5 min period. As a result, recent studies have reported sensitivities and specificities that rival that of CT and MRI[5].

US CONTRAST MEDIA AND SPECIFIC IMAGING TECHNIQUE

Ultrasound contrast agents consist of microbubbles of gas with a protein, lipid or polymer shell. The microbubbles are approximately 1 to 10 μm, which is the size of a red blood cell. These particles are too large to pass through the vascular endothelium and, as such, are considered pure blood pool agents[6]. After several minutes in the circulation, the microbubbles dissolve, the gas is exhaled and the shell is metabolized, mainly in the liver[7]. Furthermore, the microbubbles are well tolerated by patients after intravenous injection and there are very few contraindications to their use.

When subjected to an US wave, the microbubbles respond by changing their size: they expand during the rarefaction phase and contract during the pressure phase. These changes are much greater than the minor changes that occur in the soft tissues. The bubbles, like every oscillating system, have a natural frequency (the resonance frequency) at which their response is maximal.

Fortunately, the bubbles resonate at frequencies used for diagnostic US imaging. This coincidence accounts for their high reflectivity, even when they are present in a small concentration. Furthermore, the expansion of these bubbles during the rarefaction phase exceeds their contraction during the pressure phase. This asymmetric oscillation produces a returning signal (echo) that contains harmonics, i.e. multiples of the driving frequency[8]. The first microbubble-specific imaging technique exploited this property by sending an US pulse into the tissue and selectively detecting echoes at twice that frequency, such that one could theoretically image only the bubbles. However, in practice, soft tissues also produce harmonic echoes. Consequently, the images produced with this technique were of poor quality, in part due to poor tissue suppression.

Subsequently, another mode of imaging with microbubbles was developed using high-power colour Doppler US. When submitted to a high-energy US beam, the microbubbles often break up into much smaller bubbles that dissolve rapidly. As they are disrupted, the bubbles emit a strong, brief echo which is easily detected, a phenomenon known as stimulated acoustic emission (SAE)[9]. The drawback of this technique, however, is that the microbubbles are destroyed by the US beam and therefore real-time imaging is not feasible.

At the present time, the most common imaging technique is based on the principle of phase-inversion, in which two US pulses, 180° out of phase, are sent sequentially. The returning echoes are added up by the US machine[10]. The linear echoes returned by the tissues nullify each other, while the non-linear echoes returned by the microbubbles produce a detectable signal. There are two main advantages of this technique. First, excellent tissue suppression is obtained. Second, a detectable signal is obtained even when a very low-power US beam is employed, consequently, the bubbles are not destroyed. The first generation contrast media, such as Levovist® (Schering, AG, Berlin, Germany), produced a very weak signal when submitted to low mechanical index US beam owing to its fragility and lent itself to use with SAE[11]. Since then, contrast media, such as SonoVue® (Bracco, Milan, Italy), Definity® (Bristol-Myers-Squibb, Billerica, Mass, USA) and Optison® (Nycomed/Amersham, Little Chalfont, UK), which have a strong, non-linear, harmonic response, even when insonated with low acoustic power, were able to provide real-time imaging using low mechanical index (MI) modes[12].

In the following sections we review the common enhancement patterns of the most frequent focal liver lesions seen with the second generation contrast agents.

CONTRAST-ENHANCED US PATTERNS OF FOCAL LIVER LESIONS

The characterization of a hepatic lesion with microbubbles requires careful examination through all phases of contrast enhancement, i.e. arterial (10-20 to 25-35 s after injection), portal (30-45 to 120 s) and late parenchymal (> 120 s) phases[13]. Simply put, the late phase is useful to determine the benign or malignant nature of a lesion while the arterial phase helps in predicting its histology[14-19]. Between 86% to 93% of benign lesions retain the contrast in the late phase, while 78% to 98% of the malignant ones demonstrate wash out of the contrast[14,16,18]. The persistence of the second generation contrast agent in a healthy liver is thought to be the result of a very slow flow through the sinusoid[20]. Consequently, lesions devoid of normal sinusoids do not retain the contrast.

HAEMANGIOMAS

Haemangiomas are the most common solid benign lesion of the liver, with a prevalence ranging from 1% to 20% in the general population[21]. These lesions are more common in females and are frequently located peripherally or adjacent to a large hepatic vein branch. The most common sonographic appearance of haemangiomas is a homogeneously hyperechoic focal lesion, less than 3 cm in size[22]. These characteristic features, when present in a patient at a low risk for malignancy, are usually sufficient to allow a confident diagnosis. In a significant number of patients however, further imaging is required.

The characteristic early arterial nodular enhancement with delayed centripetal fill-in described on CT or MRI[23-25] is the most common appearance of haemangiomas during the arterial phase of contrast-enhanced US, seen in 52% to 88% of cases (Figure 1)[15,16,18,19]. Sustained enhancement has been reported in 83% to 100% during the late phase[16-19]. The real time nature of contrast-enhanced US is particularly useful in diagnosing small, rapidly perfusing (flash-filling) haemangiomas, where the typical enhancement pattern can be appreciated[6]. Complete enhancement does not always occur, especially in lesions larger than 3 cm, which often undergo central thrombosis or fibrosis[6,26,27].

FOCAL NODULAR HYPERPLASIA

Focal nodular hyperplasia (FNH) is the second most common solid benign hepatic lesion, with a prevalence between 0.9% and 3%[28-30]. This lesion occurs in all age groups and both sexes, but is found predominantly in women (80%-95% of the cases) during the 3rd to 5th decade of life. Oral contraceptive use has been incriminated, but a definite relationship has not been established[31,32]. FNH is not a dysplastic or neoplastic tumour, but is a hyperplastic lesion, probably occuring in response to a pre-existing arterial malformation[33]. FNH does not have a malignant potential, nor is it likely to bleed or rupture[34]. Consequently, differentiation from other lesions, particularly hepatocellular adenoma and carcinoma (especially the fibrolamellar form), is essential since FNH is managed conservatively, whereas the other lesions require surgery. FNH is often discovered incidentally.

The most common sonographic appearance of FNH is that of a homogeneous, near isoechoic lesion; some lesions are detected only because of their mass effect on adjacent blood vessels[32]. A central hypoechoic scar is detected in 20% to 45% of the cases[22,35,36]. In larger lesions, colour Doppler may show a central feeding artery with a spoke-

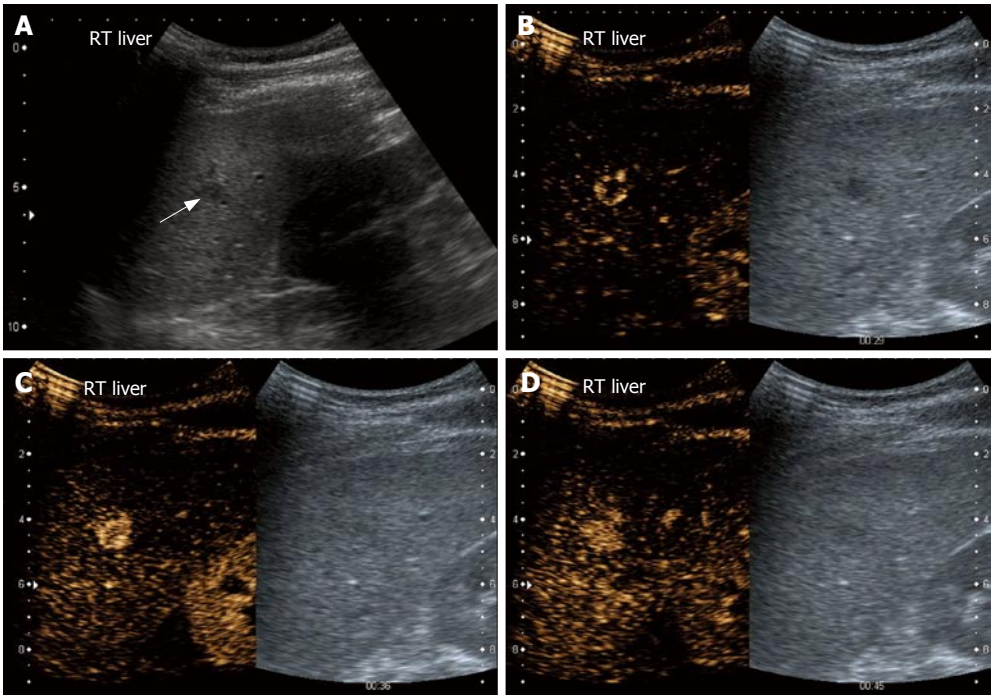

Figure 1 Haemangioma. Gray-scale US image (**A**) and split-screen display images of contrast-enhanced US scan using a low MI technique (**B-D**). The left panel shows the contrast sensitive image while the corresponding gray-scale image is on the right. On gray-scale US, the liver is hyperechoic, consistent with fatty infiltration and an ill-defined hypoechoic lesion is seen (arrow, **A**). On contrast enhanced US, the lesion demonstrates peripheral nodular enhancement during the arterial phase (**B**). At 36 s, the lesion has almost completely filled in (**C**). At 45 s, the lesion is completely enhanced (**D**) and sustained enhancement was observed in the late phase scan (not shown).

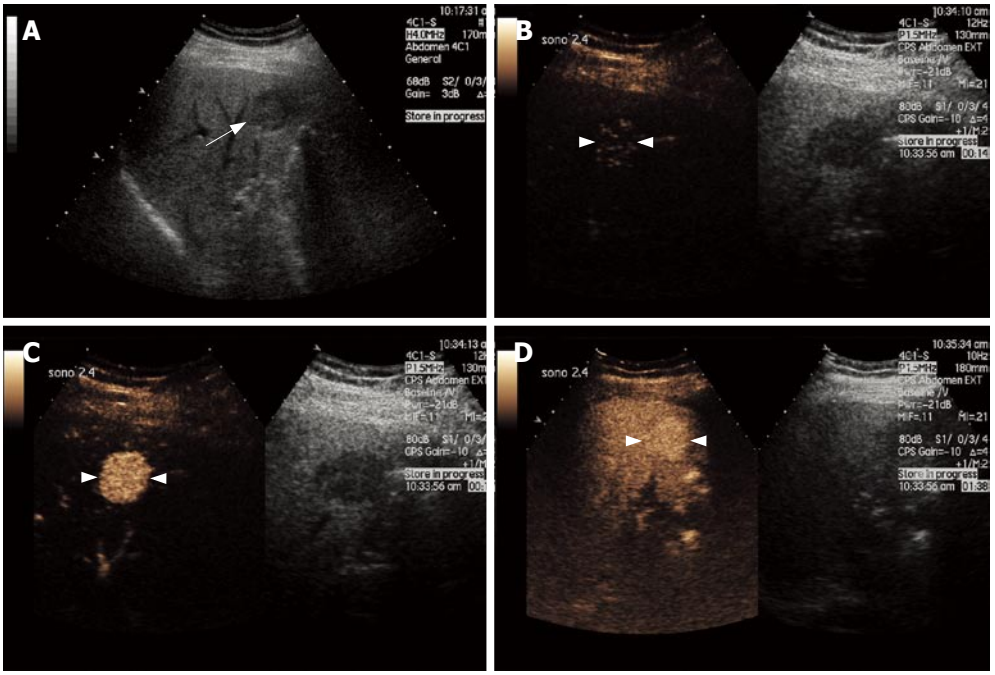

Figure 2 Focal Nodular Hyperplasia. Gray-scale US image (**A**) and split-screen display images of a contrast-enhanced US scan using a low MI technique (**B-D**). The gray-scale US image shows a focal hypoechoic lesion (arrow, **A**) in a diffusely hyperechoic liver in keeping with fatty infiltration. After contrast injection, the lesion enhances avidly in the arterial phase with filling seen from a central feeding vessel, demonstrating the classical spoke-wheel appearance (arrowheads, **B** and **C**). The lesion remains slightly hyperechoic during the portal and late phases (arrowheads, **D**).

wheel pattern of vessels radiating to the periphery.

FNH is a hypervascular tumour and consequently manifests as a strongly and homogeneously enhancing lesion during the arterial phase of contrast enhanced US in nearly 100% of the cases (Figure 2). The central spoke-wheel type of contrast enhancement can be demonstrated in 45% to 89% of FNH. These lesions become isoechoic or slightly hyperechoic, compared with the surrounding liver parenchyma during the portal and late phases of enhancement in 87% to 100% of the time[15-19,37]. The central scar is seen in 23% to 31% of cases[15,17]. However, in contradistinction to the pattern seen on CT or MRI, the central scar stands out as a defect instead of the late enhancement seen with the other imaging modalities. This finding can be explained by the fact that microbubbles are purely intravascular agents and therefore do not diffuse into the interstitium, unlike iodine and gadolinium-based contrast agents used with CT and MRI[6].

In patients with chronic liver disease, caution should be used since well-differentiated hepatocellular carcinoma (HCC) may mimic the enhancement pattern of FNH (see below). In these patients, all hypervascular lesions should be regarded with suspicion.

HEPATOCELLULAR ADENOMA

Hepatocellular adenoma (HA) is a rare, benign neoplasm of hepatocellular origin. Approximately 90% of HAs occur in young women[38]. Up to 90% of females with HA have reported the use of oral contraceptives[39]. HA is also

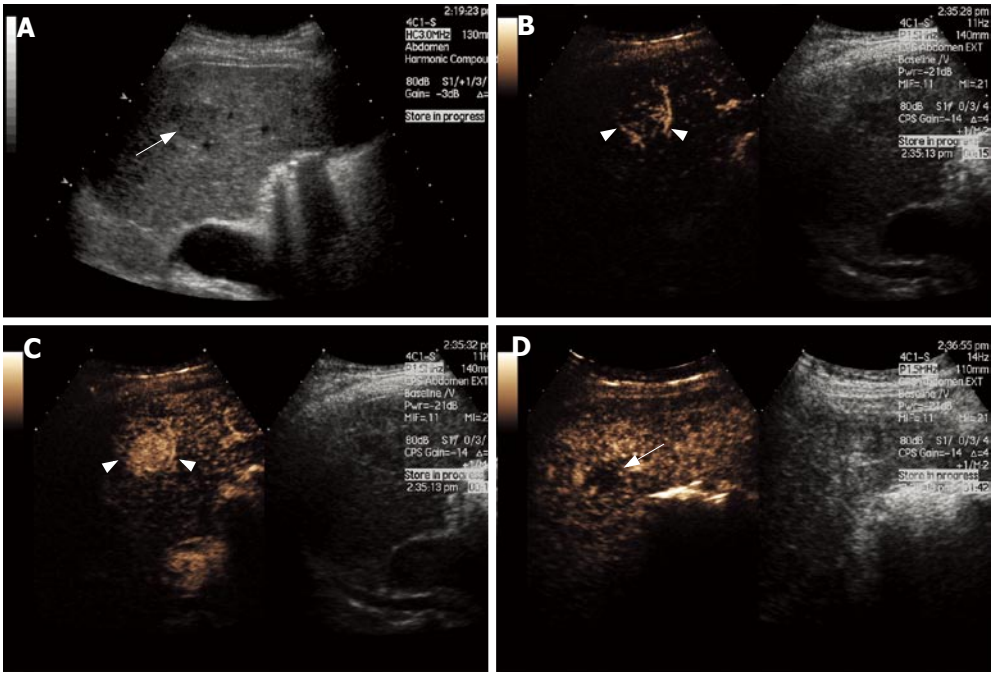

Figure 3 Hepatocellular Carcinoma. Gray-scale US image (**A**) and split-screen display images of a contrast-enhanced US scan using a low MI technique (**B-D**). The gray-scale US image shows a slightly hypoechoic lesion in segment 2 of the liver (arrow, **A**). After contrast injection, the lesion shows marked hypervascularity in the arterial phase with a basket-pattern peripheral network of vessels (arrowheads, **B** and **C**). The lesion becomes isoechoic and finally washes out to leave a defect in the late phase (arrow, **D**).

associated with the use of anabolic steroids in men and in some storage diseases[40]. In contrast to FNH, HA is a true neoplasm.

Management of HA is by surgical resection, in contrast to FNH, because of the risk of malignant degeneration and haemorrhage[41]. Patients with HA may present with pain secondary to its mass effect (40%) or intratumoral/intraperitoneal haemorrhage (40%). Alternatively, the tumour may be discovered incidentally (20%)[22].

The most common sonographic appearance of HA is a well-defined, large, solitary, hyperechoic mass owing to its high lipid content. Since the lesions have a propensity to bleed, HAs are usually heterogeneous in appearance. Colour Doppler findings are non-specific. The typical spoke-wheel pattern of FNH is absent.

The contrast-enhanced US characteristics of HA are relatively non-specific, but these lesions usually enhance during the arterial phase. Smaller lesions are likely to show homogeneous enhancement whereas the larger ones will be heterogeneous owing to previous intratumoral haemorrhage or necrosis. In one study with Sonovue, HAs were iso- or more often hypoechoic in comparison with the surrounding liver parenchyma in the portal venous and late phases of enhancement[37]. Unfortunately, this pattern of enhancement is not unique to HA, as it is also a common appearance of HCC on contrast-enhanced US (see below). In some cases, even the histopathological differentiation of HA from well-differentiated HCC is difficult[42]. The differential diagnosis should include hypervascular metastases which can exhibit similar enhancement characteristics.

HEPATOCELLULAR CARCINOMA

HCC is the most common primary malignancy of the liver. It usually occurs in patients with chronic liver disease, particularly in those with chronic hepatitis B and C infection where the risk is approximately 100 times that of patients with cirrhosis of other aetiologies. Men are affected three times more frequently than women[43]. Early detection is crucial for curative treatment, since patients with small HCC (< 2 cm) who are treated with liver transplantation have a survival rate of about 80%[44], whereas the 5-year survival of untreated HCC is less than 5%.

The gray scale US appearance of HCC is variable and non-specific. Hyperechoic foci related to the presence of fibrosis, haemorrhage and necrosis are found in approximately 50% of large HCCs[22]. On colour Doppler, approximately 75% of HCCs have a fine peripheral network of vessels, surrounding and penetrating the lesion (the so-called basket pattern)[45]. Non-invasive imaging diagnosis of HCC is often based on CT or MRI detection of a hypervascular mass in a patient with chronic liver disease because of the lack of specificity of conventional US.

With the use of contrast-enhanced US, more than 90% of HCCs behave like other hypervascular lesions and enhance avidly during the arterial phase (Figure 3)[15,17-19]. The basket pattern is reportedly seen in about one-third of the cases[19]. Also, like other malignant lesions, the majority (83% to 97%) of HCCs washout the contrast and appear as a defect during the late phase. However, caution should be used since well-differentiated HCCs may not show this washout very reliably. Moreover, it has been observed that the more differentiated a lesion, the more slowly it is likely to washout[46]. Consequently, in a patient with known chronic liver disease, a hypervascular lesion in the arterial phase should not be considered as benign on the sole finding of persistent enhancement during the portal and late phases.

METASTASES

The liver is a frequent site of metastases of extrahepatic tumours, and metastatic disease is one of the most common indications for imaging the liver. The gray-scale sonographic appearances of metastases are varied

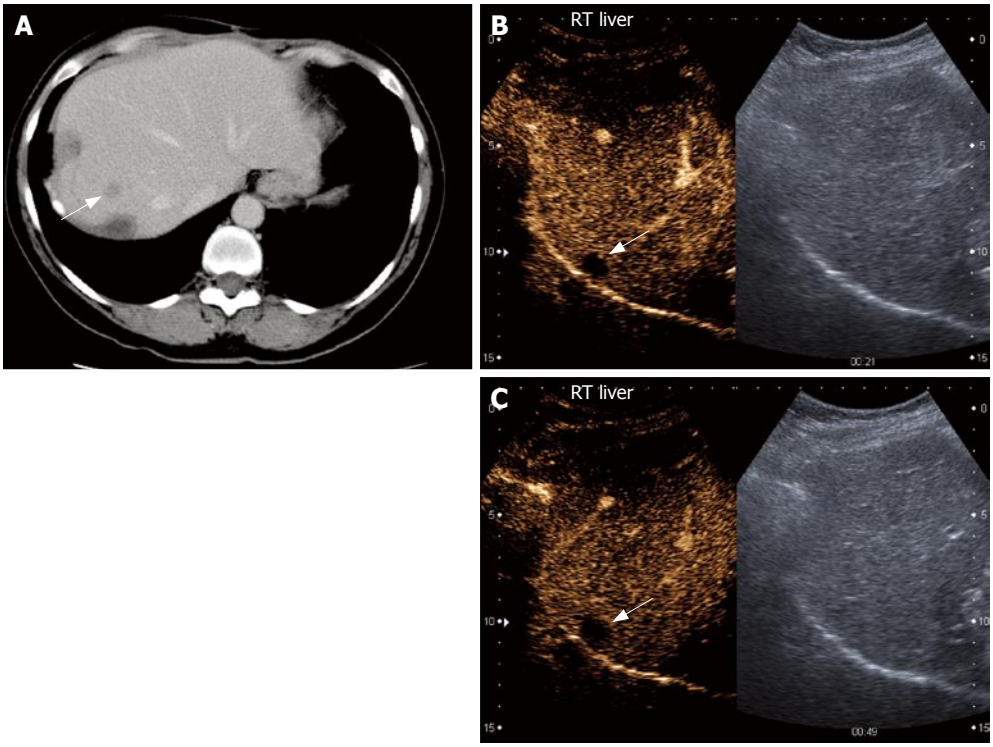

Figure 4 Liver metastasis in a patient with known metastatic colon carcinoma. The patient previously had liver resection and radiofrequency ablation. Contrast-enhanced CT scan image (**A**) and split-screen display images of a contrast-enhanced US scan using a low MI technique (**B** and **C**). On CT, a 1cm lesion is seen in segment 8 (arrow, **A**). The lesion was not visualized on gray-scale US. After injection of microbubbles, a 1 cm hypoechoic rounded lesion is seen as a defect in all the phases of enhancement (arrow, **B** and **C**). These findings are suspicious for a metastatic deposit.

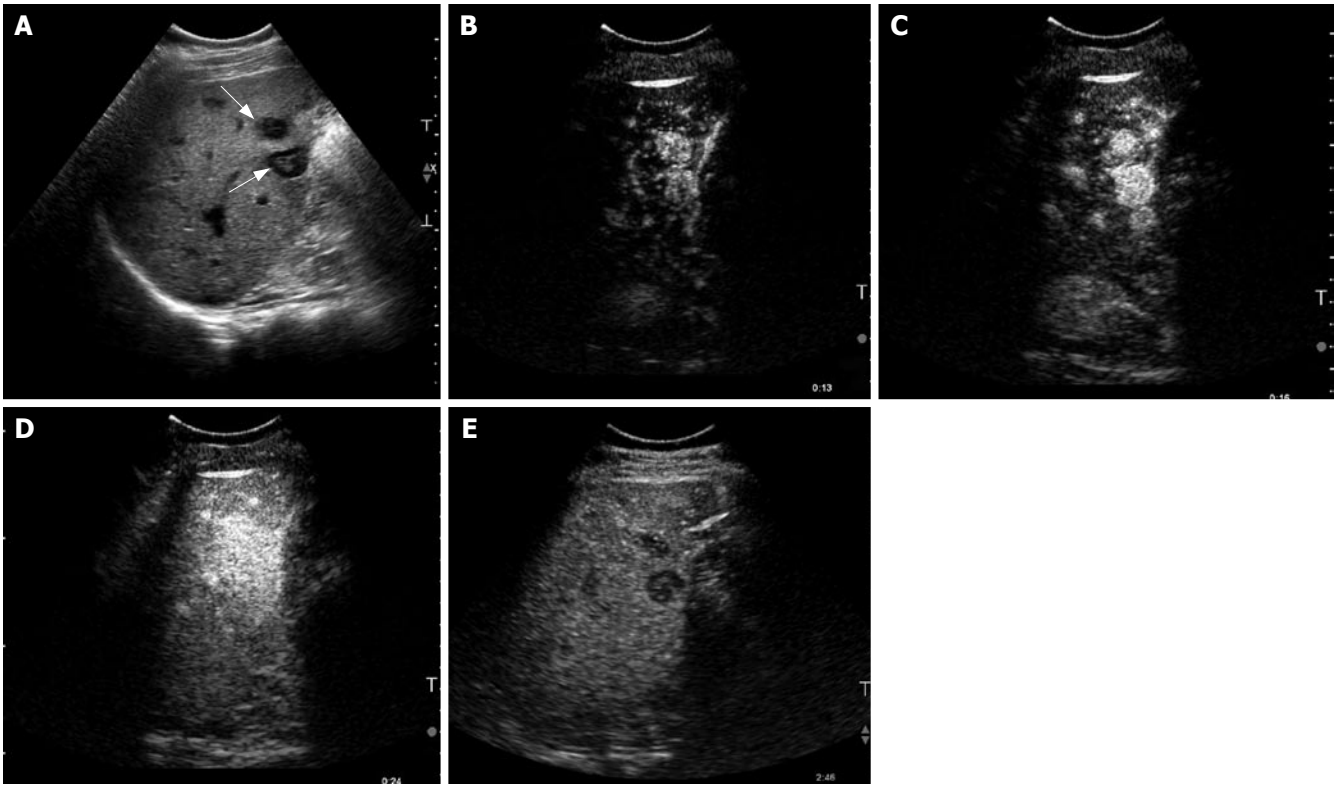

Figure 5 Hypervascular metastases in a patient with a known carcinoid tumour. Gray-scale US image (**A**) and contrast-enhanced US scan images (**B**-**E**) using a microbubble sensitive technique. On the gray-scale image, two hypoechoic target-like lesions are seen (arrows, **A**). After microbubble enhancement, an avid uptake of the contrast medium was seen in the early phase (**B**-**D**). The contrast washed out in the later phases leaving the metastases as defects (**E**).

depending on several factors such as the histology of the primary tumour and the treatment received by the patient. In a patient with a known malignancy with interval development of hepatic masses, the diagnosis is straightforward and characterisation is not an issue. However, when there is no history of malignancy or no previous imaging for comparison, characterisation becomes essential.

The accuracy of US for the assessment of liver metastases is lower than that of CT or MRI[47]. However, the use of first generation US contrast media, improved significantly the sensitivity for the detection of liver

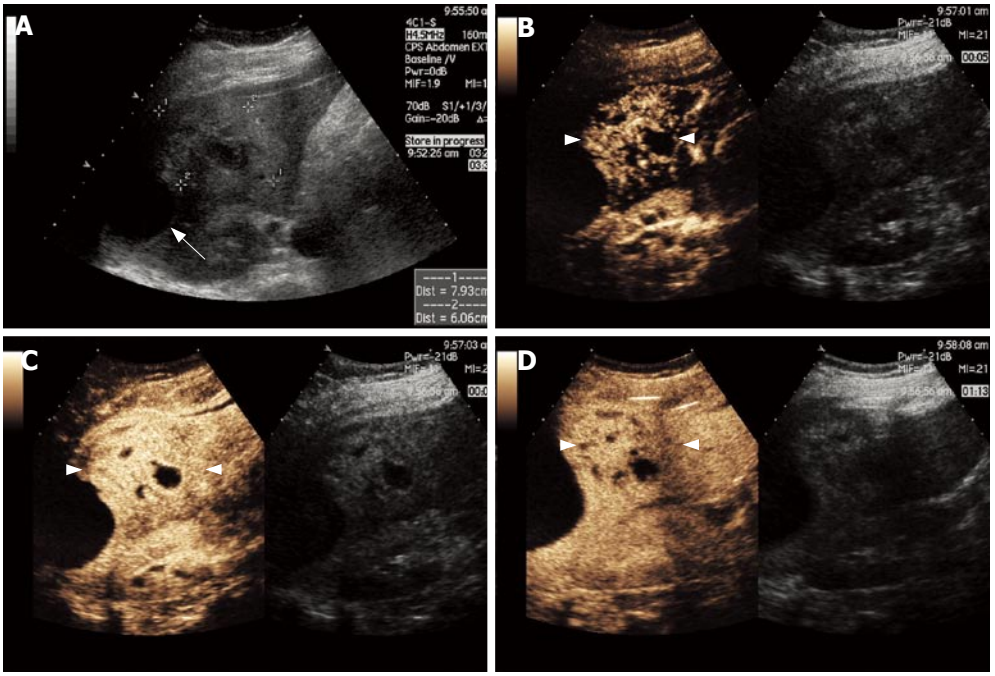

Figure 6 Hepatic abscess. Gray-scale US image (**A**) and split-screen display images of a contrast-enhanced US scan using a low MI technique (**B**-**D**).The gray-scale image shows an ill-defined heterogeneous mass in the left lobe of the liver (between callipers, **A**). A subcapsular anechoic fluid collection is also seen (arrow, **A**). After microbubble injection, regional hypervascularity during the arterial phase is shown (arrowheads, **B**-**D**). The abscess appears as a cluster of non-enhancing collections separated from each other by enhancing septations (**B** and **C**). In the late phase scan (**D**), there is no enhancement of the fluid collections and no wash out of the enhancing portions.

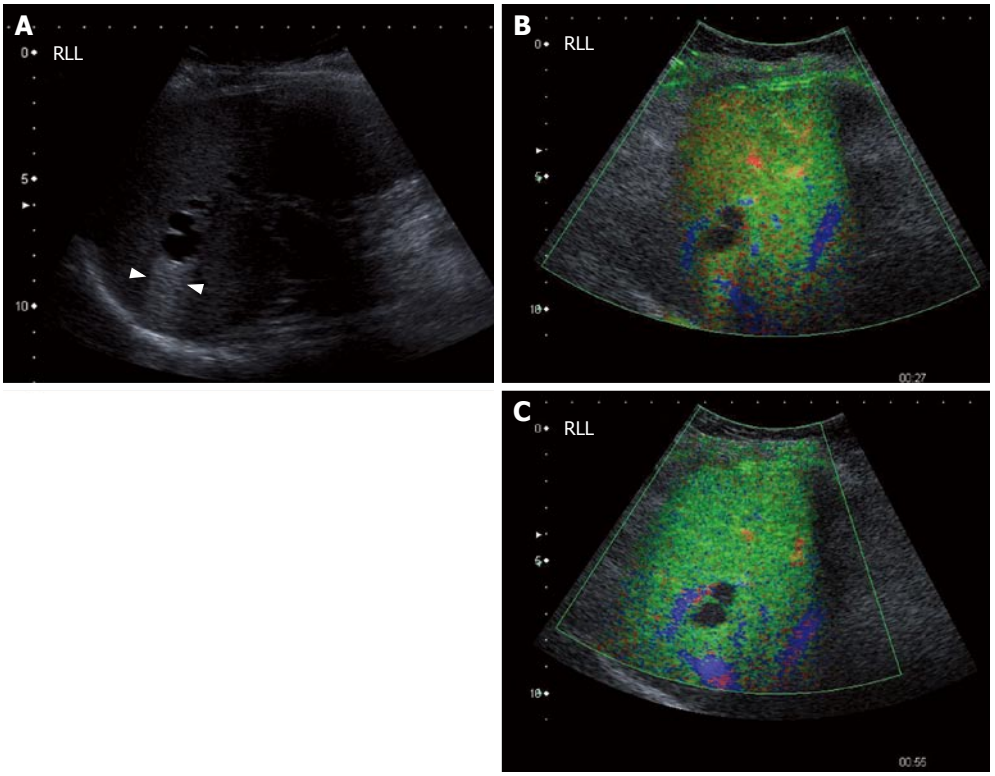

Figure 7 Potential pitfall: simple cysts. Gray-scale US image (**A**) and contrast-enhanced US scan images using a low MI technique (**B** and **C**). The gray-scale image (**A**) shows a typical simple hepatic cyst which is completely anechoic, has a thin wall and posterior acoustic enhancement (between arrowheads, **A**). After microbubble injection, no enhancement of the cyst is seen throughout all phases of enhancement (**B** and **C**). The diagnosis is straightforward if the lesion was recognised prior to contrast injection. If not, it may be misinterpreted as an enhancement defect and be categorized as a malignant lesion.

metastases[48]. It has been proposed by the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB) that any US staging study of the liver should be contrast enhanced, although the cost and feasibility of such a recommendation remains a big hurdle[13].

On contrast-enhanced US, metastases show different patterns of enhancement during the arterial phase, depending upon the vascularity pattern of the primary tumour. Regardless of their behaviour during the early phase, metastasis consistently show rapid and complete contrast washout and appear as enhancement defects on late phase scans (Figure 4)[15-17,19]. Recent publications have shown that, with the second generation US contrast media, the vast majority (> 85%) of metastases show some arterial enhancement, often more pronounced in the periphery[15,16,19,49]. This phase of hypervascularity is often not recognized on multiphasic CT or MRI because it is very brief and the lesion starts to washout within 20 s of the injection in most cases, before the arterial phase of CT and MRI, which is around 40 s from the beginning of the injection. The arterial enhancement may be rim-like,

diffuse or mosaic-like. Rim-like enhancement and early and complete washout of the lesion are typical of metastasis, while complete enhancement with a later washout is most suggestive of a HCC. However, when a hypervascular metastasis shows complete enhancement (Figure 5), differentiation from HCC is difficult and correlation with the clinical history and alpha-fetoprotein is often helpful.

OTHER MALIGNANT LESIONS

Other primary malignant lesions of the liver, such as intra-hepatic cholangiocarcinoma and lymphoma, show the typical behaviour of malignant lesions and washout contrast rapidly and appear as defects on the portal and late phases.

Cholangiocarcinoma frequently enhances during the arterial phase[40,49]. The rapid washout of this lesion observed on contrast-enhanced US is discordant with the typical behaviour of the lesion seen on CT and MRI (hypoenhancement with mild peripheral centripetal progression of enhancement over time)[50]. Again, this may be explained by the fact that microbubbles are purely intravascular agents. Some gray-scale features may help in differentiating cholangiocarcinoma from other abnormalities such as biliary duct dilatation.

ABSCESS

Liver abscesses result from bacterial, amoebic or fungal infections. Pyogenic abscess are by far the most frequent (88%)[22]. The gray-scale US findings of pyogenic abscesses vary with the stage of the disease. During early disease, the shape of the lesion is usually irregular and the echogenicity is variable. As the abscess matures the lesion becomes more rounded and hypoechoic, with debris in the middle and thick walls on the outside. Since an abscess is a fluid-filled lesion, there is usually associated posterior enhancement. Internal septations are seen commonly. Bright punctate echoes with "dirty" shadowing are present if there is gas within the cavity.

On contrast-enhanced US, pyogenic hepatic abscesses show areas of increased enhancement relative to the surrounding parenchyma[51]. Mature lesions with fluid show an enhancing rim. The internal septations also show enhancement giving the lesion a honeycomb appearance. Early (solid-appearing) lesions usually enhance diffusely, but heterogeneously. The enhancement appears early and usually persists during the portal and late phases (Figure 6), with no contrast enhancement seen in the liquefied portions. In the arterial phase, a transient peri-lesional enhancement has been reported. In a minority of cases, this is followed by portal venous phase hypovascularity[51]. The main differential diagnosis for a hepatic abscess is a necrotic metastasis. The latter would appear as a punched-out enhancement defects in the late phase, while the former appears as an ill-defined area of decreased enhancement, although, as stated above, this is not a common finding.

LIVER CYSTS

Liver cysts are common incidental findings on liver US. The diagnosis is straightforward when their pathognomonic features (anechoic, thin-walled and posterior enhancement) are present on gray-scale US.

Liver cysts are mentioned in this review because, on contrast-enhanced US, these lesions represent a potential pitfall if they have not first been recognized on gray scale US, since their gray-scale appearances are essential for characterisation. Liver cysts present as enhancement defects on all phases of contrast-enhanced US scan (Figure 7) and can be erroneously mistaken as malignant lesions.

CONCLUSION

The introduction of second generation microbubble US contrast media has allowed real-time imaging of a liver lesion in every phase of enhancement. The ability to observe the complete pattern of enhancement of a lesion has improved significantly the specificity of US for focal liver lesions, and rivals that of CT and MRI, thus reducing the need for further investigations. As a screening tool, US is ideal owing to its relative accessibility and portability. Microbubble agents have extended the utility of US further and are applicable to most imaging departments worldwide.

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EDITORIAL

Endoscopic pancreatic duct stent placement for inflammatory pancreatic diseases

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Abstract

The role of endoscopic therapy in the management of pancreatic diseases is continuously evolving; at present most pathological conditions of the pancreas are successfully treated by endoscopic retrograde cholangio-pancreatography (ERCP) or endoscopic ultrasound (EUS), or both. Endoscopic placement of stents has played and still plays a major role in the treatment of chronic pancreatitis, pseudocysts, pancreas divisum, main pancreatic duct injuries, pancreatic fistulae, complications of acute pancreatitis, recurrent idiopathic pancreatitis, and in the prevention of post-ERCP pancreatitis. These stents are currently routinely placed to reduce intraductal hypertension, bypass obstructing stones, restore lumen patency in cases with dominant, symptomatic strictures, seal main pancreatic duct disruption, drain pseudocysts or fluid collections, treat symptomatic major or minor papilla sphincter stenosis, and prevent procedure-induced acute pancreatitis. The present review aims at updating and discussing techniques, indications, and results of endoscopic pancreatic duct stent placement in acute and chronic inflammatory diseases of the pancreas.

Key words: Chronic pancreatitis; Pancreas divisum; Pancreatic pseudocyst; Pancreatic fistulas; Idiopathic recurrent pancreatitis; Main pancreatic duct stenting; Pancreatic dorsal duct stenting

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INTRODUCTION

The role of endoscopic therapy in the management of pancreatic diseases is continuously evolving; at present most pathological conditions of the pancreas are successfully treated by endoscopic retrograde cholangio-pancreatography (ERCP) or endoscopic ultrasound (EUS), or both. After the initial courageous experimental therapy tested in a few centers, pancreatic endotherapy has become an evidence-based method for carefully selected patients; because of the high level of technical skill required and the small numbers of patients who need this approach, pancreatic endotherapy should ideally only be carried out in selected centers where a multidisciplinary team is available.

Endoscopic placement of stents has played and still plays a major role in the treatment of chronic pancreatitis, pseudocysts, pancreas divisum, main pancreatic duct injuries, pancreatic fistulae, complications of acute pancreatitis, recurrent idiopathic pancreatitis, and in the prevention of post-ERCP pancreatitis. These stents are currently routinely placed to reduce intraductal hypertension, bypass obstructing stones, restore lumen patency in cases with dominant, symptomatic strictures, seal main pancreatic duct disruption, drain pseudocysts or fluid collections, treat symptomatic major or minor papilla sphincter stenosis, and prevent procedure-induced acute pancreatitis.

On the assumption that intraductal hypertension caused by obstructive lesions of the main pancreatic duct (MPD) is one cause of the pain often present in either chronic or acute pancreatic diseases, stent insertion beyond the obstruction to decompress the hypertension has a pivotal role in their therapeutic management.

The present review aims at updating and discussing the role of endoscopic pancreatic duct stent placement in acute and chronic inflammatory diseases of the pancreas.

TECHNIQUE OF PANCREATIC STENT PLACEMENT

The technique employed for placing pancreatic stents is similar to that used to place stents in the biliary tract. Once the main or accessory pancreatic duct has been deeply cannulated, a hydrophilic 0.035" (for 5F, 7F, 10F stents) or 0.018" (for 3F stents or when the minor papilla is cannulated) guidewire is introduced into the duct and maneuvred if possible beyond the stricture or leakage. The stent is then introduced over the guidewire (Figure 1). Stents can be placed with or without pancreatic sphincterotomy; pancreatic sphincter can be ablated by using the standard sphincterome in a single step procedure or after biliary sphincterotomy. The multiple step procedure is more time consuming but permits to better control the section of the

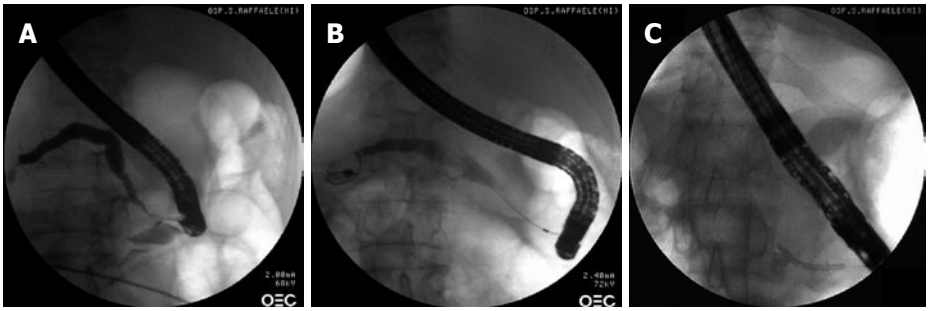

Figure 1 Chronic obstructive pancreatitis involving the head of the gland. **A**: Pancreatography showing a distal stricture of the main pancreatic duct; **B**: Insertion of the guidewire into the main pancreatic duct; **C**: Placement of a 10F plastic stent over the guidewire.

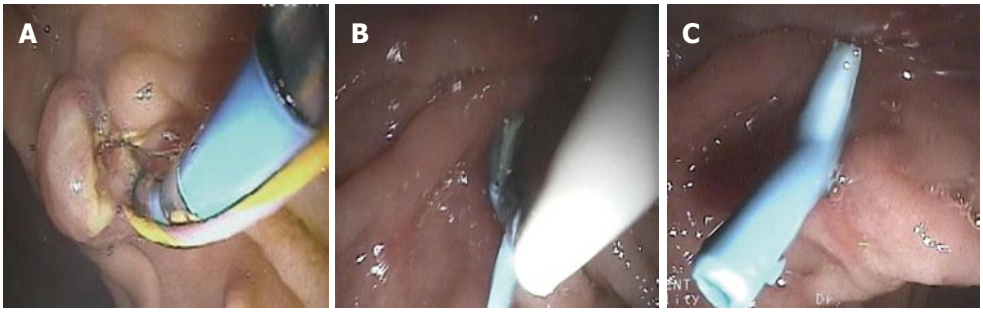

Figure 2 Placement of a pancreatic stent after pancreatic sphincterotomy. **A**: Once biliary sphincterotomy has been performed leaving a guidewire into the common bile duct, pancreatic sphincter ablation is done with the standard sphincterotome; **B**: A plastic stent is then pushed over the guidewire into the main pancreatic duct; **C**: Pancreatic stent in place.

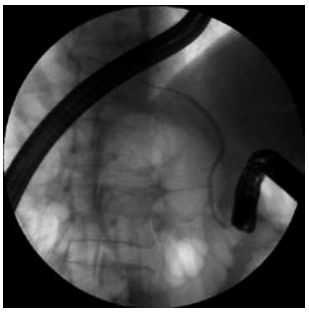

Figure 3 Chronic obstructive pancreatitis involving the head of the gland: insertion of an s-shaped stent into the main pancreatic duct.

sphincter so it is generally my preferred approach (Figure 2).

Pancreatic stents are generally made of polyethylene and are similar to biliary stents except for side holes along their length to allow flow from side branches. To prevent migration into the pancreatic duct, small-diameter stents have a J or "pig-tail" shape. For transpapillary stenting of a pseudocyst, a double pig-tail stent should be used to prevent displacement outside the cyst cavity. Recently, an S-shaped stent with many side holes has been proposed for MPD stenting in chronic pancreatitis[1]; this stent is made of ethylene vinyl acetate, which is more flexible than polyethylene. The S-shape enables the stent to adapt better to the course of the MPD and reportedly achieves a better outcome in patients with chronic pancreatitis and upstream duct dilatation than in patients treated with the standard straight polyethylene stents (Figure 3).

The diameter of the stent should not exceed the size of a normal downstream duct, so 5F and 7F stents should be used in cases with non-dilated ducts, while 10F and sometimes 11.5F can be used when the ducts are dilated, as in advanced chronic pancreatitis. Sometimes in advanced chronic disease the stricture is too tight to place a stent across it; in these cases the stricture must be dilated with a balloon or bouginage to permit insertion. In some cases the Soehendra stent retriever (5F or 8F) can be used to dilate the stricture and allow insertion.

How long stents are best left in place is not yet known. Pancreatic stents have been left in place for six months and long-term therapy requires multiple stent exchange. However, the duration of a single stent placement depends on the stent diameter: the larger the diameter, the longer the stent can be left in place.

CHRONIC PANCREATITIS

In chronic pancreatitis the MPD may be partially occluded by strictures or stones; the rise of intraductal pressure in the ductal segment above the obstruction causes dilation and obstructive pain. Pancreatic intraductal hypertension occurs regardless of the etiology and whether or not the MPD is dilated; ductal and interstitial hypertension, together with reduced acinar blood flow, may further contribute to the formation of fibrosis and progression toward more severe damage[2]. Removing the barriers to outflow of pancreatic juice may relieve chronic pain or exacerbation of chronic pancreatitis. Obstruction-related reduced outflow of pancreatic juice into the duodenum may also cause mal-digestion of nutrients even in cases with still conserved pancreatic enzyme secretion, or worsening of mal-digestion already present in advanced cases.

Although pancreatic ductal strictures can be treated by catheter or balloon dilation alone, a stent usually has to be inserted because stricture relapse is commonplace. Insertion of a stent beyond the ductal blockage achieves lasting relief of the intraductal hypertension and subsequent pain and possible mal-digestion, also restoring the lumen patency, by dilating the stricture. If a 10F stent or larger is used, the patient generally requires sphincterotomy of both the pancreatic and biliary segments of the sphincter, followed by stricture dilation (Figure 4).

The presence of both obstruction and ductal dilation is vital for predicting which patients are most likely to benefit

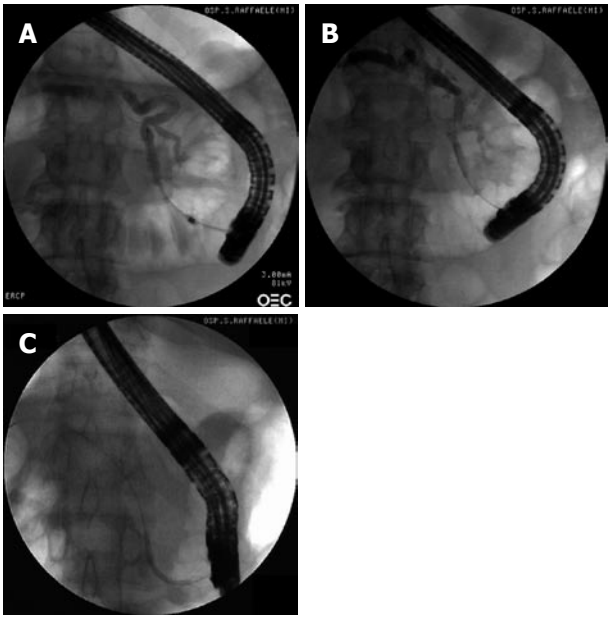

Figure 4 Symptomatic, chronic obstructive pancreatitis at early stage with moderate dilation of the main pancreatic duct. **A**: Mechanical dilation of the pre-papillary stricture by a 10F dilator; **B**: Insertion of the guidewire; **C**: Insertion of a plastic 10F stent throughout the dilated stricture over the guidewire.

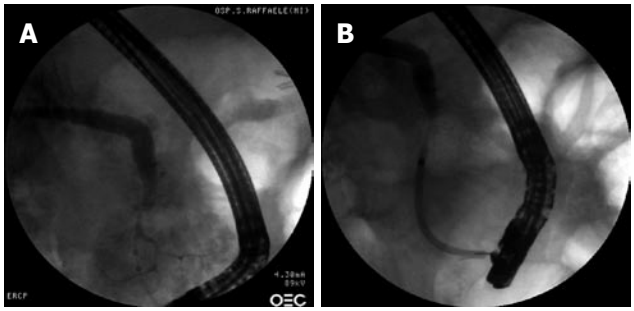

Figure 5 Chronic obstructive pancreatitis. **A**: Severe stricture of the main pancreatic duct with marked upstream dilation; **B**: Placement of a 10F plastic stent after mechanical dilation of the stricture.

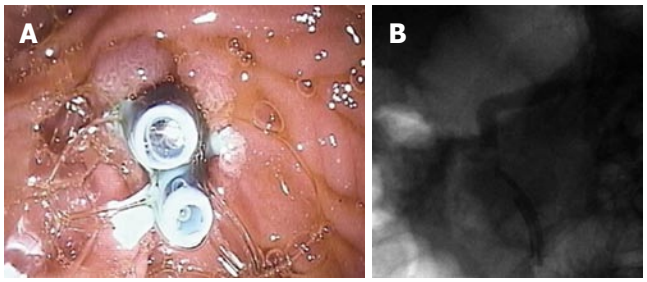

Figure 6 Refractory dominant stricture of the main pancreatic duct in chronic pancreatitis patient already treated by dilation and temporary stenting. Placement of two plastic stents. Endoscopic (**A**) and radiological (**B**) features.

from stricture therapy: the best candidates for stenting are those with a distal stricture and upstream dilation (Figure 5).

Most investigators and recent guidelines from the American Society for Gastrointestinal Endoscopy consider endoscopic management to be the preferred interventional approach for chronic pancreatitis in patients selected on the basis of anatomical changes caused by the disease; endoscopic treatment is generally safe, minimally invasive, can be repeated, and does not interfere with eventual surgery[3,4]. Other investigators, however, found surgery superior to endotherapy for long-term pain reduction. Dite *et al*[5], in a prospective randomized trial comparing endoscopic and surgical therapy for chronic, painful, obstructive pancreatitis, reported complete resolution of pain at the five-year follow-up in 37% of patients after surgery and in 14% of those after endotherapy; short-term results were similar in the two groups. Similar data have been recently published by Cahen *et al*[6].

The technical success of endoscopic stricture manipulation can range from 80% to 100% of patients with or without prior pancreatic sphincterotomy. In chronic pancreatitis patients with dominant stricture, pain relief was obtained in 52%-95% of cases over a follow-up ranging from 8 to 72 mo[1,7-19]. Stenting was also associated with weight gain and fewer hospital visits. Good clinical outcomes were related to cessation of alcohol consumption and/or smoking[15]. Early complications were reported in about 17% of cases and were related mainly to pancreatic and/or biliary sphincterotomy, stent clogging (juice infection) and inward migration.

It is not clear how long stents should best be left in place. Although the plastic 10F stents are thought to remain clinically patent for a year on average, generally they are removed and replaced every 6-9 mo. In fact, stent dysfunction leading to pancreatitis, recurrent pain or infection can occur before the scheduled exchange time in about half the cases, so repeated stent exchange is required in the long term. This may make it difficult to ensure compliance with long-term stenting treatment.

Despite encouraging medium- and long-term results, duct stricture may persist or recur after removal of a stent so definitive removal seems impracticable in a subset of patients, because of the recurrence of pain. In an intention-to-treat analysis, a German multicenter study on long-term outcomes in 1000 patients with chronic pancreatitis after pancreatic stenting reported unsatisfactory results in 35%; 16% of these patients continued with endotherapy and 24% opted for surgery[11].

A multiple stenting approach was proposed by Costamagna *et al*[20] in a subset of patients with refractory dominant MPD strictures: they reported lasting stricture dilation in 84% of their patients at 38-mo follow-up. Although placing a mean of three stents within pancreatic strictures may be difficult, this approach appears feasible and safe and could in fact dramatically reduce the need for surgery in the majority of patients with chronic obstructive pancreatitis (Figure 6).

Self-expandible metal stents have been proposed for patients with relapsing dominant strictures to achieve long-term stent patency and avoid the need for stent exchange[21]. The success rate of stent placement was 100% and patients enjoyed immediate relief of symptoms and reduction of duct diameter; however, during follow-up these patients have had high occlusion rates of the stent from mucosal hyperplasia, and it becomes impossible to remove the stent, so this approach has in fact been abandoned.

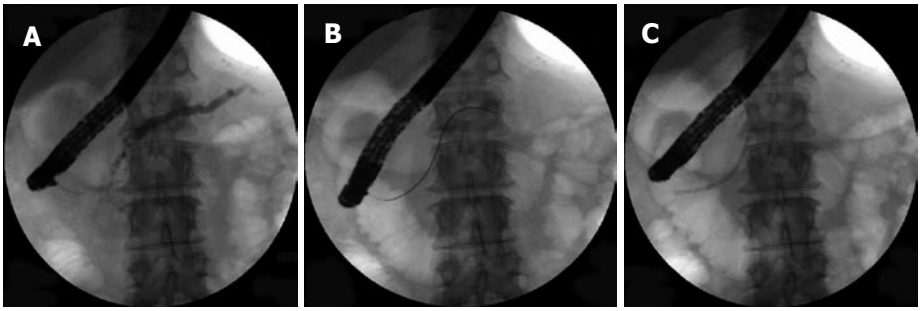

Figure 7 Pancreas divisum with chronic obstructive pancreatitis at early stage. **A**: Extensive dorsal duct stricture with moderate upstream dilation of the pancreatic duct; **B**: Guidewire insertion into the dorsal duct throughout the minor papilla, without sphincterotomy; **C**: 7F plastic stent in place. Long-term stenting rather than minor papilla sphincterotomy appears an appropriate approach in this case with extensive dorsal duct stricture.

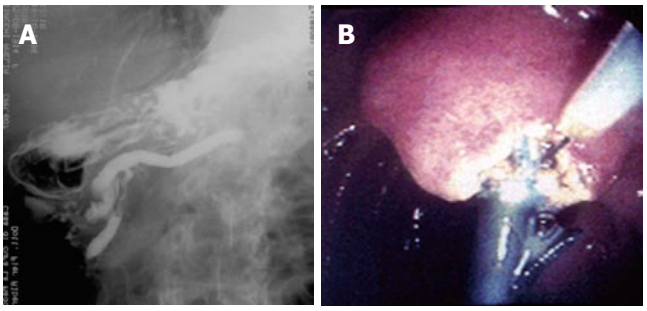

Figure 8 Chronic obstructive pancreatitis in incomplete pancreas divisum. **A**: Minor papilla stenosis associated with diffuse upstream dilation of the pancreatic duct and normal parietal morphology; **B**: Minor papilla sphincterotomy over-the-stent performed with a needle-knife sphincterotome. Minor papilla sphincterotomy rather than long-term stenting appears an appropriate approach in this case with stricture located at the level of the minor papilla.

PANCREAS DIVISUM

Pancreas divisum is present in about 7% of the population; it occurs when the ventral and dorsal ducts of the gland fail to fuse during embryological development. This anatomical variant is asymptomatic in the majority of cases but in some cases it may cause pancreatic pain due to functional obstruction at the level of the minor papilla or recurrent episodes of acute pancreatitis; persistence of the obstruction over time may lead to chronic obstructive pancreatitis. Kamisawa *et al* reported acute recurrent pancreatitis and chronic pancreatitis associated with pancreas divisum in respectively 17.1% and 28.6% of their patients[22].

Endoscopic therapy with minor papilla sphincterotomy and/or stent placement appears to be the treatment of choice at present. Critical issues concerning endotherapy in pancreas divisum are patient selection, difficulty of papillary cannulation, technique for endotherapy (minor papilla sphincterotomy or dorsal duct stenting, or both), stent-induced duct injury, and risks of post-ERCP pancreatitis. Patients with acute recurrent pancreatitis are the best candidates for endotherapy as in this group the predicted sustained response rate is around 75%; the response rate in patients with chronic pancreatitis is 40%-60%, whereas patients with recurrent or chronic abdominal pain respond poorly (20%-40%)[23].

The minor papilla is often difficult to visualize, but its orifice can be easily identified by spraying methylene blue over the duodenal mucosa in the papillary area or injecting it directly into the ventral duct, in cases with incomplete pancreas divisum[24], or by EUS[25], or by enhancing pancreatic secretion with i.v. secretin[26].

Endotheraphy of pancreas divisum includes minor papilla sphincterotomy and dorsal duct stenting with 5F, 7F and 10F stents, depending on the level of obstruction and degree of dilation (Figure 7 and Figure 8). Dorsal duct stenting without sphincterotomy was adopted by McCarthy *et al*[27], Lans *et al*[28] and Ertan[29], who reported satisfactory long-term results in respectively 89%, 90% and 76% of cases. However, Heyries *et al* reported more favorable long-term results with minor papilla sphincterotomy than with stenting[30]; they also observed fewer complications after sphincterotomy (25%) than after stenting (44%). More recently however, 45% of patients with chronic pancreatitis associated with pancreas divisum, undergone successful dorsal duct stenting and followed for a median period of 59.6 mo, required surgery after stent removal because of recurrence of symptoms[31]. Of course, stenting is the only option in cases with dorsal duct strictures proximal to the papillary orifice. A strategy of empiric 3-6 mo dorsal duct stenting may be adopted in patients with recurrent pain or pancreatitis with non-dilated dorsal duct or normal minor papilla motor function, investigated by manometry or MRCP and secretin test, in order to decide whether sphincterotomy would be appropriate. This approach in patients with non-pathological duct morphology, however, could lead to ductal changes consistent with chronic pancreatitis in about one third of cases.

PANCREATIC PSEUDOCYST WITH DUCTAL COMMUNICATION

Pseudocysts complicate acute and chronic pancreatitis in up to 20% of cases; approximately 50% of pseudocysts regress spontaneously within 6 to 12 wk. Pseudocysts that are symptomatic, or become larger on follow-up imaging, or are associated with complications, require a drainage procedure. The pseudocyst communicates directly with the MPD in up to 40%-66% of cases[32]. Pseudocysts with ductal communication can only be resolved by duct drainage[33]. This can be achieved during ERCP by a trans-papillary approach, thus avoiding the usual risks (bleeding and perforation) of endoscopic cysto-gastrostomy or cysto-duodenostomy, especially when endoscopic ultrasound (EUS) guidance is not available. Trans-papillary 5F, 7F or 10F stents can be placed beyond the strictured segment of the MPD but not into the pseudocyst in cases with duct strictures downstream of the pseudocyst, or

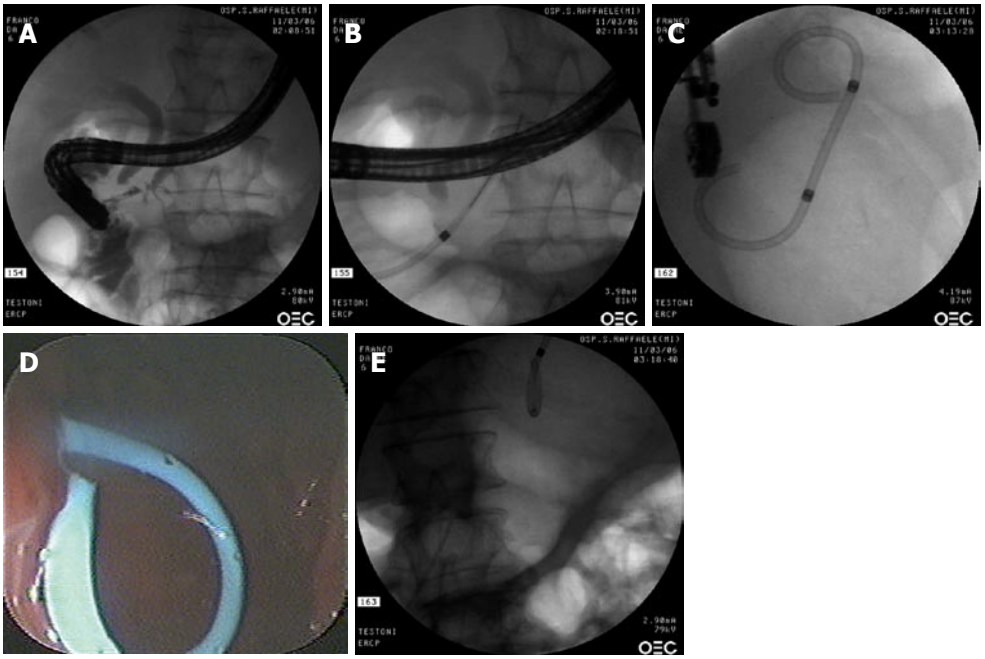

Figure 9 Large pancreatic pseudocyst communicating with the main pancreatic duct: combined endoscopic trans-papillary and trans-gastric drainage. **A**: Contrast injection into the MPD shows a stricture of the main pancreatic duct at the level of the cyst; **B**: After pancreatic sphincterotomy, the stricture is dilated, a guidewire is inserted into the cyst cavity, and a plastic stent is placed; **C** and **D**: Under EUS guidance, a double pig-tail plastic stent is also placed by a single step procedure; **D**: X-ray imaging shows trans-papillary and trans-gastric stents at the end of the procedure.

Table 1 Endoscopic transmural drainage of pancreatic pseudocyst[1]

Endoscopic centers	198
EUS-guided drainage	65%
Successful drainage	91%
Clinical success	71%
Mean number of stents inserted	2
Mean duration of stenting	6 wk
Complications rate	
Infection	12.70%
Bleeding	11.40%
Perforation	2.20%

[1]: International survey of ASGE members.

directly into the pseudocyst cavity if no MPD strictures are found[34], or into the MPD bridging the communication between the duct and the cyst cavity. When the stent is placed directly in the pseudocyst cavity, a double pig-tail stent should be preferred to avoid the risk of displacement. In the presence of a large and symptomatic pseudocyst, MPD drainage is generally associated with a trans-parietal drainage in order to achieve a more rapid decompression of the cyst cavity and resolution of symptoms (Figure 9). EUS-guided pseudocyst drainage has become popular in recent years because it has many advantages compared to the endoscopic approach[35]. Bulging is not required; ultrasound guidance permits assessment of the optimal area to access vascular structures, cyst content and communication with the main pancreatic duct (Table 1). Pseudocyst drainage is feasible even if the distance between the cyst and G.I. lumen exceeds one cm, and the procedure may be performed in a single step.

Stents should be changed routinely every 6-8 wk, to avoid clogging and the risk of infection or pancreatitis.

Features predictive of a successful trans-papillary approach are MPD dilation upstream of the ductal stricture when the stent is placed across the stricture and a non-dilated pancreatic duct when the stent is placed to bridge the communication of the cyst with the pancreatic duct. Placing a stent in the pseudocyst in a case with non-dilated MPD is associated with a higher risk of pancreatitis.

PANCREATIC FISTULAS

Fistulas can occur as a consequence of partial or complete rupture of the pancreatic duct caused by trauma, pancreatic surgery, or complicating severe acute pancreatitis. During an attack of acute severe pancreatitis, ERCP found a pancreatic duct leak in 37% of cases and this was significantly associated with a higher incidence of necrosis and longer hospital stay[36]; the early recognition and treatment of such leaks and eventually associated fluid collections is likely to improve outcomes[37].

At present, the diagnostic approach to pancreatic fistulas and suspected pancreatic duct leaks should be MRCP with secretin stimulation, leaving ERCP and EUS only for therapeutic purposes, once the lesion has been identified and staged. Before planning endoscopic treatment of fistulas or duct leaks several points must be clarified: the location of the lesion within the pancreatic ductal system, the presence and type of pancreatic duct strictures downstream of the lesion, whether pancreatic duct disruption is complete or incomplete, whether there is any communication with a fluid collection, and its anatomical characteristics.

Fifteen years ago Kozarek *et al* reported that bridging a pancreatic duct leakage by trans-papillary stent placement was effective for either internal or external pancreatic fistulas[38]. Transpapillary stenting of the MPD has now become the "gold standard" for the treatment of fistulas and duct leaks, with success rates ranging from 55%[39] to 100%[40], although higher than 80% in most series (Figure 10 and Figure 11). Telford *et al* reported that the position of the bridging stent was the only variable related with a good outcome (92%), while stents placed at the level of

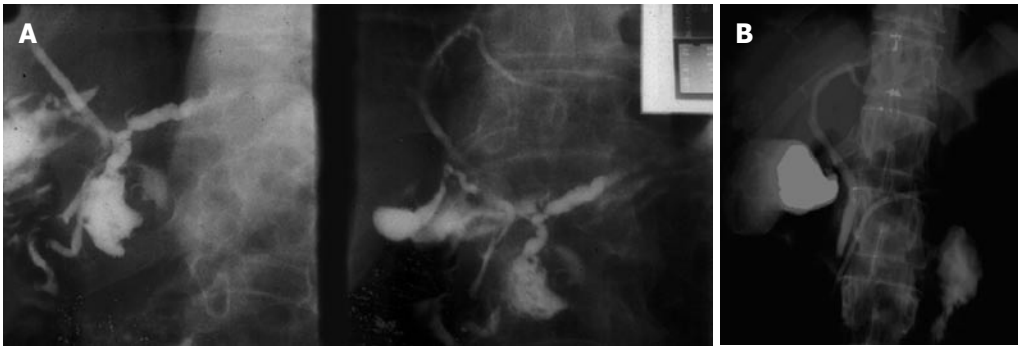

Figure 10 Main pancreatic duct disruption with pancreatic juice leakage (**A**) successfully treated by plastic stent insertion bridging the leak (**B**).

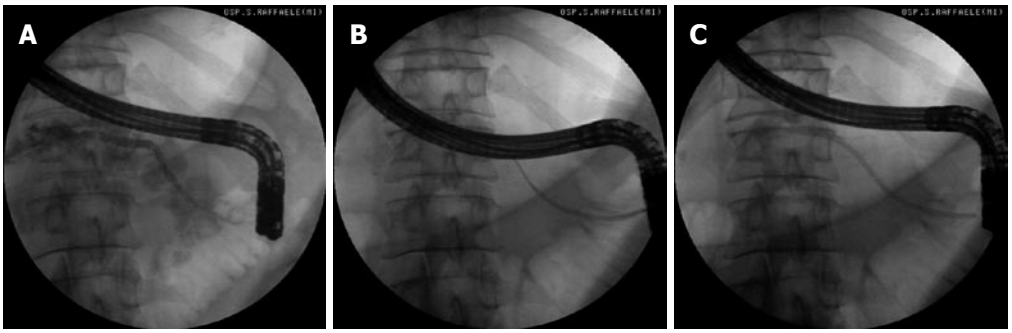

Figure 11 Pancreatic duct leakage at the level of the tail of the gland, following surgical resection of neuroendocrine tumor. **A**: The leakage site is identified by contrast injection into the main pancreatic duct; **B** and **C**: Over a guidewire, a long plastic stent is inserted at the level of the tail of the gland.

the leakage or distally were more often associated with approximately 50% of failures[41]. A partially disrupted MPD, the location of the disruption at the level of the body of the pancreas, the stent positioned to bridge the disruption, and a longer duration of stent therapy were identified as predictors of a favorable outcome in the endoscopic management of duct disruption on a large series of patients[39]. The stent should be left in place for four to six weeks. A shorter period of stenting may involve a higher rate of failure[41], while a longer period may increase the risk of stent occlusion and stent-induced alterations in ductal morphology.

SMOLDERING PANCREATITIS AND IDIOPATHIC RECURRENT PANCREATITIS

"Smoldering" pancreatitis refers to a syndrome in which patients recovering from acute pancreatitis suffer from unremitting abdominal pain, intolerance of food, persistently elevated serum levels of pancreatic enzymes, and persisting inflammatory changes in and around the pancreas at imaging studies. Functional obstruction of the papillary orifice, induced by edema or sphincter spasm, and inflammation-related fibrotic strictures of the MPD may account for the unremitting course in a subset of patients with smoldering pancreatitis. In these cases, insertion of a stent into the MPD provided permanent relief of pain in 91% of patients within a mean of nine days (range 3-20 d) and discontinuation of parenteral nutrition within a mean of 15 d (range 7-39 d); the stents were left in place for a mean of seven weeks (range 2-19 wk)[42].

Today the etiology of acute pancreatitis remains undefined in 2%-30% of cases, despite a careful diagnostic work-up including imaging techniques for pancreatic morphology (CT scan, MRCP, EUS), functional investigation of the sphincter of Oddi (manometry, secretin test), and tests for gene mutations and autoimmune disorders. In these cases the term "idiopathic pancreatitis" is generally adopted and the failure to identify the cause predisposes to further recurrences. Despite the absence of morphofunctional alterations, however, it is generally believed that biliary sludge or microlithiasis or unrecognized transient sphincter of Oddi dysfunction (Type 2) plays a causal role. In a therapeutic protocol study adopted in our institution we found that the placement of a 5F or 7F stent into the MPD in cases with pancreatitis still recurring after biliary sphincterotomy served to identify those patients with residual hypertension of the pancreatic segment of the sphincter of Oddi who benefit from pancreatic sphincterotomy, as documented during a 27-mo follow-up. In these patients with a non-dilated MPD stents were routinely changed every three months. This empiric approach can be suggested for patients with recurrent pancreatitis but no evidence of morphofunctional abnormalities, presenting at least two or three acute attacks over one year, in whom three to six months' stenting can provide a reliable basis for deciding on pancreatic sphincterotomy[43].

Jacob *et al*[44] reported the results of a prospective randomized nonblinded trial evaluating the effectiveness of pancreatic stent placement in preventing attacks of pancreatitis in patients with idiopathic recurrent pancreatitis over a five-year period. The stent group received three stents in one year while the control group underwent selective pancreatic duct opacification without stenting. Pancreatitis recurred in 53% of the control group and in 11% in the stent group. The authors concluded that unrecognized intermittent pancreatic duct sphincter dysfunction or relative outlet obstruction might be a cause of recurrent pancreatitis that can be prevented by stent

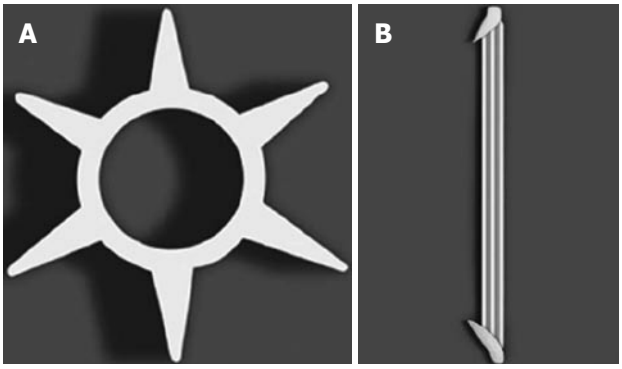

Figure 12 Experimental winged 5 and 7F stents. The new stent design with a wing shape permits an adequate flow of pancreatic juice even alongside the stent and does not compress the duct over its entire circumference, thus avoiding the risk of impaired drainage of pancreatic juice and mechanical trauma to the duct.

placement. However, long-term stenting of the pancreatic duct may in itself cause ductal damage, so only short-term stenting in patients with frequent episodes of pancreatitis is justifiable.

COMPLICATIONS OF STENT PLACEMENT

Several complications have been reported after pancreatic duct stent placement in benign diseases, ranging from 5%-39%. These include inward or outward migration of the stent, occlusion, and anatomic changes of the pancreatic duct[45,46]. The latter limits the long-term use of stents in the treatment of benign disorders especially when pancreatic ducts are non-dilated. Changes of MPD morphology consistent with chronic pancreatitis have been reported after stent placement in 36%-83% of patients; ductal changes of the pancreatogram appear as early as three months and seem not to revert to normal in some cases after removal of the stent. Pancreatic stents placed in dogs were found to induce both radiological and histological changes of chronic pancreatitis in the ductal segment treated with the stent, within eight weeks[47].

Although the mechanism by which changes are induced is not known, there is evidence that stenting the pancreatic duct leads to the formation of intraductal plugs in as little as three weeks even though pancreatograms may remain normal. These protein precipitates have the same composition as plugs removed from patients with chronic pancreatitis. Moreover, the conventional plastic stent does not provide enough side openings for unencumbered drainage at all sites where secondary ducts join the MPD; this obstruction and the pancreatic duct compression along the whole length of the stent induce a fibrotic reaction. A new pancreatic stent design with a wing shape has now been tested in dogs, with encouraging results, since this model permits an adequate flow of pancreatic juice even alongside the stent and does not compress the duct over its entire circumference, thus avoiding the risk of impaired drainage of pancreatic juice and mechanical trauma to the duct[48] (Figure 12).

In conclusion, in about the last 20 years endotherapy of pancreatic disorders has evolved from an experimental approach for some pathological conditions in selected cases where there is a fear of severe complications, to the "gold standard" for most acute and chronic inflammatory disorders involving the gland.

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REVIEW

Angiogenesis and vascular malformations: Antiangiogenic drugs for treatment of gastrointestinal bleeding

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Abstract

Treatment of gastrointestinal bleeding in patients with angiodysplasias and Osler's disease (hereditary hemorrhagic telangiectasia) is clinically challenging. Frequently, vascular malformations occur as multiple disseminated lesions, making local treatment an unfavorable choice or impossible. After local therapy, lesions often recur at other sites of the intestine. However, as there are few therapeutic alternatives, repeated endoscopic coagulations or surgical resections are still performed to prevent recurrent bleeding. Hormonal therapy has been employed for more than 50 years but has recently been shown to be ineffective. Therefore, new therapeutic strategies are required. Understanding of the pathophysiology of angiogenesis and vascular malformations has recently substantially increased. Currently, multiple inhibitors of angiogenesis are under development for treatment of malignant diseases. Experimental and clinical data suggest that antiangiogenic substances, which were originally developed for treatment of malignant diseases, may also represent long-awaited specific drugs for the treatment of vascular malformations. However, antiangiogenics display significantly different actions and side-effects. Although antiangiogenics like thalidomide seem to inhibit gastrointestinal bleeding, other substances like bevacizumab can cause mucosal bleeding. Therefore differential and cautious evaluation of this therapeutic strategy is necessary.

Key words: Angiodysplasias; Osler's disease; Angiogenesis; Gastrointestinal bleeding; Vascular endothelial growth factor

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INTRODUCTION

Diseases related to vascular malformation and pathologic vessel growth include benign conditions such as pediatric hemangioma, cutaneous angiectasias and venous malformations. They also include more severe and potentially life-threatening diseases like gastrointestinal angiodysplasias, Osler's disease (hereditary hemorrhagic telangiectasia, HHT) and cerebral arteriovenous malformations, as well as rare syndromes like hereditary dysembryoplasia and pulmonary capillary haemangiomatosis. Vascular malformations and dysfunctional vessels in these diseases result from different disturbances of the angiogenic process, which have only been partially characterized[1-3].

Among these anomalies, arteriovenous vascular malformations that are located on mucosal surfaces are of special clinical relevance because such lesions may cause intense bleeding. Other symptoms of arteriovenous vascular malformation include shunt syndromes, compression syndromes and thrombocytopenia/coagulopathy (Kasabach-Merritt syndrome).

Both Osler's disease and gastrointestinal angiodysplasias can cause recurrent bleeding, which in severe cases can require hundreds of blood transfusions over a period of years[4,5]. Despite diagnostic improvements like wireless capsule endoscopy, treatment of such patients remains a clinical challenge. Multiple lesions disseminated over the small intestine are frequently present, making local treatment an unfavorable choice or impossible[4]. However, as there are few therapeutic alternatives, repeated endoscopic coagulation or surgical resection is performed to prevent recurrent bleeding (although lesions often recur at other intestinal sites after local therapy). Therefore an effective medical treatment for these patients is urgently needed.

Although multiple drugs have been evaluated, there is currently no medical treatment with confirmed efficacy for preventing bleeding from vascular malformations. In this situation, hormonal therapy, which is still the best evaluated treatment option, is frequently employed. Based on observations of cycle-dependent severity of bleeding in some patients, hormonal therapy with estrogens and progesterones has been used for prevention of bleeding since the 1950s. However, there have been no confirming data regarding the effectiveness of hormonal therapy for prevention of intestinal bleeding. The only double-blind, placebo-controlled trial for treatment of nasal bleeding in Osler's disease suggested a moderate effect, but found no

significant reduction in epistaxis from estradiol therapy[6]. In 72 patients with non-hereditary angiodysplasias, a recent randomized placebo-controlled study failed to show an effect from a combination of ethinyl estradiol and norethisterone on the incidence of rebleeding and transfusion requirements[7]. Due to these disappointing results and substantial side-effects, especially in male patients, new therapeutic approaches are clearly required.

MECHANISMS OF BLEEDING

Understanding of the pathophysiology of angiogenesis and vascular malformation has recently substantially increased. Different mutations within the angiogenic signaling cascade indicate that two types of Osler's disease can be differentiated. Mutations of endoglin or activin receptor-like kinase 1 (ALK-1), both receptors for transforming growth factor beta (TGFβ)[1,2], are present in the majority of cases of HHT. Differential stimulation of ALK-1 and ALK-5 is thought to regulate different phases of angiogenesis, thereby activating the angiogenic process, with subsequent increased production of vascular endothelial growth factor [VEGF, initially termed vascular permeability factor (VPF)][8,9]. VEGF, so far the best characterized angiogenic molecule, stimulates proliferation of vascular endothelial cells and increases vascular permeability, and thus is a central mediator of the early phase of angiogenesis[10]. Recent studies have confirmed that both Osler's disease and nonhereditary intestinal angiodysplasias are characterized by increased production of VEGF[11,12]. High serum levels of VEGF that also correlate with severity of bleeding are found in patients with Osler's disease[11].

Intestinal angiodysplasias in patients who have undergone colonic resections because of recurrent bleeding strongly express VEGF along the endothelial lining[12], indicating a proliferative phase of angiogenesis. Expression of VEGF receptor 1 is also observed in bleeding intestinal angiodysplasias[12]. As VEGF receptor 1 is specifically up-regulated by hypoxia, this finding may indicate a role for hypoxia in the pathogenesis of angiodysplasias[13,14]. However, further detailed analyses are necessary to clarify the pathophysiology of gastrointestinal angiodysplasias.

VEGF-mediated effects are presumably also involved in bleeding in inflammatory bowel disease (IBD). The mucosal inflammation in Crohn's disease and ulcerative colitis is characterized by increased production of the proinflammatory cytokines tumor necrosis factor α, interleukin (IL)-1 and IL-6. In experimental models, proinflammatory cytokines have been shown to induce VEGF[15,16], a mechanism which is also likely present in IBD in order to supply the inflammatory infiltrate and thickened bowel wall with microvessels[17]. As a result of permanent VEGF stimulation, sprouting vulnerable vessels are located at the inflamed mucosal surface; thus possibly resulting in intermittent bleeding and so contributing to chronic anemia in Crohn's disease[18,19]. However, a small number of patients with Crohn's disease also suffers from massive recurrent bleeding[20,21]. As such episodes of severe bleeding are not associated with high disease activity[20-22], it can be speculated that the severe bleeding in such patients results from larger superficially located vessels with arteriovenous short circuits. Angiodysplasias have been documented in several of these patients; however they have not been definitely proven to cause this bleeding[20-22].

The initial phase of angiogenesis is characterized by VEGF-dependent formation and the sprouting of vessels consisting of endothelial cells. Within this process, microenvironmental concentrations of VEGF seem to determine whether normal or aberrant angiogenesis is induced[23]. Gene-therapy-induced high local concentrations of VEGF result in vascular malformations[24]. Such lesions resemble the chaotic architecture of haemangiomas or angiodysplasias and are characterized by thin-walled fragile vessels with high permeability, which lack smooth muscle cells and are susceptible to rupture[25]. To mature, such a primitive vascular plexus would have to be remodeled and vessels acquire a smooth muscle layer, a process that requires other angiogenic factors such as TGFβ, platelet-derived growth factor and angiopoietin-1 (which also stabilizes the leakage of VEGF-overexpressing vessels)[25,26]. Angiodysplasias and other vascular malformations like hemangiomas arise from massive local activation of the early stage of angiogenesis, with accumulation of VEGF, which induces primitive endothelial vessel complexes. However, these vessel precursors fail to finally differentiate into complete functional vessels and form net-like labyrinthine complexes. If surrounded by parenchymal tissue or stable epithelial structures, these complexes are generally harmless. However, when located near a mucosal surface, such fragile vessel systems are susceptible to rupture and can cause bleeding.

ANTIANGIOGENIC THERAPY

The observation that different vascular malformations, despite a distinct pathogenesis, are characterized by a pathologic accumulation of VEGF theoretically makes them an attractive target for direct or indirect VEGF-suppressive antiangiogenic therapy. Suppression of VEGF disrupts development of sprouting vessels: in experimental models VEGF withdrawal results in endothelial cell shedding and regression of primitive hemangioblastoma-like vessels[27].

Several antiangiogenic substances have been developed for treatment of malignant diseases. These include monoclonal antibodies against VEGF [Bevacizumab (Avastatin)], VEGF-trap, VEGF-receptor antibodies and antagonists [SU5416 (semaxanib), IMC-IC11, PTK 787 and SU6668], proteins (endostatin and angiostatin), matrix metalloproteinase inhibitors (Marimastat, Primostat and COL-3), thalidomide and its analogues [CC5013 (Revimid) and CC4047 (Actimid)], and several other substances that act during different phases of the angiogenic process[28]. In addition to their therapeutic potential in malignant diseases, some of these substances could also be useful for treatment of bleeding vascular malformations.

THALIDOMIDE-THE FIRST ANTIANGIOGENIC DRUG

The best known-and for four decades unrecognized as

such-inhibitor of angiogenesis is thalidomide, which tragically was used as a sedative and anti-emetic in pregnant women from 1956 until it was withdrawn from the market in 1961 after being recognized to have caused severe birth defects. During these years, in which thalidomide became the most popular sedative in Germany, about 10000 children with phocomelia and other malformations were born[29]. It was only in 1994 that thalidomide was found to inhibit VEGF-and basic fibroblast growth factor-mediated angiogenesis; a detection that resulted from a side-effect-based literature screening in search of drugs with antiangiogenic activity that D'Amato and Folkman presumed should cause both amenorrhea and fetal malformations[30]. The exact mechanism by which thalidomide acts within the angiogenic cascade remains to be determined, but appears to be located upstream of the VEGF level, i.e. reduced expression of integrin genes has been hypothesized[31]. In experimental models, the antiangiogenic activity of various thalidomide metabolites correlates with teratogenicity[30-32], indicating that antiangiogenic effects are also mainly responsible for thalidomide-related birth defects in humans. The antiangiogenic potential of thalidomide is currently being evaluated for treatment of several malignant diseases[29]. Due to promising results in patients with therapy-refractory multiple myeloma, thalidomide is now evaluated as both first-line myeloma therapy and in combination with hematopoietic-cell transplantation[33,34].

It has recently been reported in a number of case studies that thalidomide reduces the incidence of severe bleeding in different gastrointestinal diseases. Eight patients with severe bleeding related to Crohn's disease or angiodysplasias of the small intestine, who had received up to 230 blood transfusions, responded to moderate doses of 100-300 mg thalidomide daily[18,22,35]. Similarly, chronic bleeding unexpectedly resolved in patients with hereditary hemorrhagic telangiectasia, who received thalidomide as antiangiogenic cancer therapy[36,37]. In patients with angiodysplasias, rebleeding was also prevented for more than 2 years after thalidomide treatment had ended[22]. In Crohn's disease with moderate inflammatory activity, severe bleeding stopped during thalidomide treatment, partly recurred after cessation of thalidomide, but was controlled again by retreatment[18,22]. However, in patients with Crohn's disease, it is not clear whether cessation of bleeding is related to antiangiogenic or anti-inflammatory effects of thalidomide. More objective evidence that antiangiogenic effects of thalidomide are responsible for the efficacy on bleeding is found in patients with non-inflammatory diseases. In patients with angiodysplasias or Osler's disease without any evidence of inflammation[22,35,37], the efficacy of thalidomide on bleeding is unlikely to be related to anti-inflammatory or immunomodulating effects. Serum levels of VEGF were found to be suppressed by thalidomide in patients without inflammation[22]. In patients with multiple angiodysplasias of the small bowel, wireless capsule endoscopy has demonstrated that the clinical efficacy of thalidomide is paralleled by a decrease in number, size and color intensity of angiodysplasias, which indicates regression[38].

Although the results of the above case series need to be confirmed in controlled trials, present data indicate that antiangiogenic effects of thalidomide are responsible for reductions in bleeding episodes. However, the side effects of thalidomide are also substantial. Thalidomide is a potent sedative, causes severe birth defects and can also induce sensible peripheral neuropathy, especially at higher cumulative doses[39], therefore, it may not turn out to be the drug for treatment of vascular malformations hoped for by clinicians. Furthermore, in addition to its antiangiogenic activity, thalidomide exerts immunomodulating effects[29]. It is possible that other newly developed antiangiogenics will show more specific inhibition of VEGF or other steps within the angiogenic cascade, with possibly fewer side effects.

NEW ANTIANGIOGENICS

Of the currently developed antiangiogenic substances, most information regarding clinical efficacy and toxicity is available for bevacizumab (Avastatin®), a humanized monoclonal antibody against VEGF. Bevacizumab was recently shown to be effective in the treatment of colonic and renal cancer; present data indicate strong antiangiogenic activity and a favorable side-effect profile[40,41]. However, nose bleeding is frequently observed during treatment (in up to 59% of patients)[42]. The incidence of nose bleeding correlates with higher doses[40], although the reason for this bleeding is not clear. A loss of vascular integrity by bevacizumab-induced endothelial-cell shedding in highly regenerative mucosal tissues with active angiogenesis could be a possible explanation for this dose-dependent effect. Other reported side effects during treatment with bevacizumab include gastrointestinal bleeding and perforations, which were not always tumor-related[41,42].

Bleeding complications do not seem to be a specific feature of bevacizumab. A recent study with IMC-IC11, a humanized monoclonal antibody against VEGF receptor 2, also reported bleeding episodes unrelated to tumor manifestations[43]. Although most bleeding complications are probably of minor relevance in patients with malignant diseases, it is conceivable that abrupt antibody-induced withdrawal of VEGF in proliferating VEGF-dependent endothelial vessels could become critical in pre-existing vascular malformations located on mucosal surfaces.

Therefore, although VEGF-based antiangiogenic therapy is a promising and highly specific therapeutic option for preventing growth of vascular malformations, it seems questionable whether monoclonal antibodies against VEGF or its receptors are also useful for treatment of bleeding from pre-existing vascular malformations. Indeed, some highly effective antiangiogenics could even aggravate bleeding from vascular malformations.

Semaxanib (SU 5416), a small-molecule inhibitor of VEGF receptor 2 tyrosine kinase has recently been used for treatment of hemangioblastoma in patients with von Hippel-Lindau disease (vHLD). In vHLD, a loss of von Hippel-Lindau protein results in an accumulation of hypoxia-inducible factor and subsequently, induction of

VEGF[44]. Some initial studies on semaxanib have reported regression or stabilization, with improvement of macular edema, in patients with hemangioblastoma, which indicates effective inhibition of VEGF[44,45]. Frequently observed side effects of semaxanib include fatigue and headache[47,48]. To date, bleeding complications, like those found for bevacizumab, have not been reported for semaxanib (neither have they been reported for thalidomide, for which side effects have been well documented since its reevaluation for malignant and inflammatory diseases).

In summary, it is remains unclear why some antiangiogenic substances like bevacizumab can cause mucosal bleeding and others like thalidomide do not. This effect may be related to the phase of angiogenesis that is antagonized, or might reflect a particular strong antiangiogenic activity. Detailed analyses of the angiogenic cascade and how thalidomide and other antiangiogenics act within this process will be needed to resolve this issue.

SIDE-EFFECTS AND TOXICITY

Currently, only limited data are available regarding the general toxicity of antiangiogenic therapy. Arterial hypertension has been reported for several agents and is thought to be at least partially related to reduction of VEGF-mediated vascular permeability. As VEGF is also centrally involved in neuroregeneration, neurotoxicity is another possible concern for antiangiogenic treatment[49]. Experimental peripheral neuropathy is reversible by VEGF gene transfer[50]. Reduction of VEGF levels by 25% results in motor neuron degeneration reminiscent of amyotrophic lateral sclerosis[51]. Thalidomide's well-documented neurotoxicity is therefore possibly not drug-specific but could be a general effect of long-term antiangiogenic therapy. Until now, significant neuropathy has not been reported for the new antiangiogenics; however, only very limited data regarding long-term toxicity are available. Furthermore, as these drugs are generally just one part of a polychemotherapeutic regimen, it is often unclear to what extent antiangiogenics contribute to clinically observed neurotoxicity[52].

Finally, VEGF is also crucial for embryonal angio- and vasculogenesis. Loss of a single VEGF allele causes severe embryonic vascular defects[53]. Therefore, not only thalidomide, but any inhibitor of VEGF that crosses the placenta has to be considered a potential teratogen. As antiangiogenics are primarily developed as anticancer agents and designated to be used in combination with chemotherapy, this subject is generally regarded as of minor relevance. However, if antiangiogenic therapy is expanded to benign diseases like angiodysplasias, Osler's disease, and inflammatory diseases like Crohn's disease (which may also improve due to inhibition of angiogenesis)[54-57], and young women are candidates for treatment, teratogenicity becomes a critical issue. Indeed, a single dose of thalidomide is sufficient to cause birth defects[30]. Angiogenesis has a central role in embryo growth and pregnancy-prevention programs cannot completely prevent the birth of children with fetal malformations[58]; therefore, antiangiogenics should only be used under strict surveillance in non-malignant diseases.

CONCLUSION

In summary, antiangiogenic substances like thalidomide hold promise to be not only useful for treatment of malignant diseases, but may also represent the drugs that have been long awaited for the treatment of bleeding from vascular malformations. However, as some antiangiogenics can cause mucosal bleeding, a differential therapeutic approach and careful evaluation are necessary. Moreover, antiangiogenic therapy is also teratogenic and therefore has to be very cautiously considered in women of child-bearing potential.

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REVIEW

Updates on abdominal desmoid tumors

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Abstract

Desmoid tumor is a monoclonal, fibroblastic proliferation arising in musculoaponeurotic structures. This connective tissue hyperplasia infiltrates locally, recurs frequently after resection but does not metastasize. Abdominal desmoid occurs sporadically, in association with some familial syndromes and often represents a clinical dilemma for surgeons. The enigmatic biology and anatomical location of abdominal desmoids make treatment recommendations difficult. This distinct pathological entity is reviewed with a specific focus on aetiology and management.

Key words: Desmoid; Abdomen; Fibromatosis; Familial adenomatous polyposis; Gardner's syndrome

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INTRODUCTION

Desmoid tumor, also known as aggressive fibromatosis or musculo-aponeurotic fibromatosis[1], is a monoclonal[2,3], fibroblastic proliferation arising in musculoaponeurotic structures.

Although Mueller in 1838 coined the term desmoid tumor[4] (derived from the Greek *desmos* that means tendon-like), the first description of the tumor is credited to McFarlane, who reported the disease occurring in the abdominal wall of a young woman after delivery in 1832[5].

Histologically, these tumors consist of spindle-shaped cells in a collagenous matrix without the pleomorphic, atypical, or hyperchromatic nuclei of malignancy[1]. The connective tissue hyperplasia infiltrates locally, recurs frequently after resection but does not metastasize[6].

Desmoid tumors have been recently subdivided according to their location into extra-abdominal, abdominal and intra-abdominal, and the latter have been subclassified further into mesenteric fibromatosis and pelvic fibromatosis[7]. This tumor may occur at the site of any fascia, but in particular in muscle, hence the descriptive term musculo-aponeurotic fibromatosis. The most frequent sites involved by these tumors are the torso and the extremities. Many studies have shown that between 37% and 50% of desmoids arise in the abdominal region[6,8,9]. Abdominal desmoid occurs sporadically[8], in association with some familial syndromes[9] and often represents a clinical dilemma for surgeons. Most surgical reports emphasize the difficulty in achieving adequate resection margins, while maintaining acceptable function and cosmesis[10,11]. These are major factors contributing to the high rates of relapse after surgery, especially after conservative resections[12,13].

The enigmatic biology and anatomical location of intra-abdominal desmoids make treatment recommendations difficult. A significant factor limiting the attempted generalization concerning management is the small number of cases available for analysis, reflecting the relative rarity of the disease.

EPIDEMIOLOGY AND ETIOLOGY

Desmoid tumor is a rare lesion representing < 3% of all soft tissue tumors with an estimated incidence of 2-4 new cases per million per year[14].

These tumors have been well characterized from a morphologic standpoint, but their nature and pathogenesis have remained obscure for many years, to the point that Stout[15] defined it as "the most incomprehensible group" of fibromatosis. They have been considered non-neoplastic processes by some authors and well-differentiated low-grade sarcomas by others[2].

An association with familial adenomatous polyposis of the colon (FAP) and Gardner's syndrome has been well documented. Abdominal and extra-abdominal desmoids occur more frequently in FAP patients, with an incidence of 3.5%-32%. In the original Gardner kindred the incidence was 29%[9,16].

The etiology of desmoids has not been well defined. Numerous factors are acknowledged to be strongly associated with their development. An antecedent history of trauma to the site of the tumor, often surgical in nature, may be elicited in approximately 25% of cases[17,18].

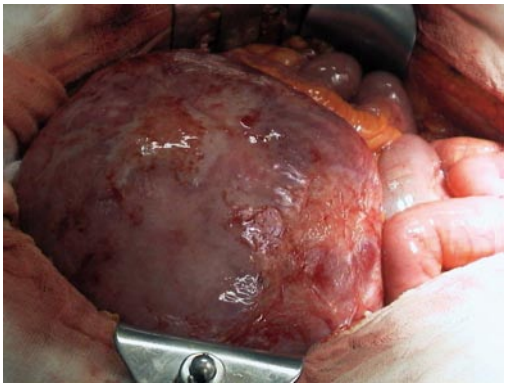

Figure 1 Intraoperative finding of giant retroperitoneal desmoid tumor.

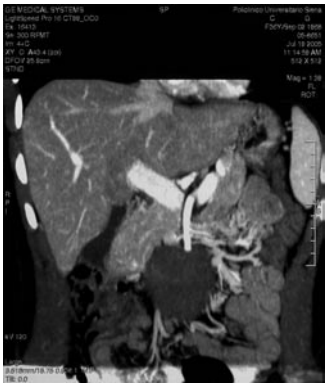

Figure 2 Coronal view of computed tomography scan shows a mesenteric desmoid tumor.

Within the FAP population, there is a strong correlation between prophylactic procto-colectomy and the subsequent development of desmoid tumours[19-22].

Other forms of trauma, such as physiologic trauma associated with pregnancy, are also thought to contribute to the development of desmoid tumors and several papers report an increased association between pregnancy and desmoid tumors[10,14,16,17].

A predominance of the disease in the female population has been reported, with a female to male ratio ranging from 1.4 to 1.8. The peak of incidence is between the ages of 25 and 35 years, even though cases occurring in patients younger than 10 years have been described[10,17,23,24]. The preponderance of cases described afflicting women in reproductive age shows a clear association of this disease with the endogenous hormonal environment and exogenous sex hormones[10,16,17].

Anecdotal reports of tumor regression during menopause[25,26], the development of desmoids in patients taking oral contraceptives[19,27], and reports of tumor regression with tamoxifen treatment[28], serve to underline an evident role of estrogen in the multifactorial pathogenesis of desmoid.

While most of the cases are sporadic, some are associated with familial syndromes (FAP, Gardner's syndrome) and these are most often intra-abdominal[19,29]. There are also cases of familial desmoid tumors at multiple sites, in patients without FAP, often involving one extremity. In both FAP and familial non-FAP tumors, mutations of the adenomatous polyposis coli (APC) gene on the long arm of chromosome 5 have been incriminated. The resultant loss of ability to degrade beta-catenin and elevated beta-catenin levels promotes fibroblastic proliferation through a nuclear mechanism[30].

CLINICAL PRESENTATION AND DIAGNOSTIC EVALUATION

Desmoid tumors most frequently present as a slowly enlarging mass (Figure 1). Symptoms depend on the location of the tumor. Patients with intra-abdominal desmoid may have asymptomatic masses, symptoms of intestinal, vascular and urinary obstruction or neural involvement[6,31,32]. The diagnosis of desmoid is based on clinical suspicion. A history of FAP or of similarly affected family members is frequently elicited. A history of trauma or recent pregnancy is also common.

The role of imaging (computed tomography and magnetic resonance) is to define the degree of extension to local structures and tumor relationship to neurovascular structure[33,34]. This tumor does not metastasize to regional nodes or distant sites so that search for metastatic disease is unnecessary. Biopsy is not usually necessary but does not seem to induce further growth, if performed. At the moment there is no accepted staging system for this disease[35].

MANAGEMENT

The management of desmoid tumors requires special attention. A strong family history of desmoid tumors and a high-risk location of the mutation on the APC gene increase the risk for the development of desmoid tumors[30,36]. Due to a reported 85% recurrence rate of desmoid tumors after surgical excision, surgery should be performed only when absolutely necessary[37,38].

Tamoxifen, toremifene, and sulindac have been used as treatments, but the results are controversial. Reports have suggested that therapy might be associated with an initial benefit but the long-term clinical improvement is minimal[39-41]. Cytotoxic chemotherapy (doxorubicin, dacarbazine, and carboplatin) may be effective in treating aggressive, rapidly growing and unresectable abdominal desmoids[42,43]. Radiation therapy might be effective in selected cases but it is frequently limited by the presence of the small bowel in the radiation field in mesenteric and pelvic desmoids[44,45].

Intra-abdominal desmoid tumors usually involve the mesentery and often involve the mesenteric vessels. They invade the mesentery diffusely, kink loops of bowel and can cause ureters obstruction (Figure 2). This feature requires a complex surgery and often radical resection is impossible to achieve[11]. Therefore the management of intra-abdominal desmoid tumors is complex and is dependant on their clinical behavior.

Given the problems related to the treatment of desmoids, there is a good case for simple observation of many tumors, particularly if asymptomatic. Following diagnosis, a small tumor which is not encroaching on any nearby structures may be followed up by regular clinical

examination (every 6 mo) with or without imaging, usually by CT. Desmoids that are growing slowly or are mildly symptomatic can be treated with sulindac and tamoxifen or with vinblastine and methotrexate, since these are less toxic regimens. Aggressive desmoids are treated with anti-sarcomachemotherapy such as doxorubicin and dacarbazine.

It would thus seem that surgery is a reasonable first-line treatment for abdominal wall tumors, since they are easier to excise than intra-abdominal desmoid tumors, recurrence rates are lower, and morbidity rates associated with the procedure are lower. The excision should be completed with a one cm margin. A mesh can be used to cover the defect if required[46].

For intra-abdominal desmoids surgery should only be used in specific circumstances. These would include tumors which do not appear to involve vital organs and vessels on preoperative imaging, those resistant to drug treatment and in cases where a risky operation is the only possible option in the case of a rapidly growing, life-threatening tumor. High rates of recurrence should be expected and patients must be counseled pre-operatively about the risks of death. Such cases should only be attempted in specialist centers with sufficiently experienced staff.

At the moment one center has also reported a technique where the tumor and small bowel are removed en bloc, perfused and cooled, and the tumor resected on the bench in a bloodless field with subsequent autotransplantation of the small bowel back into the patient[47]. Recently a report has been published where two desmoids (one intra-abdominal) were treated with percutaneous chemical ablation with acetic acid under-radiological guidance[48]. In both cases there was substantial regression of the tumours within a few months.

Unfortunately, despite any treatment, some patients deteriorate, become dependent on TPN, and have life-threatening complications develop. Intestinal transplantation is the only remaining option for these patients[49].

In conclusion the optimal treatment protocol has not yet been established and, in many cases, a multidisciplinary approach including surgery, chemotherapy, and radiation therapy has been employed. The rarity of cases in even major tumor centers has traditionally limited the ability to study this disease. The notion that a specific genotype can predict the development of an aggressive desmoid tumor in a given patient could prove to be valuable in allowing appropriate patient selection for early therapy or even a chemopreventive strategy. Several novel pharmacologic and biologic treatment approaches are actively being developed, although long-term follow-up is needed for their substantiation[50].

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BASIC RESEARCH

Protective effects of medical ozone combined with traditional Chinese medicine against chemically-induced hepatic injury in dogs

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Abstract

AIM: To investigate the protective effect of medical ozone (O_3) combined with Traditional Chinese Medicine (TCM) Yigan Fuzheng Paidu Capsules (YC) against carbon tetrachloride (CCl_4)-induced hepatic injury in dogs.

METHODS: Thirty healthy dogs were divided randomly into five groups (n = 6 in each group), namely control, oleanolic acid tablet (OAT), O_3, YC and O_3 + YC, given either no particular pre-treatment, oral OAT, medical ozone rectal insulfflation every other day, oral YC, or oral YC plus medical ozone rectal insulfflation every other day, respectively, for 30 consecutive days. After pre-treatment, acute hepatic injury was induced in all dogs with a single-dose intraperitoneal injection of CCl_4. General condition and survival time were recorded. The biochemical and hematological indexes of alanine aminotransferase (ALT), aspartate aminotransferase/alanine aminotransferase (AST/ALT), serum total bilirubin (TBIL), prothrombin time (PT), blood ammonia (AMMO), and blood urea nitrogen (BUN) were measured after CCl_4 injection. Hepatic pathological changes were also observed.

RESULTS: Compared to the other four groups, the changes of group O_3 + YC dogs' general conditions (motoricity, mental state, eating, urination and defecation) could be better controlled. In group O_3 + YC the survival rates were higher ($P < 0.05$ *vs* group control). AST/ALT values were kept within a normal level in group O_3 + YC. Hepatic histopathology showed that hepatic injury in group O_3 + YC was less serious than those in the other four groups.

CONCLUSION: Medical ozone combined with TCM YC could exert a protective effect on acute liver injury induced by CCl_4.

Key words: Carbon tetrachloride; Ozone; Traditional Chinese Medicine

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INTRODUCTION

Medical ozone, a mixture gas of ozone and oxygen, has been used for several decades in the therapy of diabetic foot, arthritis, arterial angiemphraxis and ulcerative colitis[1-9]. It has been used in an empirical fashion in recent years for therapy of viral hepatitis. Rectal insufflation with ozone can reduce hepatic and renal ischemia-reperfusion injury[10-15].

Yigan Fuzheng Paidu Capsules (YC), an empirical formula, is a compound preparation of traditional Chinese medicine (TCM) for treating chronic viral hepatitis. It has been demonstrated experimentally that YC can protect the liver and decrease transaminase level, has anti-lipid peroxidation activity, and *ex vivo* antiviral activity.

This study investigated the effect of medical ozone combined with YC affect on carbon tetrachloride (CCl_4)-induced acute liver injury in order to establish a reliable baseline for clinical application.

MATERIALS AND METHODS

Animals

Thirty healthy mongrel dogs aged 1-2 years, weighing 12-15 kg, were used in the experiments. These dogs received regular feeding, were inspected medically, treated with helminthicide, and acclimatized for at least 1 month prior to the experiments in the Experimental Animal Center of Nanfang Hospital, Southern Medical University. The experiments were carried out in accordance with the animal experiment regulations of the university.

Drugs and reagents

YC was manufactured in the Department of Traditional Chinese Medicine, Southern Medical University (Guang-lian-zhi-zi 2004, No. FPGZ150) with Batch No. 050715. Oleanolic acid tablets (OAT) were produced by Nanguo Biological Pharmacy Co., Guangdong Province, China (Guo-yao-zhun-zi No. H44023537) with Batch No. 040701. CCl_4 was produced by the Chemical Plant of Guangzhou with Batch No. 050317, and mixed with the same amount of peanut oil before use.

Equipment

The medical ozone generator (OZONOSAN alpha Plus 1107, Germany) was registered and licensed for medical therapy by SFDA (registration No. 1570177).

Groups and administration

Thirty healthy dogs were divided randomly into five groups (n = 6 in each group): group control, which received no preconditioning treatment; group OAT, treated with oral OAT at 10 mg/d; group O_3, treated with 8.1 mL/kg medical ozone at 20 μg/mL by transrectal insulfflation every other day; group YC, treated with oral YC at 0.2 g/d; and group O_3 + YC, treated with oral YC at 0.2 g/d plus 8.1 mL/kg medical ozone at 20 μg/mL by transrectal insufflation every other day, for a total of 30 consecutive days. After preconditioning treatment, acute hepatic injury was induced in all dogs with a single intraperitoneal injection of CCl_4 mixture at a dose of 0.9 m/kg body weight.

Measurements

The general condition of the dogs was observed before and after treatment, in terms of motor activity, mental state, eating behavior, urination and defecation. The survival time of each dog was measured accurately in hours.

For biochemical and hematological measurements, intravenous blood was sampled pre- and post-treatment at 24 h, 2, 3, 4, 7 and 14 d. All the blood samples were analyzed immediately by the clinical laboratory of Nanfang Hospital for alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), prothrombin time (PT), serum ammonia (AMMO), and blood urea nitrogen (BUN).

For histopathology, multiple liver tissues were obtained through ultrasound-guided percutaneous needle biopsy from each dog before and after the experiment. The formalin-fixed, paraffin-embedded liver sections (5 μm) were stained with hematoxylin and eosin (HE) and Gomori silver. The degree of necrosis and inflammatory cell infiltrate was evaluated on a four-point scale (Table 1), using 20 random fields at 100 × and 400 × magnification per slide, by a blinded pathologist (MIF)[16].

Table 1 Histological assessment of drug induced acute hepatitis in dogs

Score	Centrilobular necrosis	Inflammation in centrilobular areas
0	None	None
1	Isolated necrotic hepatocytes or single row of hepatocyte drop-out in perivenular areas	Mild: inflammatory infiltrate affecting < 50% centrilobular areas
2	> 1 and up to 3 rows of perivenular necrotic hepatocytes	Moderate: inflammatory infiltrate affecting > 50% and < 75% centrilobular areas
3	> 3 rows of perivenular necrotic hepatocytes with confluent and/or bridging necrosis	Severe: dense inflammatory infiltrate affecting > 75% centrilobular areas

Statistical analysis

Overall survival was evaluated by actuarial analysis using Kaplan-Meier estimates. Independent samples test for comparison of biochemical and hematological measurements, and one-way ANOVA and LSD test for histological assessment were performed by SPSS 13.0. P-values < 0.05 were considered statistically significant.

RESULTS

General condition of the dogs

Pre-treatment, all 30 dogs were in good condition. Eating behavior, feces and urine were normal. Post CCl_4 treatment, all dogs began to vomit and lost balance immediately. Except for the O_3 + YC group, all the dogs in the other four groups appeared to prefer to stay still alone or were pacing up and down restlessly, while some of them showed poor mental health and appetites, and yellow urine. In group O_3 + YC, there were no differences before and after treatment, except for two dogs with yellow urine.

Survival analysis

Log Rank, Breslow and Tarone-Ware tests were used for comparison of survival rate in the five groups of dogs. All three tests showed that the survival rate of group O_3 + YC was significantly higher than that of the control group (P < 0.05), while there were no significant differences between any other two groups (P > 0.05) (Figure 1).

Biochemical and hematological measurements

Pre-treatment, there were no significant differences (P > 0.05) between the control and the other four groups for any of the measurements (AST/ALT was not being analyzed at the time). After CCl_4 injection, all the measurement index in the five groups except for AST/ALT showed a tendency to increase during the first three days and then gradually fall. There were significant differences (P < 0.05) between groups OAT and O_3, OAT and YC, OAT and O_3 + YC for PT, and between groups

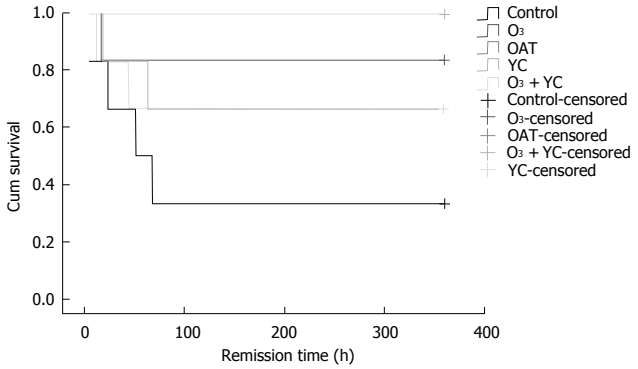


Figure 1 Survival functions of the five groups. The record of each dog's survival time starts at the injection time, and ends in post-360 h (15 d). Plots express the survival rates in different time.

OAT and O_3 + YC, O_3 and O_3 + YC for BUN. In order to compare the measurements post CCl_4 injection, all the index values for each dog were adjusted to the same level. Post CCl_4 injection, the measurement values of ALT, TBIL, PT and AMMO were significantly higher ($P < 0.05$) in the control group than in the other four groups, but there were no significant differences for BUN in all groups ($P > 0.05$). ALT was significantly lower ($P < 0.05$) in group OAT than in group YC, while ALT, TBIL, PT and BUN were significantly higher ($P < 0.05$) in group OAT than in group O_3 + YC. ALT, TBIL, PT and BUN were significantly higher ($P < 0.05$) in group O_3 and YC than in group O_3 + YC. AST/ALT in the control and YC groups showed a little below 1.0 at 24 h after CCl_4 was administrated, then increased to > 1.5 in the control group, and fell to < 0.5 on d 3-4. On d 5-14, AST/ALT in the control group continued to fall (0.1 on d 14). However, in group YC AST/ALT started to increase after 7 d (1.0 on d 14). AST/ALT in the OAT and O_3 groups was approximately 1.0 on first day, fell to approximately 0.4-0.5, and began to increase from d 4 (about 1.0 on d 14) (Table 2).

Liver histopathology

Liver sections from the five groups of dogs, after death or survival for 15 d, were examined and graded for the degree of necrosis and accompanying inflammation, as shown in Table 1. Control group livers demonstrated higher scores for necrosis ($P < 0.05$) and inflammation ($P < 0.05$) compared with the OAT, O_3, YC and O_3 + YC groups (Figure 2).

DISCUSSION

Drug-induced hepatitis is becoming a big problem in clinical practice, and proper management of this problem has not been found so far. For searching new strategy to overcome drug-induced hepatitis, medical ozone and traditional Chinese medicine were first tried in animal model. The effects of medical ozone combined with traditional Chinese medicine on CCl_4-induced acute hepatic injury in dogs were evaluated in this study. The novel method decreased the incidence of jaundice and hepatic encephalopathy and prolonged survival time. Medical ozone combined with YC showed a better effect than OAT, O_3 and YC alone in decreasing transaminases, relieving jaundice and promoting recovery of blood clotting activity.

A number of hepatotoxins have been used to induce fulminant hepatic failure (FHF) in animal models: d-galactosamine, *N*-acetyl-*P*-aminophenol and CCl_4[17,18]. CCl_4 causes serious hepatotoxicity, and has been used extensively and successfully to induce liver damage, including fibrosis[19,20]. CCl_4 is metabolized by cytochrome P450 2E1 in the liver to produce a toxic metabolite. It has been demonstrated to induce liver necrosis, as well as apoptosis[21]. Peroxidation of membrane lipids secondary to the formation of trichloromethyl radicals is believed to be the basis for the toxic effects of CCl_4.

Medical ozone is characterized by its safety and multiple effects in low dose. The main mechanisms of action of ozone pre-conditioning on hepatic injury are as follows. It enhances erythrocyte metabolism[22-26], which can promote the oxygen-carrying capacity of hemoglobin and promote liver oxygenation. Ozone can induce intracellular anti-oxidant enzymes of the liver and scavenging free radicals[14,27-29]. When hepatocytes are equipped with strength against peroxidative radicals by the preconditioning of medical ozone, they can become prone to survival under the attack of toxic CCl_4 radicals. The range of ozone concentrations within which it can exert a therapeutic effect without toxicity is wide from 10 mg/mL to 80 mg/mL[25,30]. YCs have been prescribed clinically for several years. *Astragalus mongholicus*, *Hedyotic diffusa* and *Radix polygoni* prepared as a prescription can promote production of lymphoblasts, regulate the ratio of T-lymphocyte subsets, strengthen immune function, increase activity of lymph active cells and natural killer cells, and induce interferon. Giant knotweed rhizome, *Hedyotic diffusa*, *Rhizome* and *Phyllanthus amatusniruri* have also shown antiviral effects. Honeycomb of paper wasps, red peony root and *Salvia miltiorrhiza* can promote blood flow, mitigate liver injury, and improve hepatocyte oxygenation and promote their recovery. Therefore, the combined effects of O_3 and YC were to up-regulate immune function and promote liver oxygenation. This study may spur the use of a new strategy for the clinical management of some drug-related hepatitis. Clinical trials are needed to confirm the effects of this novel regime.

COMMENTS

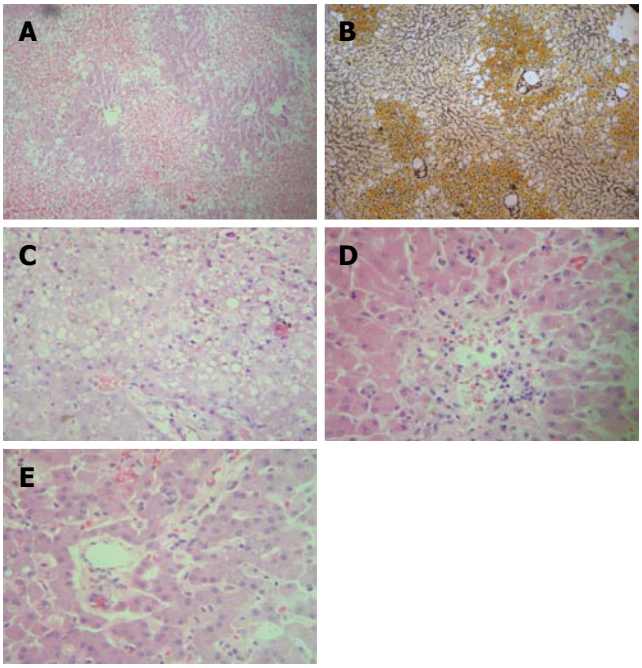
Background

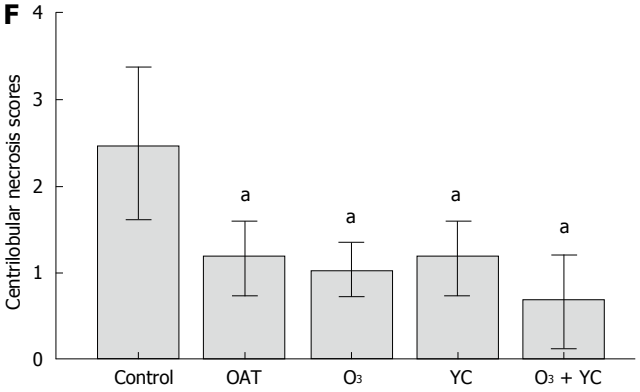
Medical ozone has been wildly used, especially in Europe, in surgery and medicine for 50 years. The technique of ozonetherapy, such as major autohemotherapy, rectal insufflation and topical ozonetherapy, were gradually developed in recent decades and served for more than 10 million patients who suffered from skin ulcerative lesion or trauma, diabetic foot, arthritis, arterial obstruction, ulcerative colitis and spinal disk herniation, *etc*. The property of ozone and its therapeutic effects were partially illuminated. Ozone has opposite faces toward human body. It is harmful gas in high concentration or administration by respiratory tract but therapeutic in low concentration. The milestone fact sheets were that: (1) the ozone reacts with red blood cells (RBC) and reactivates RBC metabolism by increasing 2, 3-DPG and ATP concentration; (2) ozone reacts with immunocompetent cells and induces cytokins release, including IL-2, 4, 10, IFN-β, γ, TGF-β1; (3) ozone activates tissue cells' antioxidative enzymes and enhances capacity of radical scavenger. Each discovery mentioned above will mould scientific base for the novel application scope of ozone. For example, the finding of effect of ozone on immune system is the scientific foundation of using

Table 2 Changes of biochemical and hematological indexes of the five groups (Data represents median ± quartile)

Group	Pre-injection	Post-injection CCl_4					
		Post-24 h	Post-2 d	Post-3 d	Post-4 d	Post-7 d	Post-14 d
ALT/(U/L)							
Control	26.00	1156.00	43.50	1018.00	1790.50	1349.50	297.00
	(16.50-33.75)	(245.00-1641.75)	(13.25-4399.75)	(66.00-1970.00)	(1763.00-1818.00)	(1307.00-1392.00)	(244.00-350.00)
OAT	24.50	113.00	95.00	173.00	135.00	465.00[a]	77.50[a]
	(17.75-26.25)	(26.50-439.00)	(43.50-427.50)	(61.25-918.50)	(52.50-1390.50)	(109.75-875.00)	(16.00-155.50)
O_3	25.50	564.00	474.00	209.00	259.00	139.00[a]	32.00[a]
	(12.00-37.25)	(75.00-1054.50)	(132.00-1025.50)	(126.50-1136.00)	(119.00-1377.00)	(57.50-538.00)	(25.00-70.00)
YC	18.00	1310.00[c]	662.00[c]	1610.50	1064.50	465.00[a]	77.50[a]
	(11.75-52.50)	(919.75-1740.25)	(355.00-2034.50)	(158.75-1682.50)	(235.25-2394.00)	(109.75-875.00)	(16.00-155.50)
O_3 + YC	26.50	28.50[f,h]	34.00[c,f,h]	33.50[a,d,f,h]	30.50[a,d,f,h]	23.50[a,d,f,g]	12.00[a,d,f,g]
	(20.00-32.75)	(23.50-41.75)	(27.25-40.25)	(27.75-37.75)	(26.50-38.75)	(20.00-36.00)	(3.75-12.50)
AST/ALT							
Control		0.78	1.36	5.71	0.06	0.05	0.10
		(0.45-1.38)	(0.28-6.94)	(0.13-11.29)	(0.03-0.09)	(0.03-0.07)	(0.09-0.10)
OAT		0.80	0.49	0.41	0.43	0.57	0.99[a]
		(0.50-2.97)	(0.17-0.85)	(0.18-0.70)	(0.10-0.77)	(0.15-0.79)	(0.29-1.70)
O_3		1.02	0.81	0.45	0.62	0.71[a]	1.52[a]
		(0.77-1.35)	(0.38-1.36)	(0.15-0.65)	(0.22-0.94)	(0.33-1.05)	(0.75-2.24)
YC		0.73	1.09	0.43	0.21	0.24	1.00[a]
		(0.48-1.11)	(0.62-7.86)	(0.26-0.78)	(0.07-0.45)	(0.07-0.87)	(0.20-2.02)
O_3 + YC		2.39[b,f,h]	2.08[d,f]	1.09[d,f,h]	1.39[a,d,f,h]	1.43[a,d,f,g]	2.08[a,c]
		(1.56-2.59)	(1.43-2.17)	(1.00-1.70)	(1.26-1.79)	(1.04-1.92)	(1.77-3.79)
TBIL/(μmol/L)							
Control	2.10	7.15	9.80	6.65	6.10	4.95	1.80
	(0.18-4.73)	(2.73-31.83)	(6.15-33.85)	(6.60-6.70)	(5.30-6.90)	(4.70-5.20)	(0.60-3.00)
OAT	3.80	5.20	5.30	6.55	3.50[a]	5.45	3.30
	(0.85-4.70)	(2.50-6.40)	(1.90-14.30)	(3.35-21.15)	(2.05-4.20)	(1.88-10.90)	(1.20-6.08)
O_3	1.55	4.30	4.30	3.30	1.90[a]	2.50[a]	3.00
	(0.20-4.85)	(2.45-5.65)	(3.05-7.05)	(1.95-6.45)	(1.10-4.35)	(1.85-4.20)	(0.80-4.95)
YC	2.65	3.40	7.00	3.40[a]	4.45	1.95[a]	2.70
	(0.35-5.18)	(1.60-26.15)	(2.95-33.65)	(1.15-3.55)	(1.55-5.78)	(0.80-3.93)	(0.98-10.73)
O_3 + YC	4.20	1.95[a,c,e]	1.00[b,c,e,h]	1.45[a,c]	1.30[a,d,g]	0.85[a,c,e]	3.50
	(3.83-4.33)	(0.10-2.40)	(0.30-2.38)	(0.80-5.10)	(0.70-1.55)	(0.50-2.13)	(0.50-4.28)
PT/s							
Control	6.80	13.20	18.90	12.25	8.70	7.80[a]	8.55[a]
	(6.50-13.05)	(8.93-38.93)	(12.40-60.70)	(11.70-12.80)	(8.40-9.00)	(7.60-8.00)	(8.20-8.90)
OAT	6.50	7.20[a]	7.85[a]	7.95	6.50	6.50	6.50
	(6.50-7.28)	(6.50-8.35)	(7.60-9.83)	(6.50-9.40)	(6.50-8.70)	(6.50-6.50)	(6.95-7.93)
O_3	7.30	8.60	9.50	7.80[a]	7.30	7.50	7.10[a]
	(6.95-8.03)	(7.30-11.35)	(8.65-16.35)	(7.50-10.00)	(6.90-8.45)	(7.00-7.70)	(6.80-7.30)
YC	7.70	12.55	9.90	7.40[a]	7.20	7.55	6.90
	(7.18-8.20)	(8.23-24.75)	(8.70-36.40)	(7.20-10.60)	(6.68-8.70)	(6.68-8.20)	(6.50-8.13)
O_3 + YC	7.65	7.25[a,c,g]	7.40[b,c,f,h]	7.30[a]	7.30[a]	6.85[a]	7.45[a]
	(7.35-8.15)	(6.50-8.13)	(7.20-8.65)	(7.18-8.28)	(7.28-7.40)	(6.50-7.50)	(7.03-7.70)
AMMO/(μmol/L)							
Control	69.55	96.50	114.80	132.80	143.40	53.00	91.40
	(35.47-106.35)	(53.20-161.33)	(80.90-133.90)	(82.30-183.30)	(67.40-219.40)	(32.50-73.50)	(40.60-142.20)
OAT	41.20	54.70	44.35[a]	49.85[a]	55.10[a]	49.65	33.30
	(35.45-54.50)	(43.70-79.70)	(25.93-83.10)	(38.48-62.28)	(31.35-64.53)	(38.55-75.45)	(13.40-52.50)
O_3	49.75	54.70	51.50[a]	65.70[a]	48.30	65.40	47.10
	(39.33-62.03)	(52.40-76.65)	(43.00-73.50)	(52.20-72.40)	(44.85-74.05)	(44.50-94.80)	(32.63-63.75)
YC	47.50	53.80	54.70[a]	55.70	77.35	45.40	45.50
	(34.25-73.23)	(49.45-173.23)	(43.70-79.70)	(45.83-86.95)	(57.63-168.25)	(39.18-97.68)	(23.35-110.85)
O_3 + YC	63.50	77.35	55.30[a]	59.65[a]	58.40	67.10	41.90
	(15.60-74.78)	(56.73-158.20)	(44.90-69.58)	(41.70-63.45)	(45.95-69.25)	(51.20-93.95)	(37.08-61.48)
BUN/(mmol/L)							
Control	3.30	5.50	3.80	2.40	3.60	3.65	5.15
	(2.45-4.45)	(3.85-7.15)	(2.70-6.20)	(1.60-3.20)	(3.10-4.10)	(3.20-4.10)	(1.20-9.10)
OAT	3.85	6.20	4.50	3.70	4.65	3.20	2.75
	(3.53-4.38)	(5.20-8.30)	(4.10-6.25)	(3.25-4.60)	(3.10-7.33)	(2.38-6.65)	(2.10-4.00)
O_3	3.75	5.70	5.80	4.00	3.90	2.60	2.60
	(3.18-5.55)	(4.65-8.70)	(4.75-7.05)	(3.20-5.80)	(2.40-6.45)	(1.90-6.35)	(2.20-3.30)
YC	3.50	7.55	4.30	4.10	3.00	2.00	3.20
	(2.88-5.73)	(3.90-13.58)	(3.55-13.00)	(1.98-7.13)	(2.55-6.68)	(1.68-6.75)	(2.83-3.20)
O_3 + YC	2.85	3.10[d,e,g]	3.50[e]	3.75	3.45	2.20	1.80[g]
	(2.28-3.65)	(1.50-3.98)	(2.70-4.15)	(2.30-4.68)	(3.30-3.95)	(1.80-3.28)	(1.70-2.40)

[a]$P < 0.05$ *vs* Control; [b]$P < 0.01$ *vs* Control; [c]$P < 0.05$ *vs* OAT; [d]$P < 0.01$ *vs* OAT; [e]$P < 0.05$ *vs* O_3; [f]$P < 0.01$ *vs* O_3; [g]$P < 0.05$ *vs* YC; [h]$P < 0.01$ *vs* YC. ALT of each dog was within the normal level (ALT < 60.0 U/L)[5] pre-injection, and thus the values of AST/ALT made no sense at that time.





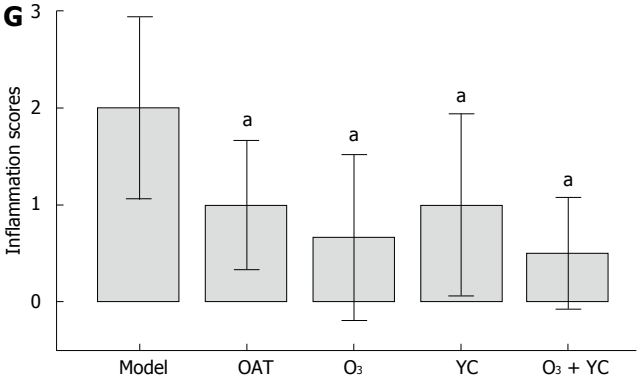


Figure 2 Histological assessment of livers following death or living for 15 d (original magnification, × 100, **A**, **B**; × 400, **C**, **D**, **E**). Hepatocellular bridged necrosis, mononuclear and lymphoid inflammatory infiltration, necrosis and hemorrhage (**A**) accompanied reticular fibers structures collapsing in control group (**B**). Increased mononuclear and lymphoid inflammatory infiltration, and necrosis in groups OAT, O_3, and YC (**C**, **D**) compared with group O_3 + YC (**E**). Inflammation and necrosis were graded on a 4-point scale (Table 1) by a blinded pathologist in 20 random high power fields per animal (n = 6 in each group), represented graphically in panels (**F**) errorbars: 95.00% CI and (**G**) errorbars: 95.00% CI. Data represent mean ± SE; $^aP < 0.05$ *vs* Control.

medical ozone to treat infectious diseases, including viral hepatitis B, and to treat rheumatic diseases. Although effort has been done to elucidate the mechanism of ozonetherapy, but its practices in whole still remained in an empirical fashion and have not been accepted by sutra legitimacy medicine.

Research frontiers

Drug-induced liver injury is an increased important problem in modern medical practice. Apart from avoiding using hepatoxic drugs, none of effective methods to prevent drug induced hepatitis are confirmed so far. There are several articles that introduced using medical ozone to prevent liver or renal ischaemia and reperfusion (I/R) injury in animal models. Free radical and reactive oxygen species during I/R process are the main cause of organ I/R injury. The mechanism of some drug-induced hepatitis is the same way as I/R injury. So, the ozone's property of inducing antioxide enzymes and enhancing capacity of radical scavenger should confer preventive effect for protecting liver from hepatoxic drug-induced injury.

Innovation and breakthroughs

This study gives us a new knowledge of medical ozone and impels us to have following deduction, i.e. medical ozone and herb medicine rectal administration may be a new strategy to prevent drug-induced hepatitis. From design point of view, big animal as dog as animal model in this study imitates human being more likely than small canine as mouse. Rectal administration of ozone and herb medicine was proved effective in this experiment, and provided convenient way for future clinical application.

Application

The results from this animal study confirm the protective effect of ozone and prescription of traditional Chinese medicine on hepatoxic drug-induced liver injury. It encourages us to apply medical ozone in preventing some drug-induced hepatitis in the future clinical trials.

Termiology

Medical ozone is a mixture gas of ozone and oxygen. It is made from pure oxygen by an ozone generator. This machine used in medical therapy must have capacity to adjust ozone concentration in a precision and stabilization pattern.

Peer review

This study further confirmed the protective effect of medical ozone and herb medicine on drug-induced hepatitis and was a steady step toward its clinical application. Medical ozone and traditional Chinese medicine are both ancient antiques, its worthiness was not staying in ancient history and its merits are also useful in modern medicine, just simply needs to be added modern decoration, i.e. the evidence based medicine (EBM).

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CLINICAL RESEARCH

Selective decrease in colonic CD56$^+$ T and CD161$^+$ T cells in the inflamed mucosa of patients with ulcerative colitis

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Abstract

AIM: To investigate the role of local colonic mucosal NK receptor-positive T (NKR$^+$ T) cells in the regulation of intestinal inflammation, we analyzed the population and function of these cells in ulcerative colitis (UC).

METHODS: Colonic mucosal tissues were obtained from colonoscopic biopsies of the descending colon from 96 patients with UC (51 endoscopically uninflamed, 45 inflamed) and 18 normal controls. Endoscopic appearance and histologic score at the biopsied site were determined by Matts' classification. A single cell suspension was prepared from each biopsy by collagenase digestion. Two NKR$^+$ T cell subsets, CD56$^+$ (CD56$^+$CD3$^+$) T cells and CD161$^+$ (CD161$^+$CD3$^+$) T cells, were detected by flow cytometric analysis. Intracellular cytokine analysis for anti-inflammatory cytokine interleukin-10 (IL-10) was performed by *in vitro* stimulation with phorbol-myristate-acetate (PMA) and ionomycin.

RESULTS: CD56$^+$ T cells and CD161$^+$ T cells are present in the normal human colon and account for 6.7% and 21.3% of all mononuclear cells, respectively. The populations of both CD56$^+$ T cells and CD161$^+$ T cells were decreased significantly in the inflamed mucosa of UC. In contrast, the frequency of conventional T cells (CD56$^-$CD3$^+$ cells and CD161$^-$CD3$^+$ cells) was similar among the patient and control groups. The populations of NKR$^+$ T cells were correlated inversely with the severity of inflammation, which was classified according to the endoscopic and histologic Matts' criteria. Interestingly, approximately 4% of mucosal NKR$^+$ T cells expressing IL-10 were detected by *in vitro* stimulation with PMA and ionomycin.

CONCLUSION: Selective reduction in the population of colonic mucosal NKR$^+$T cells may contribute to the development of intestinal inflammation in UC.

Key words: Natural killer T cells; Ulcerative colitis; Interleukin-10

Shimamoto M, Ueno Y, Tanaka S, Onitake T, Hanaoka R, Yoshioka K, Hatakeyama T, Chayama K. Selective decrease in colonic CD56$^+$ T and CD161$^+$ T cells in the inflamed mucosa of patients with ulcerative colitis. *World J Gastroenterol* 2007; 13(45): 5995-6002

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INTRODUCTION

Human ulcerative colitis (UC) is a chronic relapsing disorder of ill-defined immunoregulatory dysfunction that leads to inflammation or ulceration of the intestinal tract[1]. Increasing evidence suggests that dysregulation of mucosal T cells may play a key role in the pathogenesis of UC, which results in secretion of proinflammatory mediators, accumulation of inflammatory cells, and tissue damage[2-4]. The pathogenesis of UC remains obscure, but it is now believed that dysfunction of immunoregulatory T cells is considered one of the mechanisms by which intestinal inflammation persists in UC[5].

Human T cells that express natural killer (NK) markers, including CD56 and CD161, were originally discovered in the liver. They differentiate extrathymically and are considered immunoregulatory T cells[6,7]. Hepatic CD56$^+$ T cells and CD161$^+$ T cells can rapidly produce Th1-type and Th2-type cytokines, suggesting that these cells have roles in the regulation of both innate and adoptive immune responses[8-10]. They also have cytotoxic activity against some cancer cell lines[11]. Several investigators reported that hepatic CD56$^+$ T cells and CD161$^+$ T cells are depleted significantly in liver with chronic hepatitis C virus infection[12,13], suggesting that these cells may be involved in the development of hepatic inflammation.

These NK receptor-positive (NKR$^+$) T cells include both conventional major-histocompatibility complex (MHC)-restricted T cells that recognize peptide antigens[14]

and so-called NKT cells, which recognize glycolipid antigens presented by a non-classical antigen-presenting molecule, CD1[15-17]. The latter cell type frequently expresses invariant T cell receptor α-chains (Vα24JαQ in humans and Vα14Jα18 in mice)[9,18].

Recently it was reported that these NKR^+ T cells reside in the human intestine[19], and these populations are reduced in the colonic mucosa of patients with colorectal cancer[20]. However, to date, there have been no reports of the roles of these cells in modulation of intestinal inflammation in UC. Here, we investigated the relation between these cell populations and the severity of colonic inflammation in patients with UC by assessing colonic mononuclear cells in biopsy specimens. We found that a selective decrease in the population of colonic NKR^+ T cells may be involved in the progression of local colonic mucosal inflammation in UC.

MATERIALS AND METHODS

Study groups

Demographic features of the patients and history of drug therapy up to the time of colonoscopy are summarized in Table 1. The diagnosis of UC was based on established endoscopic and histopathologic criteria[21]. Colonic mucosal tissues were obtained from colonoscopic biopsies of 96 patients with UC (45 inflamed, 51 uninflamed). The control group consisted of 18 patients. Ten of these control patients presented with a chief complaint of abdominal pain in which a histological diagnosis was not established. The remaining eight control patients were evaluated endoscopically for hematochezia and found to have solitary adenomatous polyps. All control colonic mucosal samples were taken from a histologically normal portion of the biopsied specimen at least 10 cm away from the involved sites with the polyps. The inflammatory activity of these areas was evaluated endoscopically and histologically according to Matts' criteria with some modifications[22,23]. Inflamed and uninflamed areas were defined as grades 2-4 (and 5 for histologic Matts' criteria) and grade 1, respectively. All biopsy specimens were obtained from the descending colon. Standard 2.8 mm biopsy forceps (Olympus Optical, Tokyo, Japan) were used through all colonoscopes. All samples were obtained with informed consent in accordance with the Helsinki Declaration.

Table 1 Demographic features of participating patients with UC

		Ulcerative colitis	
	Normal	Inflamed	Uninflamed
No. of patients (cases)	18	45	51
Sex (male/female)	11/7	31/14	35/16
Age (yr), mean ± SD	47.5 ± 20.1	35.3 ± 13.6	39.3 ± 14.1
Disease duration (yr)		6.5 ± 6.6	6.2 ± 5.5
Types of colitis			
Total	-	31	10
Left-sided	-	9	14
Proctitis	-	0	24
Others	-	5	3
Treatment			
Prednisolone (PSL) (no/yes)	-	15/30	29/22
Azathioprine (AZA) (no/yes)	-	31/14	42/9

Isolation of lamina propria mononuclear cells

All specimens were weighed prior to isolation of colonic mononuclear cells (MNCs). Five biopsy samples for purification of MNCs and two biopsy samples for evaluation of histology were taken from the same region of each colon, and MNCs were purified as described previously[24]. Briefly, specimens were digested with 150 U/mL of collagenase in RPMI medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS, Sigma), 100 mg/L of gentamicin (Gibco, Gaithersburg, MD), 500 μg/L of penicillin, and 500 μg/L of streptomycin (Gibco) at 37℃ for 90 min. The cells were pelleted and washed with cold phosphate-buffered saline (PBS). Cells were resuspended in 44% isotonic Percoll (Sigma) underlaid with 66% isotonic Percoll and centrifuged for 20 min at 2200 r/min at room temperature. Cells at the interface were collected and washed twice with cold PBS. Cell viability was determined by 0.1% trypan blue dye exclusion, and it was consistently > 90% in all of the patient groups.

Intestinal histology

Intestinal biopsy specimens were fixed immediately in 10% formalin in sodium phosphate buffer and sent to the Department of Pathology at Hiroshima University for processing. Biopsies were embedded in paraffin, and histological sections were stained with hematoxylin and eosin for evaluation. Inflammation was graded according to Matts' classification[22]. The pathologist was blinded to cell surface analysis data.

Flow cytometric analysis

Cells were incubated with a saturating amount of FITC-conjugated anti-human CD3 (UCHT1) mAb and phycoerythrin (PE)-conjugated anti-CD56 (B159) mAb or anti-CD161 (DX12) mAb. All staining reagents were obtained from Becton Dickinson (BD, San Jose, CA, USA). After cells were washed twice with PBS, the stained cells were analyzed on a FACScan (BD), and data were processed with Cell Quest software (BD). The relative proportions of the lymphocyte subpopulations were determined as percentages of the total numbers of cells in a lymphogate defined by forward and side scatter properties. Non-viable cells were excluded by detection of propidium iodide uptake.

Stimulation of cells and staining for intracellular cytokines

Freshly isolated MNCs isolated from a patient with active UC were suspended in complete RPMI medium at a density of 1×10^6 cells/mL and stimulated for 6 h in 96-well plates (Microtest™ 96, BD) at 37℃ in 5% CO_2. Cells were stimulated with 50 ng/mL phorbol-myristate-acetate (PMA) plus 500 ng/mL ionomycin. As controls, unstimulated cells were treated similarly. Interleukin-10 (IL-10) production by NKT cells was examined by a combination of cell-surface and intracytoplasmic mAb staining for IL-10 (JES3-19F1, BD) with Cytofix/Cytoperm Plus™ (BD) and analyzed by flow cytometry.

Statistical analysis

Data were analyzed with StatView software (Japanese version, Hulinks, Tokyo, Japan) on a Macintosh Computer

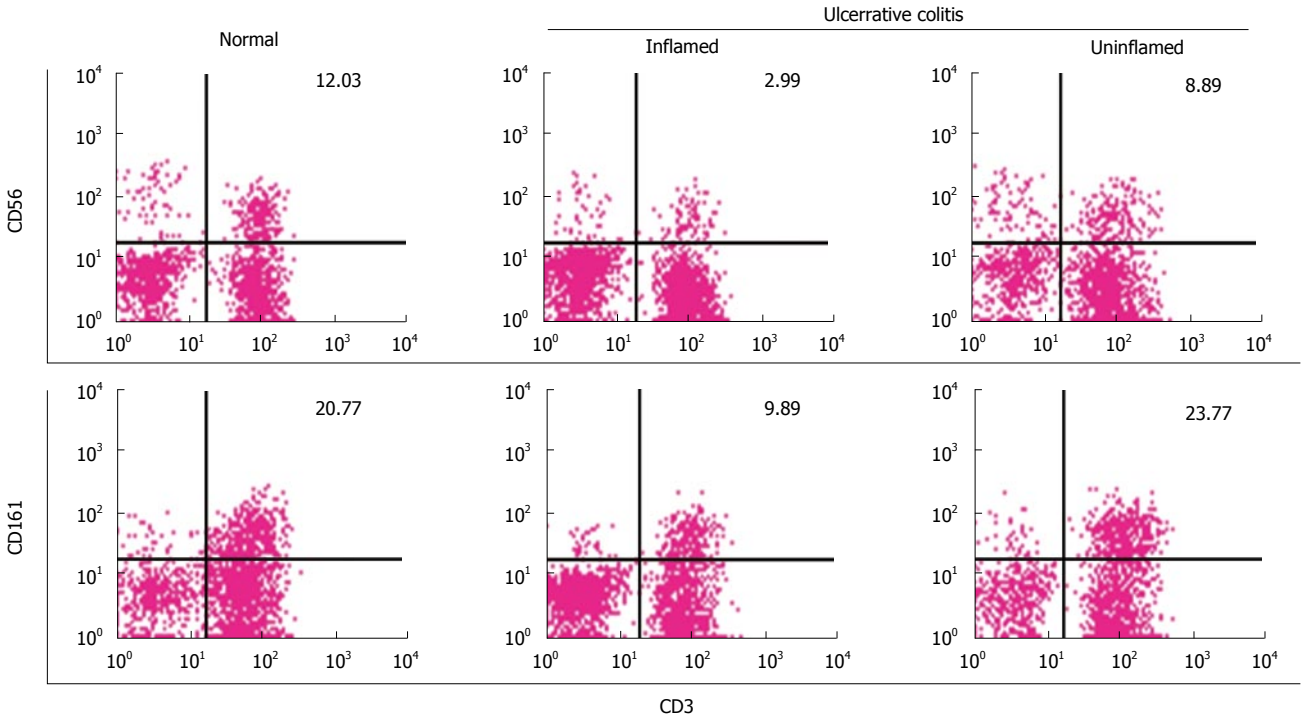

Figure 1 Representative FACS profiles of human colonic mucosal NKT cells. Flow cytometric analysis of CD3 and CD56/CD161 expression on MNCs isolated from colonic samples showing normal mucosa (left), inflamed (Matts' grade 3b) UC mucosa (middle), and uninflamed (Matts' grade 1) UC mucosa (right). The numbers in the top right quadrants denote the percentages of $CD56^+$ T and $CD161^+$ T cells.

(Apple Computer, Cupertino, CA). Data are expressed as mean ± SD. Differences between groups were examined for statistical significance with Student *t* test after analysis of variances (ANOVA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Decreases in $CD56^+$ T cells and $CD161^+$ T cells in the inflamed colonic mucosa of patients with UC

Representative FACS patterns of NKR^+T cells are shown in Figure 1. Flow cytometric analysis of freshly isolated colonic LPLs revealed that the proportion of NKR^+T cells expressing CD56 was significantly lower in patients with active UC (3.3% ± 1.7%, $P < 0.0001$; Figure 2A) in comparison with controls (6.7% ± 3.2%) and patients with inactive UC (7.5% ± 3.9%). Similarly, the proportion of NKR^+T cells expressing CD161 was significantly lower in patients with active UC (13.4% ± 6.4%, $P < 0.0005$; Figure 2B) in comparison with controls (21.3% ± 7.7%) and patients with inactive UC (21.4% ± 1.5%). The proportion of $CD3^-CD56^+$ NK cells was also reduced significantly in colonic LPL from patients with active UC in comparison with controls and patients with inactive UC (data not shown). No significant differences in the proportions of $CD56^-CD3^+$ and $CD161^-CD3^+$ conventional T cells were observed among these groups (Figure 2C and D), suggesting that mucosal NKR^+ T cells were selectively decreased in the inflamed UC mucosa. Furthermore, when we considered the inflamed mucosa by endoscopic and histologic classifications of Matts' grade, the populations of these NKR^+ T cells decreased as the degree of inflammation increased (Figures 3 and 4A). In contrast, the proportions of $CD56^-CD3^+$ and $CD161^-CD3^+$ conventional T cells were not influenced by the degree of histologic intestinal inflammation (Figure 4B). These results suggest that a relative decrease in the population of colonic NKR^+ T cells may exacerbate intestinal inflammation.

Prednisolone and azathioprine therapies did not affect the percentage of colonic NKR^+ T cells

The percentages of colonic NKR^+ T cells were compared between UC patients treated with or without prednisolone (PSL). We separated the groups into patients with inflamed and uninflamed mucosa because inflammation affects the proportion of NKR^+ T cells. PSL treatment did not influence the proportion of colonic NKR^+ T cells (Figure 5A and B). PSL dose did not influence the population of NKR^+ T cells (data not shown). Treatment with azathioprine (AZA) also did not affect the proportion of NKR^+ T cells (Figure 5C and D).

In vitro stimulation with PMA and ionomycin results in IL-10 production by colonic NKR^+ T cells

To further explore the functional role of NKR^+ T cells, analysis of expression of the anti-inflammatory cytokine, IL-10 was performed. As shown in Figure 6, a subset of colonic NKR^+ T cells from normal colonic mucosa produced intracellular IL-10 when stimulated *in vitro* with PMA^+ ionomycin.

DISCUSSION

In the present study, we observed that the populations of $CD56^+$ T cells and $CD161^+$ T cells were decreased significantly in inflamed lesions on the colonic mucosa

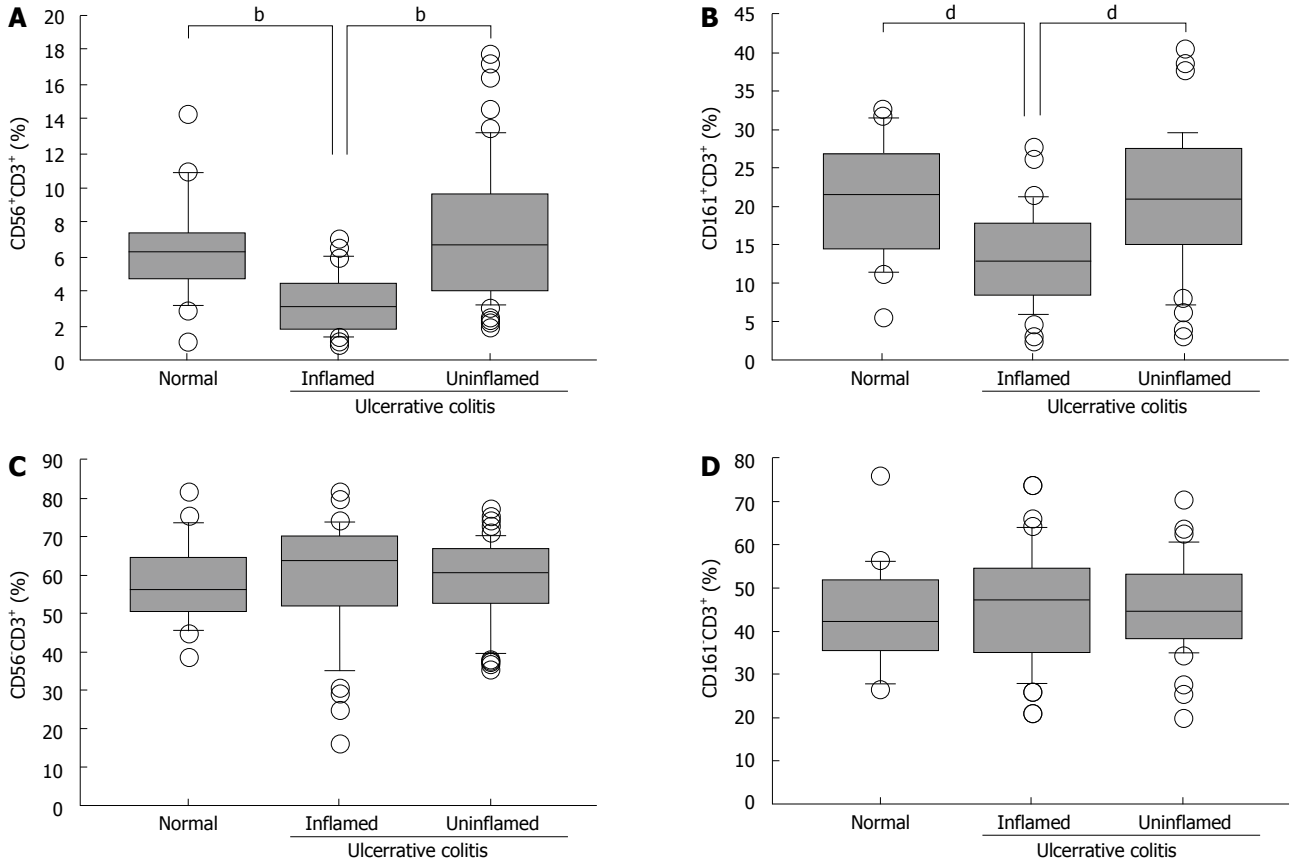

Figure 2 Selective decreases in the proportions of $CD56^+CD3^+$ (**A**) and $CD161^+CD3^+$ (**B**) cells in inflamed colonic mucosa of patients with UC. Box plot graphical representation of the percentage of colonic NKR^+ T cells from normal mucosa of non-UC patients (n = 18), uninflamed mucosa (endoscopic Matts' grade 1, n = 51), and inflamed mucosa (Matts' grades 2-4, n = 45). $^bP < 0.0001$, $^dP < 0.0005$; inflamed mucosa *vs* normal or uninflamed mucosa. (**C, D**) No relation was observed between the percentage of conventional T cells ($CD56^-CD3^+$ or $CD161^-CD3^+$ cells) and the degree of endoscopic inflammation.

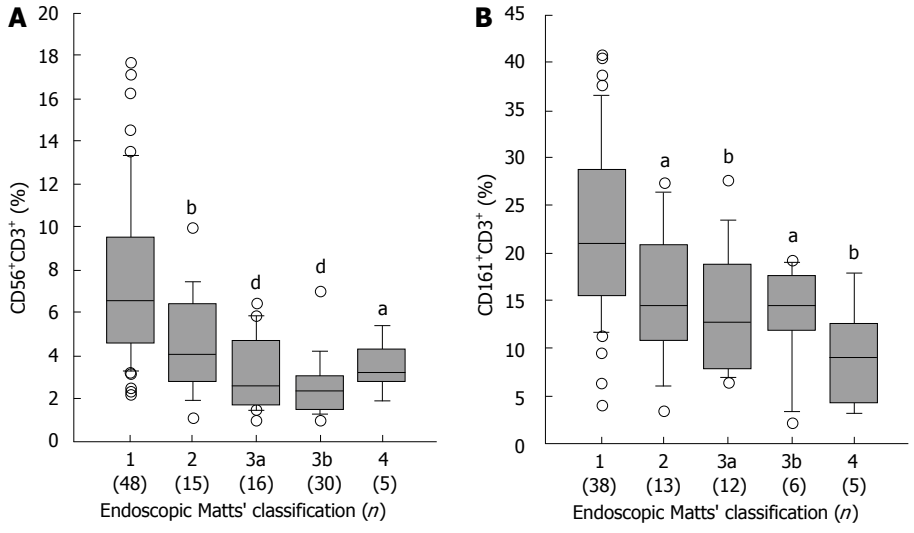

Figure 3 Relation between Matts' scores for endoscopy and the percentage of NKR^+ T cells. Box plot graphical representation of the percentage of colonic NKR^+ T cells in UC patients. The percentage of colonic NKR^+ T cells was compared with the Matts' scores. "n" indicates the number of cases. $^aP < 0.05$, $^bP < 0.01$, $^dP < 0.0001$, *vs* Matts' 1.

in patients with UC. The proportions of these cells were inversely well correlated with the degree of endoscopic and histologic intestinal inflammation. In contrast, the percentages of conventional T cells were similar among our study groups. Our findings suggest that selective decreases in levels of colonic NKR^+ T cells may contribute to the progression of intestinal inflammation.

$CD56^+$ T cells and $CD161^+$ T cells were originally identified in human liver. These cells have an extrathymic origin and have properties of innate lymphocytes[6,7]. These cells are uniquely capable of rapidly producing Th1 and Th2 cytokines upon stimulation[8-10], indicating a broad role for these cells in the activation and regulation of multiple arms of the immune responses. Although the functions of these $CD56^+$ T cells and $CD161^+$ T cells are currently unknown, they express memory T cell phenotypes[15] and homing chemokine receptors[25], suggesting that they may be memory T cells. The proliferation and function of these

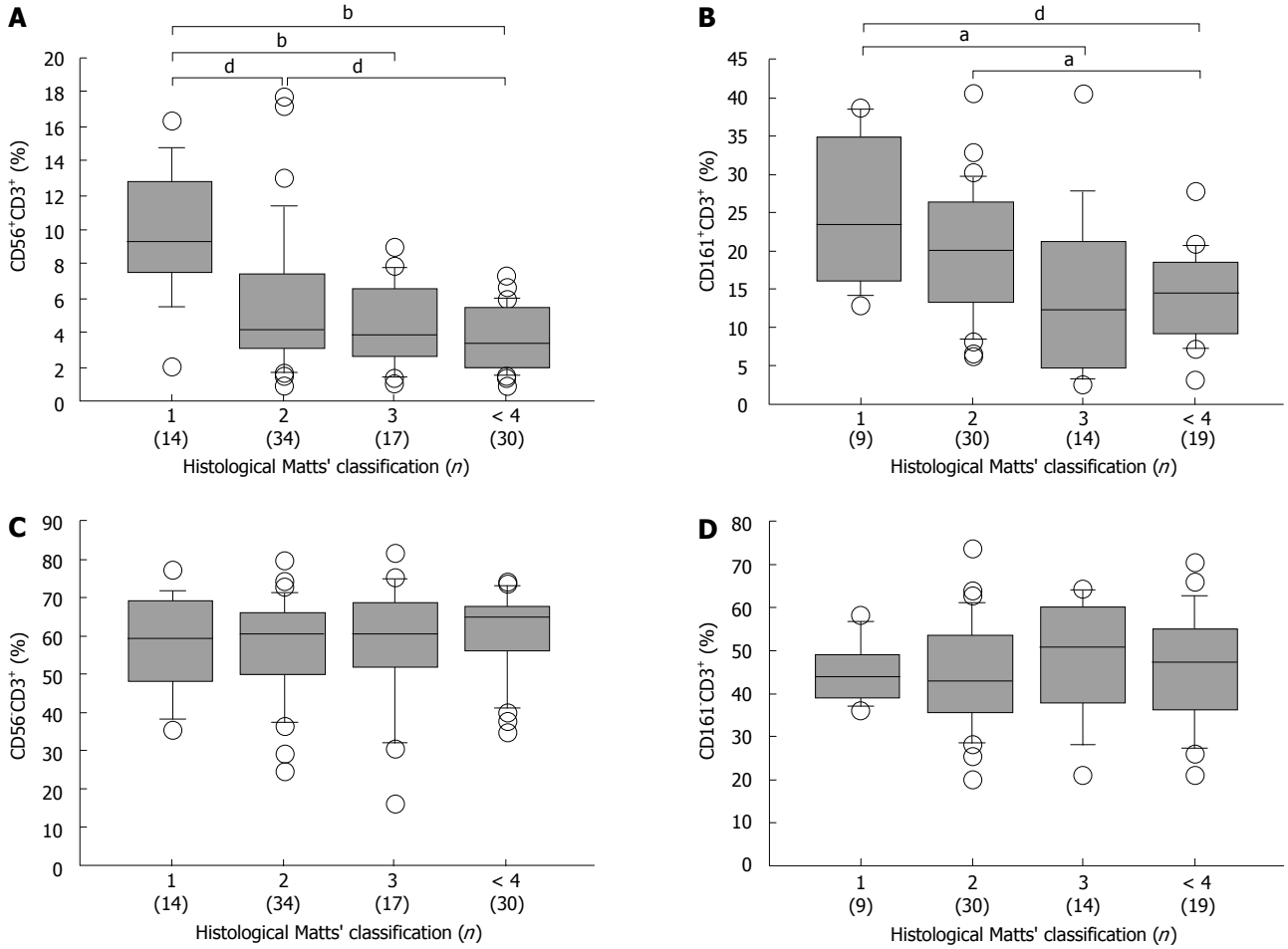

Figure 4 Relation between the histologic Matts' scores and the percentage of NKR$^+$ T cells. Biopsy samples for evaluation of histology were taken from the same regions as samples for MNC purification were taken. The percentage of colonic NKR$^+$ T cells was compared with the histologic Matts' score. Levels of CD56$^+$ T cells (**A**) and CD161$^+$ T cells (**B**) in the colon decreased significantly as a function of the severity of inflammation. "*n*" indicates the number of cases. $^b P < 0.0001$, $^d P < 0.005$ *vs* Matts' 1, $^a P < 0.05$ *vs* Matts' 1 or Matts' 2. No relation was detected between the percentages of conventional T cells (CD56$^-$CD3$^+$ or CD161$^-$CD3$^+$ cells) in the colon and the severity of histological inflammation (**C**, **D**).

cells are regulated by various cytokines, including IL-15 and granulocyte-macrophage colony-stimulating factor[26,27]. These NKR$^+$ T cells include invariant NKT cells that recognize glycolipid antigens presented by the non-classical antigen-presenting molecule CD1[15-17]. CD1d, which is an MHC Class I-like molecule, is expressed by human intestinal epithelial cells[28,29]. Bendelac *et al* reported that Vα24 NKT cells recognize CD1d molecules and act as immunoregulatory cells by producing various cytokines[30]. Blumberg *et al* showed that the CD1d molecule expressed by intestinal epithelial cells is functional because ligation with antibody against CD1d induces production of IL-10 by an intestinal epithelial cell line, T84[31]. Therefore, the interaction between CD1d on intestinal epithelial cells and mucosal CD1d-restricted T cells may be important for maintaining intestinal homeostasis. Whether these NKR$^+$ T cells are CD1d-restricted is currently under investigation.

The role of NKR$^+$ T cells in the development of intestinal inflammation is not clear. A mouse study revealed that CD1d-α-galactosylceramide (αGalCer)-restricted NKT cells are critical for protection against the development of dextran sulfate sodium-induced colitis[32]. The findings of another study suggested that IL-13-producing NKT cells are involved in the development of oxazolone-induced colitis in mice[33]. These data suggest that NKT cells are crucial for eliciting protective immunity against intestinal inflammation.

Although NKT cells are known to have an anti-inflammatory role[34], what is the mechanism by which these cells regulate the immune system? Sonoda *et al* showed that about 5% of NKT cells have the capacity to produce the immunosuppressive cytokine IL-10[35]. We have shown here that some colonic mucosal NKR$^+$ T cells can produce IL-10 when stimulated *in vitro*. IL-10 expression is also important for mucosal immunologic homeostasis. IL-10-deficient mice show enhanced production of colonic proinflammatory cytokines, including IFN-γ, and develop spontaneous chronic enterocolitis[36,37]. The subset of regulatory T cells that produce IL-10 suppresses the development of experimental intestinal inflammation[38]. Furthermore, mice with a macrophage/neutrophil-specific disruption of the Stat3 gene show impaired IL-10-mediated functions and develop chronic enterocolitis[39]. These observations support the idea that mucosal immune homeostasis involves localized production of molecules that promote IL-10 expression by resident immunoregulatory cells in the mucosa. Therefore, decreased IL-10 production due

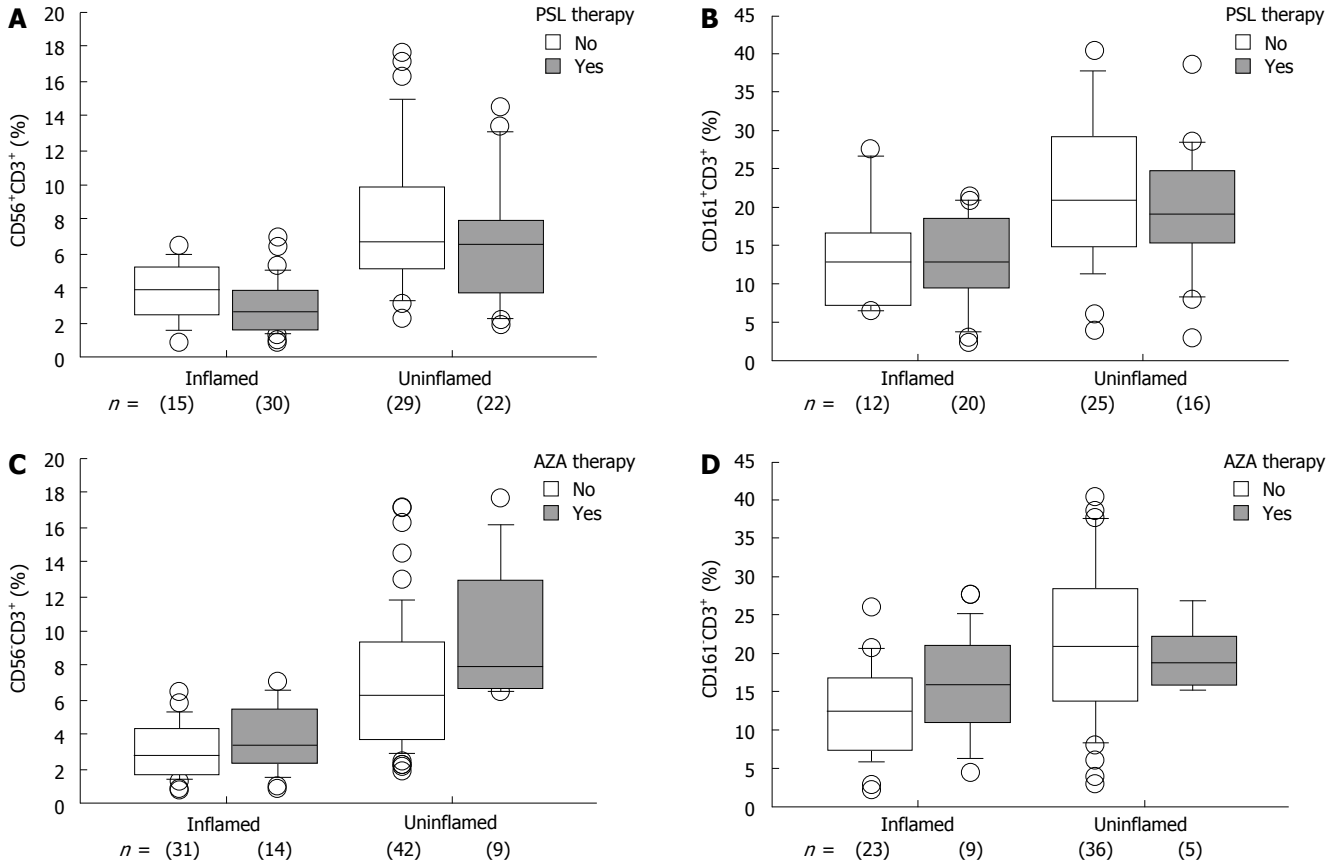

Figure 5 The percentage of colonic NKR$^+$ T cells was not affected by treatment with PSL or AZA. The percentages of colonic NKR$^+$ T cells were compared between untreated UC patients and those treated with PSL (**A, B**). The level of NKR$^+$ T cells was compared between untreated UC patients and those treated with AZA (**C, D**). "*n*" indicates the number of cases.

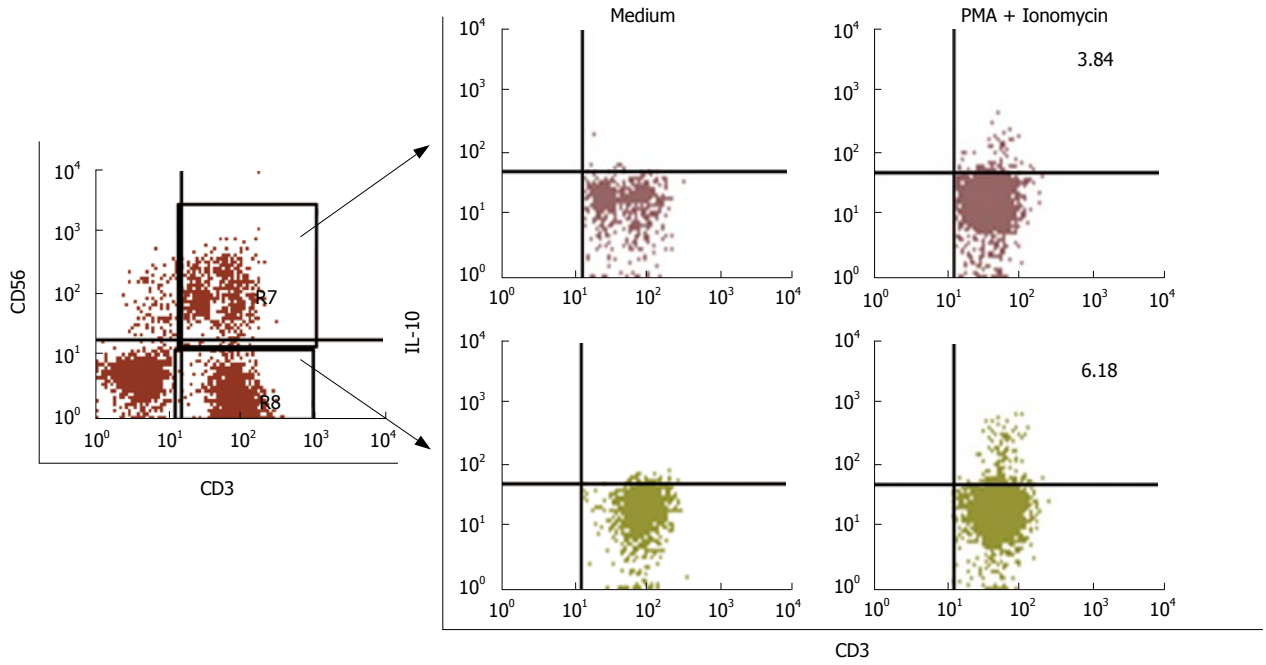

Figure 6 Detection of IL-10-producing NKR$^+$ T cells in the colonic mucosa. Flow cytometric analysis of IL-10 production by *ex vivo*-stimulated colonic MNCs isolated from a patient with active UC. FACScan was gated on CD56$^+$CD3$^+$ cells. The numbers indicate the percentages of CD56$^+$CD3$^+$ cells producing cytokines relative to unstimulated control cells.

to depletion of NKR$^+$ T cells in the colon of human UC as observed in the present study may result in insufficient inhibition of pathologic T cells and activated macrophages. Further studies are needed to compare the percentage of IL-10-producing cells between normal and UC colons.

Several mechanisms may account for the decreased

proportions of NKR^+ T cells in UC. First, the observed depletion of the local colonic NKT cell population may be the result of a continuous process of activation-induced cell death[40]. Second possible mechanism may be the loss of surface NK markers. It was recently reported that NKT cells activated by glycolipid antigens down-regulate NK receptors[41]. Third mechanism may be impaired recruitment of NKR^+ T cells from the peripheral circulation. $CD56^+$ T cells express chemokine receptors such as CCR5 or homing receptors such as $\alpha4\beta7$, a ligand for MAdCAM1 expressed specifically on the intestinal high endothelial venules[25]. These issues are currently under investigation in our laboratory.

In conclusion, human colonic $CD56^+$ T cells and $CD161^+$ T cells are thought to play important roles as anti-inflammatory cells, and the decrease in the proportions of these cells in inflamed lesions of the colon may be one mechanism by which colonic inflammation progresses in UC.

ACKNOWLEDGMENTS

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COMMENTS

Background

Human T cells that have natural killer markers are mainly located in the liver and considered immunoregulatory T cells. Recently it was reported that these natural killer receptor (NKR^+) T cells reside in the human intestine. But the exact significance of these cells in the intestine is unknown. This study was aimed at investigating the changes and significance of these cells in human ulcerative colitis (UC).

Research frontiers

Previous studies have demonstrated the significance of NKR^+T cells in human colorectal cancer. However, the role of these cells in the chronic intestinal inflammation is undefined.

Innovations and breakthroughs

The populations of both $CD56^+$ T cells and $CD161^+$ T cells were decreased significantly in the inflamed mucosa of UC. The populations of these NKR^+ T cells were correlated inversely with the severity of inflammation, which was classified according to the endoscopic and histologic Matts' criteria. In contrast, the frequency of conventional T cells ($CD56^-CD3^+$ cells and $CD161^-CD3^+$ cells) was similar among the patients with UC and healthy groups.

Applications

Selective reduction in the population of colonic mucosal NKR^+ T cells may contribute to the development of intestinal inflammation in UC.

Terminology

CD56 and CD161 are known as natural killer cell surface antigens. CD56 was demonstrated to be a neural cell adhesion molecule-1 (NCAM-1). $CD56^+$T cells are believed to be major players in immunosurveillance and antitumor responses. CD161 is a human NKR-P1 family and is recognized as an analogue of murine NKR-P1C. Moreover, $CD161^+$ T cells are also known to play an important role for antitumor immunity. CD161 is also expressed by invariant natural killer T cells that are restricted to CD1d molecule on antigen presenting cells.

Peer review

In this study, the authors demonstrate that the proportion of NKR^+ T cells is selectively decreased in the colonic mucosa of UC patients and that this reduced cell number correlates well with the severity of the disease. These cells are capable of producing an anti-inflammatory cytokine, IL-10. This work adds important information regarding cellular subsets that might be involved in the pathogenesis of UC.

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CLINICAL RESEARCH

*NAT2*6A*, a haplotype of the *N*-acetyltransferase 2 gene, is an important biomarker for risk of anti-tuberculosis drug-induced hepatotoxicity in Japanese patients with tuberculosis

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Abstract

AIM: To investigate an association between *N*-acetyltransferase 2 (*NAT2*)-haplotypes/diplotypes and adverse effects in Japanese pulmonary tuberculosis patients.

METHODS: We studied 100 patients with pulmonary TB treated with anti-TB drugs including INH. The frequencies and distributions of single nucleotide polymorphisms, haplotypes, and diplotypes of *NAT2* were determined by the PCR-restriction fragment length polymorphism method, and the results were compared between TB patients with and without adverse effect, using multivariate logistic regression analysis.

RESULTS: Statistical analysis revealed that the frequency of a variant haplotype, *NAT2*6A*, was significantly increased in TB patients with hepatotoxicity, compared with those without hepatotoxicity [P = 0.001, odds ratio (OR) = 3.535]. By contrast, the frequency of a wild-type (major) haplotype, "*NAT2*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity (P < 0.001, OR = 0.265). There was no association between *NAT2*-haplotypes and skin rash or eosinophilia.

CONCLUSION: The present study shows that *NAT2* is one of the determinants of anti-TB drug-induced hepatotoxicity. Moreover, the haplotypes, *NAT2*4* and *NAT2*6A*, are useful new biomarkers for predicting anti-TB drug-induced hepatotoxicity.

Key words: Tuberculosis; Anti-tuberculosis drugs; Drug-induced hepatotoxicity; *NAT2*-haplotype; DNA-based diagnosis

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INTRODUCTION

Tuberculosis (TB) is a re-emerging infectious disease that was declared a global health problem by the World Health Organization in 1993[1]. Since there were 9 million new TB cases and approximately 2 million TB deaths in 2004, and more than 80% of all TB patients live in sub-Saharan Africa and Asia, the epidemiology and control of TB remain important public health issues[1,2]. However, the management of TB is associated with serious problems, including disease relapse in elderly patients, occurrence

in acquired immunodeficiency syndrome, development of adverse effects of anti-TB drugs, and increase in the prevalence of multidrug-resistant *Mycobacterium tuberculosis*[2-5]. In particular, poor compliance or non-compliance with anti-TB drugs because of adverse effects, such as hepatotoxicity, skin rash, drug fever, peripheral neuritis, eosinophilia, and/or hyperuricemia, may lead to decrease in the quality-of-life of TB patients and appearance of multidrug-resistant *M. tuberculosis*. An important focus of previous studies was drug-induced hepatotoxicity, because it constitutes a major and severe adverse effect in the treatment of tuberculosis. Although the common risk factors for hepatotoxicity include advanced age[6,7], gender[7-10], malnutrition[6,9], complications of diseases[8,10,11], and alcohol intake[6,8,12], genetic factors also have an important impact on the likelihood of the development of drug-induced hepatotoxicity. Case-control studies with candidate genes in the affected populations have identified several possible susceptibility genes, e.g., *N*-acetyltransferase 2 (*NAT2*)[13-17], cytochrome P450 2E1 (*CYP2E1*)[16,18], glutathione *S*-transferase M1 (*GSTM1*)[16,19], glutathione *S*-transferase T1 (*GSTT1*)[16,19], and HLA-DQA1/-DQB1[20].

We focused our research on *NAT2* as a candidate gene associated with drug-induced hepatotoxicity because *NAT2* is the main enzyme involved in isoniazid (INH) metabolism, and is expressed in the liver. Diminution or disturbance of *NAT2* activity could result in the accumulation of precursors, such as hydrazine and acetylhydrazine in the liver, leading to hepatotoxicity[21-23]. Furthermore, the degree of metabolism with regard to *NAT2* varies among individuals, suggesting that genetic variations contribute to the metabolic activation capacity. Although studies on the association between *NAT2* phenotype (slow acetylator)[24] and anti-TB drug-induced hepatotoxicity have been reported from Taiwan[15,18], India[6,16], and Japan[13,14,17], no study has examined the association between hepatotoxicity and haplotypes/diplotypes that are composed of single nucleotide polymorphisms (SNPs). In the present study, we report our findings of the association between *NAT2* haplotypes/diplotypes and anti-TB drug-induced adverse effects, especially hepatotoxicity, in Japanese TB patients.

MATERIALS AND METHODS

Subjects

The study subjects comprised of 100 patients with new onset of pulmonary TB treated with a INH- (400 mg/d) and rifampicin (RFP, 450 mg/d)-containing regimen for six or nine months, between the years of 2003 and 2005 (Table 1). All subjects were Japanese who were recruited randomly from four general health clinics in the Nagasaki area of Japan. The study protocol was approved by the Committee for the Ethical Issue on Human Genome and Gene Analysis in Nagasaki University, and written informed consent was obtained from each patient.

The diagnosis of pulmonary TB was made on the basis of symptoms, chest radiographic infiltrates, and presence of acid-fast bacilli on sputum smear and *M. tuberculosis* on sputum culture. Patients with liver cirrhosis, chronic and acute hepatitis, alcoholic liver disease, and other chronic liver diseases were excluded from the study.

Table 1 Characteristics of pulmonary TB patients included in the study

Characteristics	TB
Number of patients	100
Age range (yr)	22-94
Age (mean ± SD)	64.0 ± 17.4
Gender (male/female)	56/44
Body mass index (kg/m^2)	20.3 ± 2.9

Diagnosis of drug-induced adverse effects

Patients with TB were classified into the following two subgroups: those with adverse effects such as hepatotoxicity, skin rash, and eosinophilia, and those without any side effects. Drug-induced hepatotoxicity was defined according to the criteria of the International Consensus Meeting[25], i.e., development of a two-fold or more increase in serum alanine aminotransferase (ALT) level above the upper limit of the normal range: N ($\leqslant$ 42 IU/L), or a combined increase of over 2 N in serum aspartate aminotransferase (AST, $N \leqslant$ 33 IU/L) and total bilirubin (TB, $N \leqslant$ 1.5 mg/dL). The presence of > 450 eosinophils/mL was defined as eosinophilia.

Determination of NAT2 polymorphisms

Genomic DNA was extracted from peripheral blood leukocytes of each patient using the DNA Extracter WB-Rapid Kit (Wako, Osaka, Japan), according to the manufacturer's protocol. SNPs of *NAT2* deposited in the SNP-database[26] were determined with PCR-restriction fragment length polymorphism (RFLP) method as described previously[27,28]. PCR was performed in a 25-μL reaction mixture containing 20 ng of genomic DNA, 20 mmol/L Tris-HCl (pH 8.4), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L dNTP, 0.4 μmol/L each of sense and antisense primers, and 1.5 U Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) with a DNA thermal cycler, GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA), according to the following protocol: initial denaturation at 95℃ for 5 min; 35 cycles of denaturation at 95℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for 30 s; and a final extension at 72℃ for 5 min. Subsequently, the PCR product was digested by restriction enzyme (TaKaRa Biomedical, Shiga, Japan) for detection of each SNP. A SNP, C282T, was detected by digestion with *Fok* I. Likewise, C481T, G590A, and G857A were detected by *Kpn* I, *Taq* I, or *Bam* HI, respectively. These fragments were subjected to electrophoresis on a 2% agarose gel, and visualized with UV transilluminator (Alpha Innotech, San Leandro, CA, USA) after ethidium bromide (Invitrogen) staining. Haplotypes were determined to be based on a combination of four SNPs (Table 2)[26,28].

Statistical analysis

Data obtained are shown as mean ± standard deviation (SD). Clinico-pathological parameters were compared between TB patients with and without adverse effect, using the Mann-

Table 2 Five Haplotypes composed of four SNPs in *NAT2*

Haplotype	SNP			
	C282T	C481T	G590A	G857A
*NAT2*4*	-	-	-	-
*NAT2*6A*	+	-	+	-
*NAT2*7B*	+	-	-	+
*NAT2*11*	-	+	-	-
*NAT2*13*	+	-	-	-

Plus or minus symbols for C282T, C481T, G590A, and G857A indicate the presence or absence of SNPs.

Whitney *U* test, χ^2 test with Yates' correction, and Fisher's exact test. Expected allele frequencies were calculated from respective single allele frequencies according to the Hardy-Weinberg equilibrium. The observed and expected allele frequencies were compared by a χ^2 test using SNP Alyze 6.0 standard (Dynacom Inc., Chiba, Japan). To evaluate odds ratio (OR) with 95% confidence interval (95% CI) for the susceptibility to anti-TB drug-induced adverse effects, haplotype and diplotype frequencies were compared between TB patients with and without adverse effects, using multivariate logistic regression analysis. A *P* value of 0.05 or less was considered statistically significant. SPSS 14.0 (SPSS Japan Inc., Tokyo, Japan) program package was used for all statistical analyses.

RESULTS

Frequency of drug-induced adverse effects, and clinico-pathological parameters for susceptibility to the effects

Out of the 100 TB patients enrolled in the study, 50 (50%) patients had anti-TB drug-induced adverse effects, 18 hepatotoxicity, 25 skin rash, and 34 eosinophilia. There were no differences in the clinical characteristics and baseline laboratory data (before chemotherapy) between TB patients with and without adverse effects (Table 3). However, eosinophilia developed less frequently in female patients than male patients ($P = 0.0186$). During TB chemotherapy, patients with hepatotoxicity had 8-times higher serum levels of ALT and AST than those without hepatotoxicity ($P < 0.0001$). Likewise, during therapy, ALT values and eosinophil counts were significantly higher in patients with skin rash compared to those without skin rash ($P = 0.0245$ and $P = 0.0058$, respectively). Moreover, eosinophils in patients with eosinophilia were increased in number compared with those without this complication ($P < 0.0001$).

NAT2-haplotype susceptible to adverse effects

In the 100 TB patients examined, we identified three haplotypes composed of four SNPs (Table 4). One haplotype, "*NAT2*4*" is a wild-type (major type), while the other haplotypes are variants (minor types). Distribution of SNPs and haplotypes among patients corresponded well with the Hardy-Weinberg equilibrium, implying that our samples had a homogeneous genetic background, and was consistent with previous observations[13,14,17]. However, since the frequencies of two haplotypes, *NAT2*11* and *NAT2*13*, were very low, they were not used for further statistical analysis.

Multivariate logistic regression analyses revealed that the frequency of a variant haplotype "*NAT2*6A*", which is composed of two SNPs (C282T and G590A), was significantly increased in TB patients with hepatotoxicity, compared with those without hepatotoxicity ($P = 0.001$, OR = 3.535, 95% CI: 1.648-7.585) (Table 4). By contrast, the frequency of the wild-type (major) haplotype, "*NAT2*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity ($P < 0.001$, OR = 0.265). There were no significant differences in the frequency of *NAT2*-haplotypes between TB patients with and without skin rash or eosinophilia (Table 4).

NAT2-diplotype susceptible to adverse effects

We identified six diplotypes composed of three haplotypes (Table 5). Distributions of the diplotypes in our study population were consistent with previous observations [13,14,17]. Of a total of 18 TB patients with hepatotoxicity, 3 (16.6%) had a diplotype, "*NAT2*6A/*7B*"; using multivariate logistic regression analyses, the frequency was significantly higher than in patients without hepatotoxicity (2/82, 2.4%; $P = 0.029$, OR = 8.000, 95% CI: 1.230-52.023) (Table 5). On the other hand, the frequency of another diplotype, "*NAT2*4/*4*", was significantly lower in TB patients with hepatotoxicity than those without hepatotoxicity ($P = 0.032$, OR = 0.272). There was no difference in the frequency of *NAT2*-diplotypes between TB patients with and without skin rash or eosinophilia (Table 5).

DISCUSSION

We have shown that a variant haplotype, *NAT2*6A*, of *NAT2* is associated with susceptibility to anti-TB drug-induced hepatotoxicity, and a wild-type (major) haplotype, *NAT2*4*, is associated with non-susceptibility to hepatotoxicity. These findings suggest that *NAT2* is one of the genetic factors responsible for predisposition to anti-TB drug-induced hepatotoxicity. However, since the number of TB patients in the present study was relatively small, it remains to be confirmed whether this association can be reproduced in a larger number of Japanese TB patients with and without hepatotoxicity as well as in other ethnic populations. Although previous reports have shown a positive association in Japanese TB patients between drug-induced hepatotoxicity and *NAT2* variants with phenotypic activities of *NAT2*, such as rapid, intermediate, and slow acetylators[13,14,17], the present study is the first report demonstrating an association with *NAT2*-haplotype variation.

Three *NAT2* haplotypes, *NAT2*5B*, *NAT2*6A*, and *NAT2*7B*, are believed to be associated with slow acetylators[24,29,30]. We did not detect *NAT2*5B* in our samples, probably because of its low frequency in the Japanese population as described in our previous study[28]. Since *NAT2* is the main enzyme involved in the metabolism of INH and *NAT2*6A* is functionally related to the low activity of *N*-acetylation in the INH metabolic pathway[30], TB patients possessing *NAT2*6A* may fail to metabolize toxic substances, such as hydrazine and acetylhydrazine, generated by INH metabolism in the liver, which therefore accumulate in the body, leading to drug-induced hepatotoxicity[21-23,31,32].

Table 3 Clinical characteristics and laboratory data of TB patients with or without adverse effect

Clinical data	Hepatotoxicity		*P*	Skin rash		*P*	Eosinophilia		*P*
	Present (*n* = 18)	Absent (*n* = 82)		Present (*n* = 25)	Absent (*n* = 75)		Present (*n* = 34)	Absent (*n* = 66)	
Age (mean ± SD)	60.8 ± 17.7	64.7 ± 17.3	0.3942	63.6 ± 18.1	64.7 ± 17.3	0.9028	63.3 ± 19.5	64.4 ± 16.3	0.7598
Gender (M/F)	9/9	47/35	0.6081	12/13	44/31	0.3640	25/9	31/35	0.0186
Body mass index (kg/m^2)	19.6 ± 2.3	20.5 ± 3.1	0.2721	19.8 ± 2.5	20.6 ± 3.1	0.2626	19.8 ± 2.9	20.6 ± 3.0	0.2684
Baseline values									
ALT (IU/L)	18.0 ± 10.4	21.1 ± 16.6	0.4527	23.1 ± 25.8	19.7 ± 10.4	0.3392	25.9 ± 24.4	17.8 ± 6.8	0.6571
AST (IU/L)	29.1 ± 26.8	26.8 ± 23.3	0.7188	31.3 ± 39.2	25.9 ± 15.9	0.3268	35.4 ± 38.5	23.0 ± 7.8	0.4277
TB (mg/dL)	0.48 ± 0.19	0.64 ± 0.43	0.1115	0.61 ± 0.44	0.61 ± 0.39	0.9874	0.62 ± 0.44	0.61 ± 0.38	0.9490
Creatinine (mg/dL)	0.64 ± 0.13	0.88 ± 1.10	0.3482	0.72 ± 0.28	0.87 ± 1.13	0.5121	0.82 ± 0.47	0.84 ± 1.17	0.9092
Eosinophils (/μL)	105.1 ± 120.6	115.5 ± 121.8	0.7434	104.7 ± 93.7	116.6 ± 129.3	0.6750	141.3 ± 133.4	99.3 ± 112.6	0.1011
During TB chemotherapy									
Peak ALT (IU/L)	316.2 ± 281.7	40.0 ± 19.5	< 0.0001	147.8 ± 281.7	61.6 ± 88.1	0.0245	129.6 ± 284.1	59.2 ± 75.4	0.3885
Peak AST (IU/L)	294.5 ± 353.6	36.7 ± 21.5	< 0.0001	139.7 ± 222.9	73.1 ± 128.9	0.1325	116.3 ± 175.9	76.0 ± 149.3	0.2107
Peak TB (mg/dL)	1.20 ± 1.16	0.74 ± 0.53	0.0101	0.78 ± 0.47	0.84 ± 0.77	0.7039	0.92 ± 0.89	0.77 ± 0.58	0.3241
Peak Creatinine (mg/L)	0.76 ± 0.13	0.96 ± 1.23	0.4742	0.81 ± 0.13	0.97 ± 1.28	0.5316	0.87 ± 0.31	0.96 ± 1.36	0.7098
Peak Eosinophils (/μL)	692.4 ± 929.1	461.1 ± 844.7	0.4538	668.4 ± 773.9	447.5 ± 885.1	0.0058	1028.1 ± 1327.9	232.1 ± 114.9	< 0.0001

Table 4 Distributions of *NAT2*-haplotypes in TB patients with and without adverse effect

Haplotype	Hepatotoxicity					Skin rash					Eosinophilia				
	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*
*NAT2*4*	16 (44.4)	120 (73.2)	0.265	0.129-0.546	< 0.001	34 (68.0)	102 (68.0)	1.00	0.503-1.987	1.000	45 (66.2)	91 (68.9)	0.880	0.472-1.642	0.688
*NAT2*6A*	14 (38.9)	29 (17.7)	3.535	1.648-7.585	0.001	12 (24.0)	31 (20.7)	1.22	0.570-2.607	0.609	15 (22.0)	28 (21.2)	1.052	0.517-2.138	0.889
*NAT2*7B*	6 (16.7)	15 (9.1)	2.235	0.818-6.104	0.117	4 (8.0)	17 (11.3)	0.70	0.226-2.170	0.537	8 (11.8)	13 (9.9)	1.227	0.482-3.124	0.667
Total number	36	164				50	150				68	132			

Table 5 Distribution of *NAT2*-diplotypes in TB patients with and without adverse effect

Diplotype	Hepatotoxicity					Skin rash					Eosinophilia				
	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*	Present (%)	Absent (%)	OR	95% CI	*P*
*NAT2*4/*4*	4 (22.2)	42 (51.2)	0.272	0.083-0.897	0.032	12 (48.0)	34 (45.3)	1.113	0.449-2.757	0.817	14 (41.2)	32 (48.5)	0.744	0.322-1.717	0.488
*NAT2*4/*6A*	7 (38.9)	23 (28.1)	1.632	0.564-4.726	0.366	7 (28.0)	23 (30.7)	0.879	0.323-2.394	0.801	11 (32.4)	19 (28.9)	1.183	0.484-2.894	0.713
*NAT2*4/*7B*	1 (5.6)	13 (15.9)	0.312	0.038-2.555	0.278	3 (12.0)	11 (14.7)	0.793	0.203-3.108	0.740	6 (17.6)	8 (12.1)	1.554	0.492-4.909	0.453
*NAT2*6A/*6A*	2 (11.1)	2 (2.4)	5.000	0.655-38.152	0.121	2 (8.0)	2 (2.7)	3.174	0.423-23.812	0.261	1 (2.9)	3 (4.5)	0.636	0.064-6.360	0.700
*NAT2*6A/*7B*	3 (16.6)	2 (2.4)	8.000	1.230-52.023	0.029	1 (4.0)	4 (5.3)	0.74	0.079-6.945	0.792	2 (5.9)	3 (4.5)	1.313	0.209-8.257	0.772
*NAT2*7B/*7B*	1 (5.6)	0 (0)	-	-	-	0 (0)	1 (1.3)	-	-	-	0 (0)	1 (1.5)	-	-	-
Total number	18	82				25	75				34	66			

A variant diplotype, *NAT2*6A/*7B*, is associated with susceptibility to hepatotoxicity ($P = 0.029$). Although another *NAT2*-diplotype, *NAT2*6A/*6A*, showed a trend towards susceptibility to hepatotoxicity, the results were statistically not significant ($P = 0.121$). However, if a larger number of subjects were analyzed, *NAT2*6A/*6A* as well as *NAT2*6A/*7B* may demonstrate a significant association with hepatotoxicity. Both of these diplotypes are homozygous for variant haplotypes and indicate phenotypically slow acetylators. Therefore, it is likely that some of the slow acetylators who are variant homozygotes possessing the *NAT2*6A* haplotype have susceptibility to anti-TB drug-induced hepatotoxicity. In this context, the results of the present study with regard to *NAT2*-haplotypes/diplotypes are comparable to those of previous reports on the association between *NAT2* phenotypic variation and hepatotoxicity[13-17,32]. Conversely, a wild-type homozygote, *NAT2*4/*4*, is associated with non-susceptibility and resistance to hepatotoxicity.

In conclusion, the haplotypes, *NAT2*4* and *NAT2*6A*, are new biomarkers for predicting drug-induced hepatotoxicity, and may prove useful in achieving optimal treatment of individual TB patients.

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COMMENTS

Background

Tuberculosis (TB) is a re-emerging infectious disease and has been declared a global health problem by the WHO. Adverse effect of anti-TB drugs including

isoniazid (INH) has become a serious problem in the management of tuberculosis. Risk factors associated with the development of adverse effects include both clinical and genetic factors. Recently, genome-wide screening and candidate gene-based association studies have been launched to identify the possible susceptibility genes sensitive to anti-TB drugs.

Research frontiers

Association studies with candidate gene-based approach in Asian and Caucasian patients have identified several possible susceptibility genes, e.g., *N*-acetyltransferase 2 (*NAT2*), cytochrome P450 2E1, glutathione *S*-transferase M1, glutathione *S*-transferase T1, and HLA-DQA1/-DQB1.

Innovations and breakthroughs

There are several reports on the association between *NAT2* polymorphisms and adverse effects, especially hepatotoxicity, of anti-TB drugs from Japan, Taiwan, and India. However, *NAT2* polymorphisms have been analyzed as phenotypic activities of *NAT2*, such as rapid, intermediate, and slow acetylators, but not as *NAT2*-haplotypes. The present study has shown that some phenotypically slow acetylators who are variant homozygotes possessing *NAT2*6A* haplotype have increased susceptibility to anti-TB drug-induced hepatotoxicity. This is the first report on the association with *NAT2*-haplotypes and hepatotoxicity in Japanese TB patients.

Applications

Our findings can be used for DNA-based diagnosis of TB patients before initiating treatment with anti-TB drugs, using *NAT2*6A* as a biomarker. Since patients possessing *NAT2*6A* haplotype have higher susceptibility to anti-TB-drug-induced hepatotoxicity, such individuals should be treated by reducing the dose of INH from 400 to 200 mg, in order to achieve optimal results.

Terminology

NAT2 is the main enzyme in the INH metabolism, and is expressed in the liver. Single nucleotide polymorphism (SNP) is a DNA sequence variation which occurs when a single nucleotide in the genome differs in paired chromosomes of an individual. Haplotype is a combination of alleles at multiple linked loci that are transmitted together. A second interpretation is that a haplotype is a set of SNPs on a single chromatid that is statistically associated. Such information is very valuable in investigating the genetics behind common diseases. Restriction fragment length polymorphism (RFLP) is a laboratory technique designed to distinguish differing nucleotide sequences from two related contexts.

Peer review

This study is well performed and the subject matter is very interesting.

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CLINICAL RESEARCH

Risk factors for gastroesophageal reflux disease, reflux esophagitis and non-erosive reflux disease among Chinese patients undergoing upper gastrointestinal endoscopic examination

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Abstract

AIM: To analyze the spectrum and risk factors of gastroesophageal reflux disease (GERD) based on presenting symptoms and endoscopic findings.

METHODS: A cross-sectional survey in a cluster random sample was conducted from November 2004 to June 2005 using a validated Chinese version Reflux Disease Questionnaire (RDQ) and other items recording the demographic characteristics and potential risk factors for GERD. Subjects were defined as having GERD symptoms according to the RDQ score (> 12). All subjects were endoscopied and the definition and severity of erosive esophagitis were evaluated by Los Angeles classification. The statistical analysis was performed with SPSS13.0 programs.

RESULTS: Of 2231 recruited participants, 701 (31.40%) patients were diagnosed as having GERD while 464 (20.80%) patients had objective findings of reflux esophagitis (RE). Of those 464 patients, only 291 (13.00%) were reported as subjects with GERD symptoms. A total of 528 (23.70%) patients were found to have GERD symptoms, including 19.50% patients with grade A or B reflux esophagitis, 0.90% with grade C and 0.40% with grade D. On multivariate analysis, old age, male, moderate working burden, divorced/widowed and strong tea drinking remained as significant independent risk factors for erosive esophagitis. Meanwhile, routine usage of greasy food and constipation were considered as significant independent risk factors for non-erosive reflux disease (NERD).

CONCLUSION: GERD is one of the common GI diseases with a high occurrence rate in China and its main associated factors include sex, anthropometrical variables and sociopsychological characteristics.

Key words: Gastroesophageal reflux disease; Reflux esophagitis; Non-erosive reflux disease; Prevalence; Risk factors; Endoscopy

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INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common disorder with a high incidence rate of 10%-38% of adults in the Western population occurring at least once a week[1,2]. The prevalence of GERD has been increasing[3]. The diagnosis and treatment of GERD are therefore, important because the disease, in addition to the highly disturbing typical symptoms, has a series of known consequences. The presence of GERD may affect the patients' quality of life[4], decrease functional activity[5], increase the economic burden[6] and the risk of esophageal carcinoma in the cases of Barrett's esophagus[7]. With an emphasis on morphological diagnosis, endoscopy has become a major tool to assess the final consequences of GERD, which is especially useful for population-based screening.

Although many investigators have reported the prevalence of erosive esophagitis[8], the prevalence of NERD has not been investigated in China. Our study was designed to analyze the spectrum of GERD subjects based on presenting symptoms and endoscopic findings. In order to determine the risk factors for such disease in outpatients from Zhejiang Province of East China, a cross-sectional survey in a cluster random sample was conducted from November 2004 to June 2005.

MATERIALS AND METHODS

Subjects

From November 2004 to June 2005, outpatients visiting departments of medicine in 10 hospitals from island, mountainous area, plain, city, countryside and suburb, in the Zhejiang Province of East China were recruited to the study. Subjects were excluded if they were not permanent inhabitants of East China, less than 18 years old, and had major psychotic episodes, mental retardation, dementia, severe visual or hearing abnormalities or other illnesses that might render them unable to complete the questionnaire or undergo the endoscopy (e.g. stroke). Excluding criteria also contained a history of peptic ulcer disease and receiving proton pump inhibitors or H_2-blockers over the preceding 2 wk. A total of 2278 individuals who had GI endoscopy were recruited, of which 2231 were eligible with a response rate of 97.9%.

Questionnaire

The gastroesophageal reflux questionnaire, a self-report instrument that to evaluate reflux-associated symptoms during the prior month, included the Chinese version of the Reflux Diagnostic Questionnaire (RDQ)[9] and items concerning the demographic characteristics and probable risk factors for GERD. It comprised the following parts: (1) General information: gender and age. (2) The Chinese version of the Reflux Diagnostic Questionnaire (RDQ): its framework of the RDQ was based on a validated questionnaire previously published[3]. The Chinese version of the RDQ was designed to measure symptoms suggestive of GERD appearing during the previous month. The intraclass correlation coefficient of the Chinese version of the RDQ was 0.9, thus it was validated and found to be a useful screening test for GERD-associated symptoms in China[9]. The symptoms suggestive of GERD in the RDQ included heartburn, substernal chest pain, acid eructation and food regurgitation. The following definitions were used to identify the symptoms in the RDQ: (1) heartburn, a burning sensation located beneath the sternum; (2) substernal chest pain: any pain felt inside in the chest but not including heartburn or any pain that is primarily originated from the abdomen; (3) acid regurgitation, a bitter- or sour-tasting fluid coming into the throat or mouth; and (4) food regurgitation, unpleasant movement of material upwards from the stomach but not vomit. Each symptom was scored according to the frequency and severity (5-point scale). The highest score for one subject was 40. The frequency was measured according to the following scale: 0, no symptom in the past month; 1, less than once a week; 2, once a week; 3, two to three days a week; 4, four to five days a week; and 5, almost daily. Symptom severity was assessed on the following scale:0, none; 1, very mild (symptoms can be easily ignored unless reminded of them); 2, mild (between 1 and 3); 3, moderate (symptoms are obvious and sufficient to influence normal activities, and occasionally need treatment); 4, severe (between 3 and 5); and 5, very severe (symptoms are obvious and sufficient to influence normal activities, and need long-term medication).

The Chinese version of the RDQ has been tested in a multicenter study including 10 hospitals in China. The specificity and sensitivity of the RDQ were evaluated by comparing the results with those of upper gastrointestinal endoscopy and esophageal 24-h pH monitoring. The RDQ score correlated positively with the severity of reflux esophagitis. Esophageal pH monitoring showed that patients with abnormal RDQ scores had higher Demeester scores than those with normal RDQ scores (20.18 *vs* 16.84). Taking 12 as the parameter for the threshold of RDQ score for GERD-associated symptoms, the study group obtained the maximal Youden index, the area under the receiver operating characteristic curve (ROC), was 0.71, the true positive diagnostic rate was 88.07% and the true negative diagnostic rate was 68.42% with a sensitivity of 94.12% and specificity of 50.00%. The subject was defined as a patient with GERD symptoms if his/her RDQ score was higher than 12[9]. Probable risk factors for GERD symptoms included life status: working burden, marital status (married, single, divorced/widowed), constipation, dietary and other personal habits: excessive consumption of acidic beverages, coffee, strong tea, spicy food, greasy food, sweet food, cigarette and alcohol. Definitions: heavy smoker (more than 20 cigarettes per day), excessive alcohol ($\leqslant$ 210 g of alcohol per week), constipation (frequently occurred during last 12 mo), routine use of coffee (more than 100 mL per day on average), acidic beverages and strong tea (more than 200 mL per day on average), dietary habits (taking the food mentioned above more than one time per day on average). The questions about probable risk factors, extra esophageal symptoms and accompanying diseases were all binary: yes or no.

Upper gastrointestinal endoscopy

Patients were examined for the presence of reflux esophagitis. Diagnosis and classification of reflux esophagitis were based on the Los Angeles classification[8]. Barrett's esophagus was diagnosed when columnar epithelium was seen to extend the Z line and confirmed histologically that showed specialized intestinal metaplasia. These criteria were consistently applied and endoscopic diagnosis was confirmed by either of the authors who were present during each endoscopic procedure. Patients who returned for endoscopic reassessment for any reason were excluded from the analysis to prevent duplication of cases.

Survey design and response rate

The cross-sectional survey in a cluster random sample was conducted from November 2004 to June 2005. The present study was based on a standard protocol including routine internal medicine counseling, endoscopy, and a self-reported questionnaire. Consecutive numbers were assigned to each registered subject and a 1:10 ratio of sample was selected using random number tables. All subjects completed the detailed questionnaire before endoscopy. Confirmed consent was obtained from all patients before the questionnaire was administered. All subjects were given the questionnaire.

A subject with GERD symptoms was defined according to the RDQ score (> 12). Patients who were

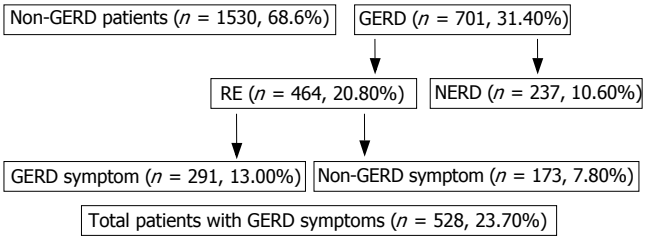

Figure 1 Diagnosis of gastroesophageal reflux disease (GERD) based on symptoms and presence of reflux esophagitis (RE).

Table 1 Grading of reflux esophagitis in 2278 patients

Grade	Total number	%
None	1767	79.20
A	333	14.93
B	102	4.57
C	20	0.90
D	9	0.40
Total	2231	100.00

Table 2 Complications of reflux esophagitis

	Total number	%
Barrett's esophagus	No 1190	98.76
	Yes 15	1.24
Esophageal stenosis	No 1191	98.76
	Yes 15	1.24
Esophagorrhagia	No 1186	98.18
	Yes 22	1.82

suspicious of having GERD but without evidence of reflux esophagitis (RE) were diagnosed as having NERD. GERD was diagnosed based on the presence of reflux esophagitis and/or the presence of predominant reflux symptoms. Because the survey explanation is made according to the RDQ score, all the questions in the RDQ must be answered without omission. In this study, a total of 2231 eligible subjects were recruited.

Ethics

The study was approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University.

Statistical analysis

The database was established with Epidata3.0. Statistical analysis was performed with SPSS13.0 programs. Univariate analysis was performed using χ^2 test for categorical variables. Univariate and multivariate logistic regression models were used to identify the potential risk factors of GERD, NERD and reflux esophagitis. The probable risk factors for GERD symptoms were selected by the univariate logistic regression, including life status (labor burden, marital status), dietary and other personal habits such as routine usage of acidic beverage, spicy food, greasy food, coffee, strong tea, sweet food, cigarette and alcohol, and constipation. All risk factors associated with GERD symptoms on univariate analysis were modeled using multivariate forward stepwise logistic regression analysis. To find the best model, a forward elimination stepwise procedure was carried out in a way that the factor would be brought into the analysis if the corresponding P value was less than 0.5. A P value $\geqslant 0.05$ was considered statistically significant and all P values were obtained by two-tailed examination.

Table 3 Comparison between upper gastrointestinal endoscopy and RDQ (χ^2 test)

RDQ	Endoscopy		*P* value
	Negative	Positive	
Negative	1530	173	0.002
Positive	237	291	

$P = 0.002$, there is significant statistical difference between the two investigations.

Table 4 Comparison between upper gastrointestinal endoscopy and RDQ (Wilcoxon rank-sum test)

RDQ	Endoscopy					Total	*U* value	*P* value
	Negative	A	B	C	D			
Negative	1530	131	34	5	3	1703		
Positive	237	202	68	15	6	528	3.60	0.000
Total	1767	333	102	20	9	2231		

RESULTS

Sample characteristics

A total of 2231 (56.6% male, 43.4% female) outpatients aged from 18 to 90 years (a median age of 43 years) were recruited to this study.

Prevalence of GERD, reflux esophagitis and NERD

As shown in Figure 1, 701 (31.40%) patients were diagnosed as having GERD and 464 (20.80%) patients were found to have objective findings of reflux esophagitis. Of the 464 patients, only 291 (13.00%) presented with GERD symptoms. Among the 2231 subjects, 528 (23.70%) presented with GERD symptoms.

Distribution of different grades and complications of reflux esophagitis

As shown in Table 1, 435 patients (19.50%) had grade A or B reflux esophagitis while 20 patients had grade C (0.90%) and 9 had grade D (0.40%). The complications of reflux esophagitis are shown in Table 2. The most frequent complication is esophagorrhagia (1.82%).

Comparison between upper gastrointestinal endoscopy and RDQ

As shown in Tables 3 and 4, there is significant difference between the two investigations. The Kappa value was 0.47, $P = 0.000$, demonstrating no predominant consistency between the two diagnostic methods.

Risk factors in GERD and non-GERD patients

The prevalence of various variables in GERD patients

Table 5 Association between variables determined using univariate analysis: GERD (n = 701) *vs* non-GERD (n = 1530) patients

Variables	GERD n (%)	P value	OR	Univariate (95% CI)	
				Low	High
Age (yr)					
> 65	106/239 (44.40)	< 0.0001	0.53	0.41	0.70
≤ 65	595/1992 (29.90)				
Gender					
Male	439/1263 (34.80)	< 0.0001	0.7	0.58	0.84
Female	262/968 (27.10)				
Working burden					
Heavy	64/147 (43.50)	0.694	1.04	0.86	1.27
Moderate	299/975 (30.70)	0.004	0.6	0.42	0.85
Mild	282/895 (31.50)				
Marital status					
Divorced/ widowed	6/14 (42.90)	< 0.0001	2.05	1.49	2.82
Single	52/261 (19.90)	0.478	0.68	0.24	1.97
Married	592/1752 (33.80)				
Excessive eating					
Yes	245/704 (34.80)	0.009	0.77	0.63	0.94
No	372/1276 (29.20)				
Routine intake of greasy food					
Yes	231/618 (37.40)	< 0.0001	0.65	0.53	0.80
No	375/1347 (27.80)				
Routine intake of spicy food					
Yes	196/512 (38.30)	< 0.0001	0.65	0.52	0.80
No	418/1463 (28.60)				
Routine intake of acidic beverage					
Yes	118/358 (33.00)	0.403	0.90	0.71	1.15
No	493/1606 (30.70)				
Routine intake of strong tea					
Yes	149/343 (43.40)	< 0.0001	0.51	0.4	0.64
No	451/1613 (28.00)				
Routine intake of sweet food					
Yes	225/683 (32.90)	0.122	0.85	0.70	1.04
No	375/1269 (29.60)				
Heavy smoking					
Yes	209/562 (37.20)	< 0.0001	0.68	0.55	0.83
No	407/1426 (28.50)				
Excessive alcohol					
Yes	185/503 (36.80)	0.002	0.71	0.57	0.88
No	434/1486 (29.20)				
Routine intake of coffee					
Yes	26/77 (33.80)	0.529	0.86	0.53	1.39
No	568/1869 (30.40)				
Constipation					
Yes	122/316 (38.60)	0.002	0.68	0.53	0.87
No	489/1641 (29.80)				

GERD: Gastroesophageal reflux disease.

Table 6 Association between variables determined using univariate analysis: Reflux esophagitis (n = 464) *vs* non-GERD (n = 1530) patients

Variables	RE n (%)	P	OR	Univariate (95% CI)	
				Low	High
Age (yr)					
> 65	77/210 (36.70)	< 0.0001	2.09	1.55	2.82
≤ 65	387/1784 (21.70)				
Gender					
Male	322/1146 (28.10)	< 0.0001	1.94	1.56	2.43
Female	142/848 (16.70)				
Working burden					
Heavy	45/128 (35.20)	0.353	0.90	0.71	1.13
Moderate	189/865 (21.80)	0.006	1.74	1.17	2.59
Mild	191/804 (23.80)				
Marital status					
Divorced/ widowed	5/13 (38.50)	< 0.0001	0.48	0.33	0.70
Single	34/243 (14.00)	0.283	1.85	0.60	5.69
Married	392/1552 (25.30)				
Excessive eating					
Yes	147/606 (24.30)	0.346	1.12	0.89	1.41
No	259/1163 (22.30)				
Routine intake of greasy food					
Yes	129/516 (25.00)	0.090	1.23	0.97	1.57
No	263/1235 (21.30)				
Routine intake of spicy food					
Yes	112/428 (26.20)	0.037	1.31	1.02	1.69
No	283/1328 (21.30)				
Routine intake of acidic beverage					
Yes	76/316 (24.10)	0.593	1.08	0.81	1.44
No	326/1439 (22.70)				
Routine intake of strong tea					
Yes	108/302 (35.80)	< 0.0001	2.28	1.74	2.98
No	284/1446 (19.60)				
Routine intake of sweet food					
Yes	131/589 (22.20)	0.769	0.97	0.76	1.22
No	265/1159 (22.90)				
Heavy smoking					
Yes	153/506 (30.20)	< 0.0001	1.75	1.38	2.22
No	252/1271 (19.80)				
Excessive alcohol					
Yes	124/442 (71.90)	0.003	1.45	1.13	1.85
No	283/1335 (21.20)				
Routine intake of coffee					
Yes	21/72 (29.20)	0.167	1.44	0.86	2.43
No	371/1672 (22.20)				
Constipation					
Yes	67/261 (25.70)	0.275	1.18	0.87	1.60
No	336/1488 (22.60)				

GERD: Gastroesophageal reflux disease.

compared to non-GERD patients is shown in Table 5. On univariate analysis, age > 65, male, moderate working burden, divorced/widowed, excessive eating, greasy food, spicy food, strong tea, smoking, alcohol, and constipation were found to be significant participants. On multivariate analysis, old age (OR, 0.57; β, -0.57; 95% CI, 0.40-0.80), male (OR, 0.78; β, -0.25; 95% CI, 0.61-1.00), moderate working burden (OR, 0.58; β, -0.54; 95% CI, 0.39-0.87), divorced/widowed (OR, 1.82; β, 0.60; 95% CI, 1.27-2.60), greasy food (OR, 0.75; β, -0.29; 95% CI, 0.60-0.95), strong tea (OR, 0.67; β, -0.40; 95% CI, 0.50-0.89) remained as significant independent risk factors.

Risk factors in reflux esophagitis and non-GERD patients

The prevalence of various variables in reflux esophagitis patients compared to non-GERD patients is shown in Table 6. On univariate analysis, age > 65, male, moderate working burden, divorced/widowed, spicy food, strong tea, smoking, and alcohol were found to be significant. On multivariate analysis, old age (OR, 1.86; β, 0.62; 95% CI, 1.29-2.70), male (OR, 1.77; β, 0.57; 95% CI, 1.32-2.37), moderate working burden (OR, 1.91; β, 0.65; 95% CI, 1.22-2.97), divorced/widowed (OR, 0.55; β, -0.60; 95% CI, 0.36-0.85), strong tea (OR, 1.62; β, 0.48; 95% CI, 1.18-2.23) were considered as significant independent risk factors.

Risk factors in NERD and non-GERD patients

The prevalence of various variables in NERD patients compared to non-GERD patients is shown in Table 7. On univariate analysis, divorced/widowed, excessive eating,

Table 7 Association between variables determined using univariate analysis: NERD (*n* = 237) *vs* non-GERD (*n* = 1530) patients

Variables	NERD *n* (%)	*P*	OR	Univariate (95% CI)	
				Low	High
Age (yr)					
> 65	29/162 (17.90)	0.08	1.46	0.96	2.25
≤ 65	208/1605 (13.00)				
Gender					
Male	117/941 (12.40)	0.198	0.84	0.64	0.10
Female	120/826 (14.50)				
Working burden					
Heavy	19/102 (8.60)	0.547	1.10	0.81	1.48
Moderate	110/786 (14.00)	0.119	1.54	0.89	2.66
Mild	91/704 (12.90)				
Marital status					
Divorced/ widowed	1/9 (11.10)	0.007	0.5	0.30	0.83
Single	18/227 (7.90)	0.762	0.73	0.09	5.83
Married	200/1360 (14.70)				
Excessive eating					
Yes	98/557 (17.60)	< 0.0001	1.71	1.27	2.29
No	113/1017 (11.10)				
Routine intake of greasy food					
Yes	102/489 (20.90)	< 0.0001	2.29	1.71	3.07
No	112/1084 (10.30)				
Routine intake of spicy food					
Yes	84/400 (21.00)	< 0.0001	2.06	1.52	2.78
No	135/1180 (11.40)				
Routine intake of acidic beverage					
Yes	42/282 (14.900	0.41	1.17	0.81	1.68
No	167/1280 (13.00)				
Routine intake of strong tea					
Yes	41/235 (17.40)	0.043	1.47	1.01	2.14
No	167/1329 (12.60)				
Routine intake of sweet food					
Yes	94/552 (17.00)	0.001	1.67	1.24	2.25
No	110/1004 (11.00)				
Heavy smoking					
Yes	56/409 (13.70)	0.802	1.04	0.75	1.45
No	155/1174 (13.20)				
Excessive alcohol					
Yes	61/379 (16.10)	0.078	1.34	0.97	1.85
No	151/1203 (12.60)				
Routine intake of coffee					
Yes	5/56 (8.90)	0.36	0.65	0.26	1.64
No	197/1498 (13.20)				
Constipation					
Yes	55/249 (22.10)	< 0.001	2.14	1.51	3.01
No	153/1305 (11.70)				

GERD: Gastroesophageal reflux disease; NERD: Non-erosive reflux disease.

greasy food, spicy food, strong tea and constipation were found to be significant. On multivariate analysis, greasy food (OR, 1.65; β, 0.50; 95% CI, 1.16-2.36) and constipation (OR, 1.51; β, 0.41; 95% CI, 1.01-2.25) were regarded as significant independent risk factors.

DISCUSSION

Traditionally, GERD is defined based on three major diagnostic parameters: (1) ambulatory 24-h esophageal pH monitoring; (2) upper gastrointestinal (GI) tract endoscopic examination for erosive esophagitis; and (3) clinical evaluation by physicians and clinical therapeutic treatment by acid suppression agents. The sensitivity of endoscopic examination is limited, as most patients with GERD do not have obvious mucosa injury. Therefore, most of their disease is categorized as non-erosive reflux disease (NERD). Ambulatory 24-h esophageal pH monitoring also has problems with sensitivity for the intermittent nature of symptoms and daily activities may disturb the placement of a pH probe. Endoscopy can be more easily applied to healthy participants than ambulatory 24-h esophageal pH monitoring. Furthermore, it is more objective in terms of finding reflux disease, which has been investigated in many previous studies. Erosive esophagitis is classified using the LA system, which appears to be the most unambiguous and simple method to apply. However, endoscopic examination alone can not rule out GERD or acid-induced epithelial injury. A variety of questionnaires designed for GERD clinical trials have been developed. The Gastrointestinal Symptom Rating Scale (GSRS)[10] comprises 15 items addressing five symptom clusters (gastroesophageal reflux, abdominal pain, indigestion, diarrhoea, and constipation). The GSRS used graded response categories from "none" to "very severe" without defining what these adjectives meant. This can produce subjective answers, reducing reliability and validity[11]. The "CarlssonDent Self-Administered Questionnaire (QUEST)"[12] had a good face validity, since it incorporated "word pictures" using simple English to describe symptoms of GERD. The GERQ[13] is a self-administered validated instrument that identifies the onset of GERD symptoms and grades the frequency and severity of symptoms over a prior year. It was a long questionnaire containing 80 questions, making it inconvenient for use in clinical trials.

The Chinese version of the Reflux Diagnostic Questionnaire (RDQ): Its framework of the RDQ was based on a validated questionnaire published before[3]. Shaw *et al*[3] found that the RDQ demonstrated validity and reliability and was responsive to change for reflux. The reliability coefficient of the RDQ scales ranged from 0.8 to 0.88, well beyond the acceptable level of 0.70. It was tested in the multicenters and found that it could accurately identify the presence of symptoms suggestive of GERD[9]. It was designed to measure GERD symptoms over the previous month, not the previous year. It was feasible to prevent the recall bias since McColl found that 1-mo was the maximum period over which patients could provide reliable data due to recall errors[14]. Four symptoms were included in the RDQ that may be somewhat different from the definition of the previous studies[10,12-13]. It would be more accurate to include substernal chest pain and food regurgitation to make a diagnosis of GERD[9,15]. Complete satisfaction of multitrait scaling criteria justifies combining the items into scales that can be scored with simple addition, thus eliminating the need for item weighting[16]. As our study confirmed there was no significant statistical difference between the two investigations. The Kappa-value was 0.4-0.75, which demonstrated no predominant consistency between the two diagnostic methods.

GERD becomes more common in Asian countries, resulting in more people coming to the gastroenterology outpatient department for treatment. A 13%-15% prevalence of reflux symptoms has been reported in

Asian GERD patients, which is comparable with results in many Western series. However, the definition of GERD may alter estimated prevalence. In the present study, data for subjects with GERD symptoms (23.70%) support previous prevalence rates[17].

We showed for the first time that the prevalence of NERD in the Chinese is 10.60% (237 of 2231 investigated persons), which is lower than that of erosive esophagitis (20.80%), while the prevalence rate of symptomatic GERD is 10%-30% in Western countries, and more than half of the patients lack endoscopically proven erosive esophagitis[18,19]. In Western countries, the majority of patients with GERD have been reported to have NERD, but not erosive esophagitis, even in cases with severe symptoms[18].

The factors that determine the form of NERD versus erosive esophagitis have not yet been clarified. However, we elucidated differences in the possible causative factors of NERD and erosive esophagitis. By multivariate logistic regression analysis, it was found that old age, male, moderate working burden, divorced and strong tea remained as significant independent risk factors for erosive esophagitis, meanwhile, greasy food consumption, constipation were regarded as significant independent risk factors for NERD.

Several risk factors associated with GERD have been reported as follows. Old age has been shown to be associated with increased risk of erosive esophagitis, Berrat's esophagus, and esophageal adenocarcinoma[20].

The previous studies showed that male gender is a risk factor for erosive esophagitis; whereas female is more likely to be associated with NERD[21,22]. Less parietal cell mass in women may be underlining reasons for the lower risk of GE[23].

Tea drinking has previously only been studied in a case series of reflux episodes[24]. While from another previous population-based study, the tea drinking does not seem to be a risk factor for GERD[25].

Coffee has been reported to be a reduced risk of reflux symptoms among coffee drinkers compared with non-coffee drinkers[25,26]. But previous cross sectional epidemiological studies have been able to establish that coffee drinking is a risk factor for GERD[24]. To accurately evaluate the long term effects of coffee drinking on the risk of reflux, an analysis of prospective exposure data would be necessary.

Smoking has often been cited as risk factors for GERD, although the findings of studies on this matter have been inconsistent[25,27]. Smoking was inversely related to NERD compared with RE[22]. Smoking decreases lower esophageal sphincter pressure and increases the frequency of reflux episodes. In addition, deleterious effects on esophageal defenses such as reduction of esophageal clearance and salivary function have been described[22].

Erosive esophagitis was positively related to alcohol consumption[28]. The mechanism is that alcohol intake induces nausea and vomiting and directly causes mucosal impairment, while food intake at late night elevates the risk of esophagitis[29].

In this cross sectional study, greasy food consumption was associated with an increased risk of GERD symptoms and erosive esophagitis. Several physiological studies of human volunteers have shown increased frequency of transient lower esophageal sphincter relaxation and increased esophageal acid exposure with greasy food consumption[30].

However, a limitation of the current study involves the subject sample. Subjects were outpatients from 10 hospitals and therefore probably represent a population of intermediate GERD severity between subjects recruited from gastrointestinal clinics and those randomly selected from the general population. In addition, other potential risk factors of GERD such as *H pylori*, hiatal hernia and BMI were not assessed in our study. Whether these factors are positively associated with GERD, calls for further observations.

In conclusion, GERD is a highly prevalent disease. Old age, male, moderate working burden, divorced and strong tea remained as significant independent risk factors for erosive esophagitis, meanwhile, midst bodily form, greasy food consumption, and constipation were considered as significant independent risk factors for NERD.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a common disorder with a high occurrence of up to 10%-38% of adults in the Western population at least once a week. The prevalence of GERD has been increasing year after year. The diagnosis and treatment of GERD are important because the disease, in addition to the highly disturbing typical symptoms, has a series of known consequences. The presence of GERD may affect the patients' quality of life, decrease functional activity, increase the economic burden associated and highlight the risk of esophageal carcinoma in the cases of Barrett's esophagus. With an emphasis on morphological diagnosis, endoscopy has become a major tool to assess the final consequences of GERD, which is especially useful for population-based screening.

Research frontiers

The definition or the diagnostic parameters of GERD and the factors that determine the form of NERD versus erosive esophagitis have not yet been clarified.

Innovations and breakthroughs

Although many investigators have reported the prevalence of erosive esophagitis, the prevalence of NERD has not been investigated in China. We showed for the first time that the prevalence of NERD in the Chinese is 10.60% (237 of 2231 investigated persons), which is lower than that of erosive esophagitis (20.80%), While the rate of symptomatic GERD is 10% to 30% in Western countries, and more than half of these patients lack endoscopically proven erosive esophagitis. The factors that determine the form of NERD versus erosive esophagitis have not yet been clarified. However, we elucidated differences in the possible causative factors of NERD and erosive esophagitis.

Applications

Our study was designed to analyze a spectrum of GERD subjects based on presenting symptoms and endoscopic findings. In order to determine the risk factors for such disease in outpatients from department of internal medicine in Zhejiang Province of East China, a cross-sectional survey in a cluster random sample was conducted from November 2004 to June 2005.

Peer review

This is a report designed to analyze a spectrum of GERD subjects based on presenting symptoms and endoscopic findings in Eastern part of China, surveyed by RDQ Chinese version. This clinical study was well designed.The limitation of the study was absence of assessment of the other important risk factors of GERD such as *H pylori*, hiatal hernia, and BMI. It is well known that negative association between *H pylori* and GORD does exist, especially in Asia (Kupcinskas

L, Malfertheiner P. Helicobacter. 2005; 10 Suppl 1: 26-33). In case the other GERD risk factors would be assessed, the results of multivariate analysis could be substantially different.

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RAPID COMMUNICATION

Breath test for differential diagnosis between small intestinal bacterial overgrowth and irritable bowel disease: An observation on non-absorbable antibiotics

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Abstract

AIM: To estimate the prevalence of small intestine bacterial overgrowth (SIBO) among patients with an earlier diagnosis of irritable bowel disease (IBS) in our geographical area, and to collect information on the use of locally acting non-absorbable antibiotics in the management of SIBO.

METHODS: A non-interventional study was conducted in 73 consecutive patients with a symptom-based diagnosis.

RESULTS: When the patients underwent a "breath test", 33 (45.2%) showed the presence of a SIBO. After treatment with rifaximin 1200 mg/d for seven days in 32 patients, 19 (59.4%) showed a negative "breath test" one week later as well as a significant reduction of symptoms, thus confirming the relationship between SIBO and many of the symptoms claimed by patients. In the other 13 patients, "breath test" remained positive, and a further cycle of treatment with ciprofloxacin 500 mg/d was given for 7 additional days, resulting in a negative "breath test" in one patient only.

CONCLUSION: (1) about half of the patients with a symptomatic diagnosis of IBS have actually SIBO, which is responsible for most of the symptoms attributed to IBS; (2) only a "breath test" with lactulose (or with glucose in subjects with an intolerance to lactose) can provide a differential diagnosis between IBS and SIBO, with almost identical symptoms; and (3) the use of non-absorbable antibiotics may be useful to reduce the degree of SIBO and related symptoms; it must be accompanied, however, by the correction of the wrong alimentary habits underlying SIBO.

Key words: Small intestine bacterial overgrowth; Irritable bowel disease; Breath test; Rifaximin; Hydrogen; Non-absorbable antibiotics

Esposito I, de Leone A, Di Gregorio G, Giaquinto S, de Magistris L, Ferrieri A, Riegler G. Breath test for differential diagnosis between small intestinal bacterial overgrowth and irritable bowel disease: An observation on non-absorbable antibiotics. *World J Gastroenterol* 2007; 13(45): 6016-6021

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INTRODUCTION

Irritable bowel syndrome (IBS) is a very common diagnosis in gastroenterology that is done on the basis of the Rome Ⅱ symptomatic criteria. The basic clinical pattern is characterized by abdominal pain and changes in bowel habit, on the basis of which three different variants of IBS are recognized (IBS with stipsis, IBS with diarrhea or IBS with alternated stipsis and diarrhea). No matter which variant is diagnosed, 92% of the patients with IBS complain of abdominal bloating, flatulence and meteorism, three symptoms that are, however, more probably related to a small intestine bacterial overgrowth (SIBO) rather than to IBS.

A close relationship exists between the changes in pattern and distribution of gastrointestinal (GI) bacterial flora, and the altered GI motility (changes in bowel habit) and sensorial physiology (abdominal pain and bloating) observed in patients with IBS. It has been demonstrated that the myoelectric activity of intestinal loops are deeply modified by the presence of SIBO, leading to the hypothesis that many of the sensorial and motorial symptoms of IBS are really determined by changes in the GI bacterial flora[1]. Moreover, it is well known that both an acute GI infection[2,3] and the use of systemic antibiotics[4,5] lead to profound changes in GI bacterial flora, and that both the conditions may result in symptoms (such as abdominal bloating and changes in bowel habit), which look like those of IBS[6-9].

Finally, it has been reported that even one single cycle of systemic antibiotics may provoke long-time sustained alterations of GI physiology[10], while a treatment with antibiotics specifically addressed to correction of intestinal

disbiosis is followed by an improvement of IBS- or SIBO-related symptoms[11]. Thus, there is ground to believe that there is a large overlapping between SIBO and IBS, and that many patients with an earlier symptomatic diagnosis of IBS are actually suffering from SIBO. However, the prevalence of SIBO among patients with an initial diagnosis of IBS is not exactly known.

Cuoco and Salvagnini[12] have recently reported in North Italy a 46% incidence of positive "breath test" (increased hydrogen concentrations in the expired air after oral lactulose administration) among 96 patients with IBS. According to USA-based clinicians, this incidence could be higher than 80%[13-15], while European investigators have reported an increased GI bacterial flora in 43% of patients with IBS compared with 12% of matched-control healthy subjects, without any relationship between degree of disbiosis and severity of altered GI motility and symptoms[16].

The different values in SIBO prevalence observed worldwide among patients with an initial diagnosis of IBS are probably due to the different methods employed to detect the bacterial colonization of the small intestine: a typical and simple clinico-laboratory test ("breath test" with lactulose) in the first two studies[14,15], a more rigorous microbiological, but also methodologically more complicated test (GI bacterial count $\geqslant 10^5$/mL) in the third study[16].

Recent studies have provided increasing support for the concept that disturbances in gut flora occur in patients with IBS and that such abnormalities may contribute to IBS-type symptoms[17]. In any case, the overlapping of SIBO and IBS and the role eventually played by SIBO in the pathogenesis of the IBS symptoms, are demonstrated by two double-blind placebo-controlled clinical studies, which have shown respectively a 75% reduction in the GI symptoms and a long-lasting (over 10 wk) clinical improvement in subjects with IBS, after treatment with non-absorbable antibiotics with a topical activity limited to the GI tract[18].

Our study is therefore aimed to estimate the prevalence of SIBO in our geographical area (Campania, South Italy) in patients with IBS diagnosed according to the Rome Ⅱ criteria; the diagnosis of SIBO is established on the basis of a positive "breath test" with lactulose. We have also gathered information on the use of locally active antibiotics in the management of SIBO.

MATERIALS AND METHODS

This study was purely observational. Within a time interval of 27 mo (January 2005-March 2007), we selected patients of both sexes who came to our medical centre for advice, and had a diagnosis of IBS, because of abdominal pain and discomfort complying with the following characteristics: (1) Three months of continuous or recurring symptoms of abdominal pain or irritation that: (a) may be relieved with a bowel movement; (b) may be coupled with a change in frequency, or (c) may be related to a change in the consistency of stools. (2) Two or more of the following present at least 25% of time: (a) change in stool frequency (> 3 bowel movements daily or < 3 bowel movements weekly); (b) noticeable difference in stool form (hard, loose and watery stools or poorly formed stools); (c) passage of mucous in stools; (d) bloating or feeling of abdominal distention; (e) altered stool passage (e.g. sensations of incomplete evacuation, straining, or urgency).

Patients with severe cardiovascular or respiratory or renal diseases and patients with cancer or under treatment with antibiotics and corticosteroids were excluded. All the patients gave their informed consent to the management of personal data according to the "privacy" regulations.

All the symptoms, either GI or not, were recorded during the first medical visit, and the patients were asked to score the global intensity of symptoms by means of Visual Analogue Scale (VAS) 10-cm long (0 = no symptom; 10 = unbearable symptom). Then, all the patients underwent a "breath test", whose concept is based on a non-invasive measurement of hydrogen (H_2) concentrations in the expired air.

In the evening before the examination, the patient was required to eat only boiled rise with no sausage or cheese, and grilled meat, to make a careful oral hygiene and to drink only no-gas water. If stipsis was present, the dietary prescriptions were extended to the three days preceding the exam. On the day of the test, the patient was completely fasted, and smoking was forbidden. Immediately before the test two samples of expired air were taken at a 10-min interval to assay the basal hydrogen concentrations in the still fasted subject; then, 75 g of lactulose were administered and the expired air was sampled every 15 min in the next 3 consecutive hours. In one subject with intolerance to lactose, the "breath test" has been performed by using 50 g of glucose and sampling expired air every 10 min for 2 h.

A positive test required an elevated breath hydrogen concentration higher than 10 ppm over basal values[19]; these concentrations are indicative of a bacterial colonization of the small intestine, where bacteria can metabolize non-absorbable sugars thus producing increased H_2 amounts which are eliminated through respiration[20].

The patients with a positive "breath test", were diagnosed as having SIBO and treated with rifaximin polimorph A (Normix®, Alfa Wassermann) at the daily dose of 1200 mg/die for 7 consecutive days. One week after the end of the treatment, the "breath test" was repeated, and the patients who still showed a positive test, received a further treatment with ciprofloxacin 500 mg/die for additional 7 d. At the end of the second cycle of antibiotic treatment the "breath test" was repeated for the third time.

The demographic characteristics of the patients were described as means and standard deviations (min-max ranges), or frequencies when appropriate. The frequencies of symptoms observed in patients with diagnosis of SIBO and IBS were compared using the χ^2 test, and the frequency of positive "breath test" was analyzed by means of the Fisher exact test.

RESULTS

A summary flow-chart of the employed methodology and the results achieved in our study is shown in Figure 1.

A total of 73 patients with IBS were selected (28 males and 45 females). They were aged between 17 and 87 (mean ± SD, 41.2 ± 15.8 years), and their weight and height were 66.8 ± 12.6 kg and 167.1 ± 9.3 cm,

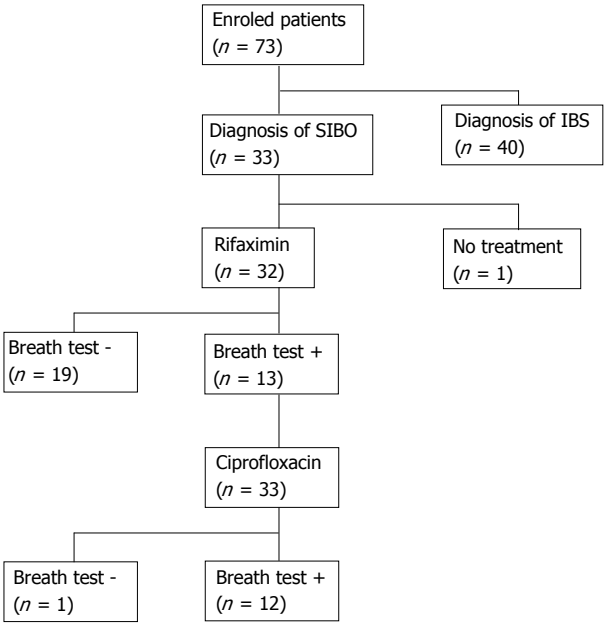

Figure 1 Diagram and synthesis of activities and results in this study.

Table 1 Results of "breath test" with lactulose or glucose in 73 patients with an initial symptoms-based diagnosis of IBS

Definitive diagnosis, *n* (%)		Breath test	Lactulose	Glucose
SIBO	33 (45.2%)	Positive	32	1
IBS	40 (54.8%)	Negative	40	0

The data are reported as frequency and percentage. SIBO: Small intestine bac-terial overgrowth; IBS: Inflammatory bowel disease.

respectively. More than 60% of males and 50% of females were younger than 40, and 10% of both males and females were older than 60 years.

The symptoms more frequently observed were abdominal bloating (83.6%), lower abdominal pain (76.7%), flatulence (65.8%), tenesmus (63.0%) and pain to palpation (50.7%), followed with lower frequencies by chronic diarrhoea, upper abdomen pain, nausea, steatorrhea, reduced body weight and stipsis. It is interesting to note that the most frequently observed symptom ("abdominal bloating") is also the most characteristic symptom of SIBO.

When the patients underwent the "breath test" with lactulose (except one patient with intolerance to lactose who received a "breath test" with glucose), 33 (45.2%) had a positive test, revealing the presence of a clinically relevant bacterial contamination of the small intestine (Table 1).

The symptoms in the patients with a confirmed diagnosis of IBS and those with a diagnosis of SIBO (positive "breath test") are shown in Table 2. The symptomatology was almost superimposable in the two groups, although some symptoms, such as reduced body weight, nausea, pain to palpation, and chronic diarrhoea, were slightly less frequent in subjects with SIBO, while other symptoms, such as tenesmus, were slightly more frequent in the patients with IBS. On the whole, the analysis of the clinical symptoms confirmed that a "breath test" is needed for a differential diagnosis between SIBO and IBS.

Table 2 Frequency of symptoms in 40 and 33 patients with a definitive diagnosis of IBS and SIBO respectively

	IBS (*n* = 40) *n* (%)	SIBO (*n* = 33) *n* (%)	*P*
Chronic diarrhoea	16 (40.0)	17 (51.5)	NS
Upper abdominal pain	17 (42.5)	14 (42.4)	NS
Lower abdominal pain	30 (75.0)	26 (78.8)	NS
Tenesmus	28 (70.0)	18 (54.5)	NS
Pain to palpation	18 (45.0)	19 (57.6)	NS
Abdominal bloating	32 (80.0)	29 (87.9)	NS
Flatulence	24 (60.0)	24 (72.7)	NS
Reduced body weight	7 (17.5)	9 (27.3)	NS
Nausea	9 (22.5)	15 (45.4)	NS
Steatorrhea	3 (7.5)	1	NS
Megaloblastic anemia	1	-	NS
Stipsis	8 (20.0)	9 (27.3)	NS
Fever	1	2 (6.1)	NS
Other (not specified)	1	0	NS

The data are reported as frequency and percentage.

Table 3 Results of "breath test" and symptom score in 32 patients with defini-tive diagnosis of SIBO treated with rifaximin 1200 mg/d for 7 d

	Before treatment	After treatment with rifaximin	
Breath test	Positive	Positive	Negative
	32 (100.0%)	13 (40.6%)	19 (59.4%)
Global symptom score	3.48 ± 0.82	3.24 ± 0.80	0.91 ± 0.06
		NS	*P* = 0.004
		After a further antibiotic treatment with ciprofloxacin	
Breath test		Positive	Negative
		12	1
Global symptom score		3.32 ± 0.95	1.00

The test was repeated one week later and, in the subjects with a still positive "breath test", a further treatment with ciprofloxacin was done (the data are re-ported as frequencies or mean ± standard deviation as appropriate).

Except one patient who refused further treatment, all the patients showing a positive "breath test" were treated with rifaximin 1200 mg/d for seven days. Among them, 19 (59.4%) patients showed the disappearance of the hydrogen peaks in expired air at the "breath test" one week after the treatment. In these patients, the symptom score was significantly reduced from 3.48 ± 0.82 (basal) to 0.91 ± 0.06 after treatment with rifaximin ($P = 0.004$), thus confirming the relationship between SIBO and many of the symptoms claimed by patients (Table 3).

On the contrary, the remaining 13 subjects still showed a positive "breath test" in spite of a treatment with rifaximin, and reported a symptom score (3.24 ± 0.80) that was almost unchanged compared with the basal values. In these patients, a further antibiotic treatment was given with ciprofloxacin 500 mg/d for 7 additional days. At the end of the treatment, only one patient showed a negative "breath test", while in the remaining 12 patients the "breath test" was still positive and the symptom score remained unchanged (Table 3).

No adverse effect or adverse drug reaction was observed in our study during the test and/or the medicinal treatment.

DISCUSSION

The GI tract is colonized by bacteria immediately after birth[21]; *Escherichia coli*, *Streptococci* and *Clostridi* are the first bacteria harboured by the colon, followed by anaerobic *Enterococci*, *Lattobacilli* and *Bacteroidi*[22]. All these bacteria are able to bind the GI mucosa by means of receptors, such as adhesin and lectin, which are expressed either on the host mucosa or other bacteria[23,24], and to resist to the antibacterial activity of many substances that are present in the GI environment, as well as to the gastric acid and GI motility[25].

Many factors affect the type and distribution of the bacteria along the GI tract, starting from the type of delivery[26] and nursing[27] in the first days of life, up to the food habits during adulthood. Normally, bacteria are scarcely present in the acid environment of the stomach while they reach the highest concentrations in the large intestine[28]. Moreover, the pattern of bacterial colonization is different among the different segments of the GI tract, the most prevalent being bacteria represented by aerobes and gram-positive in the duodenum and proximal ileum[28], gram-negative in the distal ileum, and anaerobes (*Bacteroides*, *Bifidobacteri*, *Eubacteri* and *Clostridii*) in the colon[28-30].

The role played by the bacterial flora in the normal physiology of the GI tract is known from animal studies performed many years ago[31-34]. It is quite clear nowadays that the bacterial flora affects the GI motility by means of three different mechanisms: (a) the release of substances produced or metabolized by bacteria; (b) the involvement of neuroendocrine factors; and (c) the involvement of the GI immunological tissue.

The growth of bacteria is controlled by several mechanisms, including gastric acid secretion, immunological factors, diet and bacterial competition[28,29,35]; however, the GI motility is probably the most important factor for control of bacterial growth. It is known that a large part of the bacteria may be eliminated by drugs increasing the GI motility. More importantly, a reduced GI motility leads to bacterial colonization of the small intestine, and many systemic and/or GI diseases characterized by a reduced GI motility have SIBO as one of their consequences[36,37].

In our study, slightly lower than 50% of patients with an initial diagnosis of IBS, are actually affected by a SIBO that is responsible for many of the symptoms earlier attributed to IBS. Our estimate is almost identical to the 46% observed by other clinicians in North Italy with similar methods of investigation[12].

Although other investigators have found a lower prevalence using the direct complex method of the GI bacterial count, it should be noted that the "breath test" with lactulose or glucose, with the determination of the hydrogen concentrations in the expired air, is considered an indirect but highly specific, method for diagnosis of SIBO[38,39]. On the other hand, the symptomatology in both SIBO and IBS is almost identical (Table 2) and, therefore, only a "breath test" can help in the differential diagnosis between the two disorders.

Clinicians should be encouraged to perform a "breath test" to promptly identify a SIBO, because the disorder has several systemic consequences ranging from malabsorption of lipids and liposoluble vitamins and loss of electrolytes[22,28], to a more severe translocation of bacteria (usually, gram-negative and aerobic bacteria, such as *Escherichia*, *Proteus*, *Enterobacter* and *Klebsiella*) from the GI tract to extraintestinal tissues[40], especially in the presence of a pathologically reduced epithelial barrier and immunological defences[41,42]. All these factors may lead to sepsis and multiorgan failure[42-46].

The treatment of SIBO must firstly focus on the correction of wrong food and dietary habits that usually underlie the disorder (e.g. excessive use of fast-food), and then to the reduction of bacterial colonization of the small intestine by means of antibiotics[47,48]. In this regard, the use of locally acting non-absorbable antibiotics would be particularly useful in reducing immediately the bacterial count waiting for the slow-acting beneficial effects of dietary measures.

In our study, the treatment with rifaximin for one week has determined the negativization of "breath test" in 59.4% of treated patients. Our data confirm other reports in the most recent literature: at the dose of 800 mg for four weeks rifaximin significantly reduced the symptoms in 20 patients with IBS and led to a negative "breath test" in almost half of patients[49]; in another series of 23 patients with SIBO and positive "breath test", a treatment with rifaximin 1200 mg/d for 7 d followed by a treatment with probiotics, led to a negative "breath test" in 19 (82.6%) cases and significantly reduced the peak in hydrogen concentrations in the expired air from 40.9 ± 20.4 to 4.78 ± 8.42 ppm[12]. More evidence on the efficacy of rifaximin has been reported in patients with SIBO and acute diverticulitis of the colon[50], and patients with SIBO and celiac disease[51].

It should be noted that further treatment with ciprofloxacin - an antibiotic widely used in the treatment of IBS[52,53] has not given significantly better results than rifaximin in our experience. Valuable alternatives to rifaximin that have been proven to be effective in the treatment of SIBO are represented by norfloxacin and amoxicillin-clavulanic acid[54], gentamycin[55], trimethoprim/sulfamerazin and polymyxin[56], and chlortetracycline[57].

In conclusion, (1) about half of the subjects with a symptomatic diagnosis of IBS have SIBO as a main cause of their claimed symptoms, which have been initially imputed to IBS; (2) only a "breath test" either with lactulose or with glucose in subjects with intolerance to lactose, can provide a differential diagnosis between IBS and SIBO with identical symptoms; (3) the use of non-absorbable antibiotics is useful in reducing the degree of GI bacterial contamination and related symptomatology, although the correction of wrong dietary habit remains the milestone in the management of SIBO if we want to maintain the results achieved with antibiotic treatment for quite some time.

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COMMENTS

Background

The role played by the bacterial flora in the normal physiology of gastrointestinal

(GI) tract is known, and it is quite clear nowadays that the bacterial flora affects the GI motility by means of three different mechanisms: (a) the release of substances produced or metabolized by bacteria; (b) the involvement of neuroendocrine factors; and (c) the involvement of the GI immunological tissues. Recent studies have provided increasing support for the concept that disturbances in gut flora occur in patients with irritable bowel syndrome (IBS) and such abnormalities may contribute to IBS-type symptoms.

Research frontiers

The article provides evidences that in about 50% of patients with a symptom-based diagnosis of IBS, the symptoms are provoked by a small intestine bacterial overgrowth (SIBO).

Innovations and breakthroughs

Clinicians should be encouraged to perform a "breath test" to promptly identify a SIBO, because the disorder has several systemic consequences of malabsorption of lipids and liposoluble vitamins, and loss of electrolytes.

Applications

The treatment of SIBO must firstly focus on the correction of wrong food and dietary habits that usually underlie the disorder (e.g. excessive use of fast-food), and then to the reduction of bacterial colonization of small intestine by means of antibiotics. In this regard, the use of locally acting non-absorbable antibiotics would be particularly useful in reducing immediately the bacterial count waiting for the slow-acting beneficial effects of dietary measures.

Terminology

A standard microbiological definition of SIBO: an increased bacterial count in the small intestine $\geqslant 10^5$ colonic bacteria/mL). A positive "breath test": an elevated breath hydrogen concentration within 90 min, two distinct peaks, and an increase higher than 20 ppm over basal values; these concentrations are indicative of a bacterial colonization of the small intestine, where bacteria can metabolize non-absorbable sugars, thus increasing the H_2 amounts which are eliminated through respiration.

Peer review

This report documents the incidence of small bowel overgrowth in patients with irritable bowel syndrome and how their symptoms respond to appropriate antibiotic therapy and whether or not the overgrowth (documented by serial hydrogen breath tests) is eradicated.

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RAPID COMMUNICATION

Predictive factors of tumor response to trans-catheter treatment in cirrhotic patients with hepatocellular carcinoma: A multivariate analysis of pre-treatment findings

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Abstract

AIM: To elucidate the pre-treatment clinical and imaging findings affecting the tumor response to the transcatheter treatment of unresectable hepatocellular carcinoma (HCC).

METHODS: Two hundred cirrhotic patients with HCC received a total of 425 transcatheter treatments. The tumor response was evaluated by helical CT and a massive necrosis (MN) was defined as a necrosis > 90%. Twenty-five clinical and imaging variables were analyzed: uninodular/multinodular HCC, unilobar/bilobar, tumor capsula, hypervascular lesion, portal vein thrombosis, portal hypertension, ascites, platelets count, aspartate transaminases/alanine transaminases (AST/ALT), alfa-fetoprotein (AFP) > 100, AFP > 400, serum creatinine, virus hepatitis C (VHC) cirrhosis, performance status, age, Okuda stage, Child-Pugg stage, sex, CLIP (Cancer of the Liver Italian Program) score, serum bilirubin, constitutional syndrome, serum albumine, prothrombin activity, BCLC (Barcelona Clinic Liver Cancer) stage. Prognostic factors of response were subjected to univariate analysis and thereafter, when significant, to the multivariate analyses.

RESULTS: On imaging analysis, complete response was obtained in 60 (30%) patients, necrosis > 90% in 38 (19%) patients, necrosis > 50% in 44 (22%) patients, and necrosis < 50% in 58 (29%) patients. Ninety-eight (49%) of the 200 patients were considered to have a MN. In univariate analysis, significant variables (P < 0.01) were: uninodular tumor, unilobar, tumor size 2-6 cm, CLIP score < 2, absence of constitutional syndrome, and BCLC stage < 2. In a multivariate analysis, the variables reaching statistical significance were: presence of tumor capsule (P < 0.0001), tumor size 2-6 cm (P < 0.03), CLIP score < 2 (P < 0.006), and absence of constitutional syndrome (P < 0.03). Kaplan-Mayer cumulative survival at 12 mo was 80% at 24 mo was 56%. MN was associated with a longer survival (P < 0.0001).

CONCLUSION: MN after transcatheter treatment is more common in the presence of tumor capsule, maximum diameter of the main lesion between 2 and 6 cm, CLIP score < 2 and absence of constitutional syndrome. The ability to predict which patients will respond to transcatheter treatment may be useful in the clinical decision-making process, and in stratifying the randomization of patients in clinical trials.

Key words: Hepatocellular carcinoma; Trans-catheter embolization/chemoembolization; Tumor response

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INTRODUCTION

Trans-catheter treatment is extensively used to treat hepatocellular carcinoma (HCC) not suitable for surgical resection or percutaneous ablation therapies. Transarterial chemoembolization (TACE), transarterial oily chemoembolization (TOCE) and transarterial embolization

(TAE) have been adopted[1-8]. Prognosis of patients with HCC complicated with cirrhosis mainly depends on the tumor growth, progression of the underlying liver disease, and effectiveness of anti-tumor treatment. Recent meta-analysis showed that the overall survival of patients with well preserved liver function was improved after intra-arterial treatment[9,10]. The primary goal of the trans-catheter treatment is to achieve a massive necrosis, to reduce tumor size, and prevent its dissemination and portal vein invasion. Unfortunately, the tumor response to trans-catheter treatment is heterogeneous with a wide range of necrosis that cannot be accurately predicted. The lack of well-defined prognostic indicators inspired us to perform the present study to analyze the tumor response and the pre-treatment imaging and clinical prognostic factors predictive of response in patients with HCC and compensated cirrhosis who were treated by trans-catheter treatment.

Table 1 Patient and HCC characteristics at admission

Age (mean ± SD, range)	63 ± 8.62 (35-81)
Sex distribution (male/female)	138/62
Liver cirrhosis etiology:	
Hepatitis C	158
Hepatitis B	29
Others	13
Child Pugh score A/B/C	136/59/5
Clip score (0/1/2/3/4/5)	53/79/52/11/4/1
BCLC stage (1/2/3/4)	61/115/14/0
Constitutional syndrome (yes/no)	22/178
Performance status (0/1/2/3)	177/14/8/1
HCC uninodular/multinodular	96/104
HCC unilobar/bilobar	148/52
Tumor capsula: yes/no	83/114
Portal vein thrombosis: absent/partial/complete	185/15/0
Alfa-fetoprotein: < 20/20-100/> 100 ng/mL	83/48/69

MATERIALS AND METHODS

This is a retrospective cohort study based on the analysis of 200 consecutive cirrhotic patients with single or multifocal HCC, treated with intra-arterial therapy and evaluated with follow-up imaging at a single transplant centre. Patients who had at least one image examination (16-slides helical CT scan or triphasic contrast-enhanced MRI) before and after treatment were included into the study. Diagnosis of HCC was based on radiological findings, alfa-fetoprotein level and biopsy according to the Barcelona criteria[11]. Bone metastases were ruled out by bone scintigraphy; lung and abdominal metastases were ruled out by CT scan. Liver function impairment was estimated with routine biochemical parameters reflecting liver function. The cardiac risk was evaluated by EKG and left ventricular ejection fraction (LVEF), as measured by multi-gated angiography scan (MUGA scan) or echocardiography. HCC and patient's characteristics at admission are reported in Table 1. Informed consent was not specifically required for the study, although written informed consent was obtained for each diagnostic and interventional radiology procedure.

All CT scan studies were performed with a 16-slice multidetector CT (Light speed, General Electric Medical Systems, USA) before and 4-6 wk after the intra-arterial treatment. Quadruple-phases protocol was used (unenhanced phase, arterial phase, portal venous phase and late phase).

All CT images were evaluated by at least 2 radiologists. Pre-treatment studies were analyzed without knowledge of the final outcomes of the patients. A consensus of the readers was reached in all cases. The following CT features were evaluated: number of lesions (single, multiple), size of the main lesion (maximum diameter), hepatic distribution (unilobar or bilobar), tumor extension ($\leqslant$ 3 lesions each $\leqslant$ 3 cm or > 3 lesions or one > 3 cm), vascularity of the lesions (hypervascular, hypovascular), tumor capsule, portal vein invasion (lobar, segmental, or subsegmental), signs of portal hypertension (spontaneous splenorenal shunt, patency of umbelical vein, patency of coronary vein), ascites, and necrosis. In post-treatment studies, the presence of arterial enhancement at CT imaging was considered as viable tumor. In patients who underwent TOCE, the complete necrosis was considered only if the lesion had homogeneous Lipiodol uptake without contrast enhancement in arterial phase. In case without clear results, MRI with gadobenate dimeglumine (Gd-BOPTA), using a 1.5T MR scan (General Electric Medical Systems, USA), was performed. The efficacy of intra-arterial treatment was defined according to the amount of tumor necrosis valuable on CT and/or MR follow-up imaging and the WHO recommendations[12]. The presence of non-enhanced tumor areas was defined as tissue necrosis and expressed as percent of the total tumor volume. Tumor necrosis was considered complete when no foci of enhancement were seen within the tumor or at its periphery. In patients with multiple lesions, the necrosis was computed as average of tumor necrosis in each lesion. The response to treatment was classified as: complete response, necrosis > 90%, necrosis > 50%, and necrosis < 50%. In this study, a tumor necrosis > 90% over the treatment interval was classified as massive necrosis (MN).

Intra-arterial treatment was performed in patients with multifocal HCC or single unresectable HCC and contraindications to radiofrequency thermal ablation (RFA).

In our centre, contraindications to RFA are: size of the lesion greater than 4 cm, lesion near to vital organs such as gallbladder, stomach, and colon, lesion adjacent to a big portal or hepatic vein branches at risk of bleeding, subphrenic lesion not easily accessible for RFA and lesion in subcapsular position at high risk of tumor seeding[13].

Exclusion criteria for intra-arterial treatment were HCC volume > 50% of total hepatic volume, complete thrombosis of main portal vein, AST/ALT > 300 U/L, serum bilirubin > 3 mg/dL, serum creatinine > 1.8 mg/dL, white blood cell (WBC) < $2.5 \times 10^3/\mu L$, platelets < $35 \times 10^3/\mu L$, severe ascites and performance status > 3.

TACE was performed using Epirubicine at a dose of 50 mg/m^2 of body surface; the dose was reduced to 50% if serum bilirubin level was > 1.2 mg/dL and < 2 mg/dL and/or white blood cell count (WBC) was $34 \times 10^3/\mu L$;

a dose reduction to 25% was administered if bilirubin was > 2 mg/dL and/or WBC 2.53 × 10^3/μL. Epirubicine was prepared in sterile drip and infused over 30 min using a peristaltic pump. Afterwards, the embolization was performed using Gelfoam (Pfitzer, Belgium) till a stagnation flow was visualized at the fluoroscopy. In patients with acceptable liver function and superselective catherization of the hepatic artery, 2-10 mL of Lipiodol (Lipiodol Ultrafluid, Guebert, Italy) was infused before the gelfoam embolization (TOCE)[14]. No chemotherapeutic agent (TAE) was used in the presence of WBC less than 2.5 × 10^3/μL, previous episodes of neutropenia (< 500/mL), positive HBsAg and HBV DNA[15] and ejection fraction ⩽ 45%. These patients were treated with lipiodol and/or gelfoam. In patients with single HCC, superselective catheterization of the artery supplying the lesion was performed whenever possible, in the other cases the treatment was given in the branch of the right hepatic artery or in the branch of the left hepatic artery supplying the lesion. In patients with multifocal HCC, treatments were given in the right hepatic artery (RHA) or in the left hepatic artery (LHA). In case of multifocal, bilobar HCC, no treatment was given in RHA and LHA during the same session. After the treatment, the patients were carefully observed in a conventional hospitalization room, and analgesics were administered if necessary. Oral intake was reinitiated as soon as possible according to the tolerance of the patients. Usually the day after the procedure, after confirming the absence of clinical abnormalities, the patients were discharged and followed up in the outpatient clinic.

The intra-arterial treatment was repeated every 6-12 wk according to the tumor response based on the follow-up imaging and clinical assessment.

Statistical analysis

Twenty-five clinical and imaging variables were analyzed, including uninodular/multinodular HCC, unilobar/bilobar, tumor capsula, hypervascular lesion, portal vein thrombosis, portal hypertension, ascites, platelets count, AST/ALT, AFP > 100, AFP > 400, serum creatinine, VHC cirrhosis, performance status, age, Okuda stage, Child-Pugg stage, sex, CLIP score, serum bilirubin, constitutional syndrome, serum albumine, prothrombin activity, BCLC stage.

The relationship between these variables and the tumor response was first assessed by univariate analysis using the Chi-square test or Student's *t* test when indicated. For qualitative variables, patients were grouped according to the presence or absence of each variable. For quantitative variables, the cut-off level was determined evaluating the relationship between the sensitivity and the specificity of the MN at different cut-off points from a ROC curve. To identify independent predictors of MN occurrence, all the variables reaching statistical significance in the univariate analysis were subsequently included in a multivariate analysis using the step-wise logistic regression procedure. A *P* value of less than 0.05 was considered significant. Statistical analyses were performed with the SPSS software (SPSS Institute Inc., Cary, NC). Survival curves were modelled using the Kaplan-Meier method.

RESULTS

Patients received a total of 425 sessions of intra-arterial treatments. The mean number of treatment sessions was 2.1 ± 2.0 per patient, range 1-8. Interval of treatment was 76 ± 48 d (range 16-249 d). Type of treatments performed were: TACE 243 (57%), TOCE 126 (30%) and TAE 56 (13%). Technical success was achieved in all the treatments performed. No major life-threatening complications occurred.

On imaging analysis, complete response was obtained in 60 (30%) patients, necrosis > 90% in 38 (19%) patients, necrosis > 50% in 44 (22%) patients, and necrosis < 50% in 58 (29%) patients. In this analysis, 98 (49%) of the 200 patients were considered to have MN. At univariate analysis, significant variables ($P < 0.01$) were: uninodular tumor, unilobar, tumor size 2-6 cm, CLIP score < 2, absence of constitutional syndrome, and BCLC stage < 2. In a multivariate analysis, the variables reaching statistical significance were: presence of tumor capsule (β-coefficient 1.447, $P < 0.0001$), tumor size 2-6 cm (β-coefficient 0.838, $P < 0.03$), CLIP score < 2 (β-coefficient 1.074, $P < 0.006$), and absence of constitutional syndrome (β-coefficient 1.764, $P < 0.03$). Kaplan-Mayer cumulative survival was 80% at 12 mo and 56% at 24 mo. The survival of patients with and without MN was 95% and 75% at 12 mo, and 70% and 55% at 24 mo, respectively. Massive tumor necrosis was associated with a longer survival ($P < 0.0001$).

DISCUSSION

Transcatheter treatment is the most common choice for patients with surgically unresectable HCC and contraindications to percutaneous treatment such as PEI, and RF thermal ablation. The arterial embolization with or without chemotherapy induces tumor necrosis by occlusion of the feeding artery of the HCC, and its clinical efficacy has been documented[3,4,16-21]. The goal of TAE/TACE is to deliver a high dose of chemotherapeutic drug and/or embolizing agent in the HCC, causing tumor necrosis and tumor control, preserving as much normal liver parenchyma as possible. The imaging and clinical factors affecting the tumor response after transcatheter treatment remain to be elucidated. The aim of this study was to identify, by means of multivariate analyses, the pre-treatment variables of independent predictive value of MN in patients with cirrhosis managed with transcatheter therapy.

Our multivariate analysis showed that two tumor-related factors such as tumor capsule and maximum diameter of the main tumor are the only independent factors significantly affecting the tumor response to the transcatheter treatment. The presence of a well-recognizable tumor capsule at the pretreatment CT scan was the most important independent predictive factor of MN after transcatheter treatment. According to our results, previous pathological studies performed on liver resection following transarterial chemoembolization[22,23] demonstrated that encapsulated HCCs are more responsive to the transcatheter treatment than non-encapsulated tumors. Interestingly, a significant correlation

between the thickness of the capsule and the effectiveness of tumor necrosis after chemoembolization has also been documented[24,25]. The reason for the relatively poor necrosis of tumors without capsule is not clear. Wasaka *et al* showed that barium sulfate infused into the portal vein entered into the non-encapsulated tumors but not into encapsulated tumors, suggesting that there is a difference in the type of blood supply that may greatly influence the tumor necrosis after embolization of the feeding hepatic artery[23]. The type of histologic growth pattern at the tumor-nontumor boundary may also affect the necrosis occurrence after transcatheter treatment. HCC is usually the expanding type in encapsulated tumors and replacing type in uncapsulated tumors and chemoembolization appears to be most efficacious for HCC with growth expanding pattern. The poor results expected in the replacing type of HCC may be related to the fact that in the case of tumor, cells replace hepatocytes, the blood spaces communicate with sinusoids supplied by portal blood flow[26,27]. A limitation of the pathological studies is that they were performed weeks or months after the chemoembolization, and the granulated tissue between HCC and normal liver parenchyma, secondary to the treament response, could simulate a true capsule. In this case, the psuedocapsule could be an indicator and not a predictor of tumor response[28]. Our results showed that the naturally formed capsule of encapsulated HCC resulting from condensed reticulin fibers produced by atrophic changes of noncancerous liver tissue and detected at the pretreatment CT scan, is the most important independent predictive factor to obtain MN after transcatheter treatment. However, the capsule seems to form when the tumors are small, and larger tumors are commonly uncapsulated. Finally, in absence of a very well-defined capsule, one must keep in mind the possibility that a negative CT image may not completely exclude the presence of capsule and may give partial information about its integrity. It is known the CT scan is limited in detecting capsular invasion of tumor cells, small satellite nodules, and tumor thrombi of the peripheral portal vein branches that are rather poorly responsive to the transcatheter treatment probably because they receive blood supply from the portal vein[23-25,28].

We obtained MN after transcatheter treatment more frequently in nodules with a maximum diameter of 2-6 cm. Similarly, a previous histologic study evaluating the effect of chemoembolization showed that it is effective for encapsulated HCC measuring between 2.3 and 5.5 cm in diameter[25]. TAE is usually less effective for HCC smaller than 2 cm in diameter[27,29]. The reason for the failure of a complete necrosis of the small tumors is not clear. No significant difference of the predominant lesion vascular pattern was found in this study between patients with or without MN. The apparent discrepancy from previously reported studies[30] might be due to the fact that in our series the hypovascular pattern was very uncommon.

A CLIP score < 2 and the absence of constitutional syndrome are strong independent clinical predictors of MN after transcatheter treatment because these kind of patients have a preserved liver function and multiple treatments might yield a good response.

In conclusion, pretreatment helical CT and clinical findings provide accurate prediction of tumor response in patients with HCC and compensated cirrhosis. The MN (> 90%) is more common in the presence of capsule, maximum diameter of 2-6 cm and in well compensated patients with CLIP score of 1 and without constitutional syndrome. The ability to predict which patients will respond to transcatheter treatment may be useful in the clinical decision-making process, and in stratifying the randomization of patients in the therapeutic clinical trials. The prognostic implications and the survival of patients with massive tumor necrosis require further studies.

COMMENTS

Background

Trans-catheter treatment is extensively used to treat hepatocellular carcinoma in cirrhotic patients to achieve a massive necrosis, to reduce tumor size, and prevent its dissemination and portal vein invasion. Tumor response after trans-catheter treatment cannot be accurately predicted.

Research frontiers

The aim of this study is to analyze tumor response and the pre-treatment imaging and clinical prognostic factors predictive of response in patients with HCC and compensated cirrhosis who were managed by trans-catheter treatment.

Innovations and breakthroughs

This study clearly showed that massive necrosis is more common in presence of capsule, a maximum diameter of 2-6 cm and in well compensated patients with CLIP score of 1 and without constitutional syndrome.

Applications

The ability to predict which patients will response to transcatheter treatment may be useful in the clinical decision-making process and in stratifying the randomization of patients in clinical trials.

Terminology

CLIP (Cancer of the Liver Italian Program) prognostic score includes Child-Pugh stage, tumor morphology and extension, serum alfa-fetoprotein (AFP) levels, and portal vein thrombosis. Costitutional syndrome is characterized by weight loss, malaise and anorexia.

Peer review

This is an interesting study, aiming at identifying the factors predicting tumor response to the trans-catheter treatment in cirrhotic patients with HCC. The authors concluded that presence of tumor capsule, a maximum diameter between 2 and 6 cm, CLIP score < 2 and absence of constitutional syndrome were independent predictors of massive necrosis. The study is well conducted. The results are clearly reported and support the main conclusions.

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RAPID COMMUNICATION

Evaluation of leukocyte esterase and nitrite strip tests to detect spontaneous bacterial peritonitis in cirrhotic patients

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Abstract

AIM: To investigate the diagnostic efficacy of leukocyte esterase and nitrite reagent strips for bedside diagnosis of spontaneous bacterial peritonitis (SBP).

METHODS: A total of 63 consecutive patients with cirrhotic ascites (38 male, 25 female) tested between April 2005 and July 2006 were included in the study. Bedside reagent strip testing was performed on ascitic fluid and the results compared to manual cell counting and ascitic fluid culture. SBP was defined as having a polymorphonuclear ascites count of $\geqslant 250/mm^3$.

RESULTS: Fifteen samples showed SBP. The sensitivity, specificity, positive and negative predictive values of the leukocyte esterase reagent strips were; 93%, 100%, 100%, and 98%, respectively. The sensitivity, specificity, positive and negative predictive value of the nitrite reagent strips were 13%, 93%, 40%, and 77%, respectively. The combination of leukocyte esterase and nitrite reagents strips did not yield statistically significant effects on diagnostic accuracy.

CONCLUSION: Leukocyte esterase reagent strips may provide a rapid, bedside diagnostic test for SBP.

Key words: Spontaneous bacterial peritonitis; Urinary reagent strip; Leukocyte esterase; Nitrite

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INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a serious and potentially life-threatening complication that can occur in cirrhotic patients with ascites[1,2]. The prevalence of SBP in hospitalized patients with liver cirrhosis and ascites is high, ranging between 10% and 30%. Notably, in-hospital mortality rates range between 30% and 50%[3,4].

SBP requires timely diagnosis that is usually based on cytobacteriological examination of ascitic fluid. A polymorphonuclear leucocyte (PMN) cell count in ascitic fluid > $250/mm^3$, irrespective of the ascitic fluid culture, is currently considered the standard criterion for diagnosis of SBP[1,2]. For this testing, it is of paramount importance that the results of the cytobacteriological examination of the ascitic fluid should be promptly delivered so that appropriate antibiotherapy can be initiated[4,5]. However, because of the organization of facilities in many hospitals, a bacteriological laboratory is not always available for all departments admitting cirrhotic patients with ascites. It follows that alternative methods for rapid diagnosis of SBP are an urgent requirement[6,7].

Use of reagent strip testing for leucocyte esterase has been proposed to reduce the time from paracentesis to a presumptive diagnosis of SBP from a few hours to a few seconds[7,8]. Intriguingly, reagent strips detecting leukocyte esterase activity in biological fluids have been validated for the diagnosis of urinary tract infections[9,10], peritonitis in patients on continuous ambulatory peritoneal dialysis[11], pleural infections[12] and meningitis[13]. The observation that nitrate levels in ascites fluid are raised in patients with SBP is also of interest[14]. Thus far, however, no study has specifically looked at the potential usefulness of nitrite reagent test strips as a diagnostic tool in this patient group.

Therefore, in this study we sought to determine the sensitivity, specificity, and positive (PPV) and negative predictive values (NPV) of nitrite and leukocyte esterase reagent test strips for the diagnosis of SBP in cirrhotic patients with ascites.

MATERIALS AND METHODS

Study sample

This hospital-based study was conducted at the Department of Gastroenterology, Uludag University Medical School, Bursa, Turkey from April 2005 to July 2006. Sixty-three consecutive unselected cirrhotic patients (38 men, 25 women, mean age 59.0 ± 11.6 years) with ascites were included in our study. The general characteristics of the study participants are shown in Table 1. Diagnosis of cirrhosis was established by histological criteria and/or by clinical, laboratory, endoscopic and/or ultrasonographic findings. Only subjects with serum-ascites albumin gradient (SAAG) > 1.1 g/dL were enrolled. Patients with SAAG < 1.1 g/dL were excluded. Subjects with portal hypertension (SAAG > 1.1 g/dL) due to malignancy or tuberculosis were also excluded. The local ethics committee of the Uludag University Medical School approved the study and informed consent was obtained from each participant.

Paracentesis

Paracentesis was performed at admission and during the hospital stay for treatment of ascites or with clinical signs of SBP. Immediately after the paracentesis, ascitic fluid was tested using nitrite and leukocyte esterase reagent strips (Aution Sticks 10 EA; Arkray, Kyoto, Japan). Fresh ascitic fluid was collected in a clean dry container and the strip was immediately immersed in ascitic fluid. The strips were read by two independent research physicians who were unaware of the clinical status of the participants. The strips had a colorimetric 5-grade scale (0-4). A correlation between PMN and a 5-grade scale was suggested by the manufacturer, as follows: grade 0, 0 PMN/mm^3; grade 1, 25 PMN/mm^3; grade 2, 75 PMN/mm^3; grade 3, 250 PMN/mm^3; and grade 4, 500 PMN/mm^3.

Laboratory analysis of the ascitic fluid was performed without delay in all patients. For testing a standard sterile technique was used and included total and differential cell counts, Gram stain and total protein levels. Cultures of ascitic fluid were performed at bedside for all patients using blood culture bottles, including both aerobic and anaerobic media. A minimum of 10 mL of ascitic fluid was inoculated into each bottle.

Diagnostic criteria

The diagnosis of SBP was based on a PMN cell count > 250/mm^3 in ascitic fluid, irrespective of a positive ascitic fluid culture and clinical signs of SBP, and an absence of intra-abdominal sources of infection, inflammation or tuberculosis.

Statistical analysis

Sensitivity was defined as the proportion of patients with a positive reagent strip divided by the number of those with SBP diagnosed by criteria previously defined. Specificity was defined as the proportion of patients with a negative reagent strip divided by the total number of patients without SBP. PPV was defined as the proportion of patients with a true-positive reagent strip divided by the total number of patients with a positive reagent strip. NPV was defined as the proportion of true-negative reagent strips divided by the total number of patients with a negative reagent strip. Data are presented as means ± SD for quantitative variables and as frequencies for qualitative variables. The exact 95% confidence interval for each statistic was calculated from the binominal distribution. SPSS statistical software version 14.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis.

Table 1 General characteristics of the study participants

Characteristics	Study patients (n = 63)
Male gender, n (%)	38 (60.3)
Age (yr)	59 ± 11.6
Child–Pugh classification, n (%)	
B	25 (39.7)
C	38 (60.3)
Etiology of cirrhosis, n (%)	
Chronic B hepatitis	19 (30.1)
Chronic C hepatitis	11 (17.5)
Alcohol abuse	7 (11.1)
Other causes	26 (41.3)

RESULTS

According to the Child-Pugh classification, of the 63 patients there were 25 (39.7%) with Child class B and 38 (60.3%) with Child class C. The etiology of cirrhosis was chronic B hepatitis in 19 (30.1%), chronic C hepatitis in 11 (17.5%), alcohol abuse in 7 (11.1%), and other causes (e.g., autoimmune, primary biliary cirrhosis, cryptogenic) in 26 (41.3%).

SBP occurred in 15 patients (23.8%). Culture of ascitic fluid was positive in 6 cases (40.0%) and negative in 9 (60.0%). Two patients (3.0%) were found to have infected ascites fluid, whereas the remaining 46 (73.0%) had no sign of ascites infection. The bacteria isolated from cultures of ascitic fluid were: *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus warneri*, and *Streptococcus bovis*. PMN counts in ascitic fluid in patients with or without SBP were (mean ± SD/mm^3) 4064 ± 4486 and 69 ± 63, respectively.

Among the 15 patients with SBP, the urine leukocyte esterase strip tested 1+ in the ascitic fluid of one patient and ⩾ 2+ in the remaining 14 individuals. The ascitic fluids of 2 patients with SBP tested positive by the nitrate strips. Detailed laboratory data and urine strip test results of the 15 SBP patients are shown in Table 2. The sensitivity, specificity, PPV and NPV of the leukocyte esterase reagent strips (at a ⩾ 2+ cutoff point) were as follows: 93%, 100%, 100%, and 98%, respectively. The sensitivity, specificity, PPV and NPV of the nitrite reagent strips were 13%, 93%, 40%, and 77%, respectively. The addition of nitrite strips to leukocyte esterase reagent strips did not yield statistically significant effects upon the diagnostic accuracy compared to the leukocyte esterase test alone. Thus the combination of both tests yielded a sensitivity, specificity, PPV and NPV of 93%, 100%, 100%, and 98%, respectively.

DISCUSSION

SBP continues to be an important source of morbidity and mortality in patients with cirrhosis[15]. Under such

circumstances, prompt diagnosis and treatment are crucial to ensure better clinical outcomes in this patient group. However, it has been shown that a positive bacterial culture can be obtained in just 40% of patients with SBP, and that test results may be delayed for several days[16]. Under such circumstances, reagent strips emerge as an attractive means for rapid diagnosis of this clinical entity in patients with cirrhosis and ascites. These tests have the ability to detect esterase activity of PMN cells[16]. Numerous independent studies have evaluated the diagnostic value of reagent strips in settings involving SBP[17-19]. Most have shown high sensitivity and specificity in keeping with our present results.

In a landmark study by Castellote *et al*[8], it has been shown that reagent strips are useful and inexpensive when used in the context of SBP. Specifically, when a cutoff point of 2 or more was selected, overall sensitivity and specificity were 96% and 98%, respectively[6]. More recently, Thévenot *et al*[18] have investigated the utility of two reagent strips, the Multistix test and the Combur 2 test, for the rapid diagnosis of SBP. Both tests showed a high sensitivity and specificity and permitted an accurate laboratory diagnosis of this life-threatening condition. Furthermore, a multicenter study by Sapey *et al*[19] has provided important evidence that two leukocyte esterase reagent strips (Nephur-Test and MultistixSG10) may be of clinical use in the bedside diagnosis of SBP. In light of these results, it has been suggested that in patients with cirrhosis and positive strip test results, antibiotic therapy should be started without delay[17]. Our study confirms and expands previous findings of the potential utility of the leukocyte esterase test as a simple and inexpensive means for testing in settings involving SBP[18-20]. Notably, the selection of a cutoff point $\geqslant 2$ yielded 100% specificity and 100% PPV for the diagnosis of SBP. By contrast, results of a urine strip test of 0 and 1+ yielded a 98% NPV, which excluded SBP in these individuals.

Another aim of our study was to determine whether nitrite reagent strip testing is a useful diagnostic tool in patients with SBP. Previous reports have indicated that nitrate concentration and nitric oxide levels may be raised in ascites from patients with cirrhosis and SBP[14,21]. Since nitric oxide is a diffusible, short-lived, and a reactive free radical gas that is difficult to measure *in vivo*[22,23], most studies have based their conclusions on measurement of nitrite and nitrate in biological fluids[24-26]. In line with this approach, in our study we used Aution Sticks 10EA for the nitrite test strips. This test is based on the Griess Reaction which measures the combined oxidation products for nitric oxide (nitrites and nitrates) after reduction with nitrate reductase[24]. Nonetheless, the sensitivity and PPV of the nitrite reagent strips were low, namely 13% and 40%, respectively. There are several reasons that might explain the low diagnostic performances of the nitrite reagents strips. Nitrite and nitrate levels in body fluids may vary according to a host of confounding factors including the effects of diet-derived nitrate, hepatic synthesis capacity, as well as the occurrence and prevalence of concomitant infections[27-29]. Specifically, it is posited that the limited utility of nitrite strips to screen for SBP in patients with cirrhosis may be due to the low bacterial concentration in ascites fluid[30]. Therefore, for precise bedside diagnosis of SBP in patients with cirrhotic ascites, the highly specific leukocyte esterase strip test remains the method of choice.

Table 2 Results of urine strip test, culture, and ascitic fluid PMN count in 15 patients with SBP

Case number	Reagent strip test		Culture	PMN count (/mm^3)
	Leukocyte esterase	Nitrite		
1	1+	0	Negative	490
2	4+	0	*Escherichia coli*	3860
3	2+	0	Negative	510
4	3+	0	Negative	802
5	3+	0	Negative	311
6	4+	0	*Escherichia coli*	7130
7	4+	1+	*Klebsiella pneumoniae*	16700
8	2+	0	*Staphylococcus warneri*	280
9	4+	0	*Streptococus bovis*	3810
10	3+	0	Negative	9100
11	3+	0	Negative	6120
12	3+	0	Negative	2460
13	4+	0	Negative	5330
14	3+	0	Negative	3790
15	2+	2+	*Klebsiella pneumoniae* plus *Escherichia coli*	270

In conclusion, leukocyte esterase reagent test strips were found to facilitate very rapid identification of patients with SBP and cirrhotic ascites. Specifically, this test can be performed efficiently in order to speed up the bedside diagnostics of this clinical entity. It fits easily into the work flow of a routine gastroenterology department and can be conducted in facilities that do not have the facilities to carry out biochemical and bacteriological tests for diagnosis of SBP.

COMMENTS

Background

SBP is a serious and potentially life-threatening complication that can occur in patients with cirrhosis and ascites. Timely diagnosis is of paramount clinical importance.

Research frontiers

Use of reagent strip testing for leucocyte esterase has been proposed to reduce the time from paracentesis to a presumptive diagnosis of SBP from a few hours to a few seconds.

Innovations and breakthroughs

The leukocyte esterase reagent test strips were found to enable the very rapid identification of patients with SBP in clinical settings involving patients with cirrhotic ascites. This test can be performed efficiently to speed up bedside diagnostics of this clinical entity.

Applications

Leukocyte esterase reagent strip testing fits easily into the work flow of a routine gastroenterology department and can be conducted in facilities that do not or cannot carry out biochemical and bacteriological tests for diagnosis of SBP.

Terminology

Reagent-strip leukocyte esterase: the presence of leukocyte esterase is indirect evidence of the presence of white blood cells in biological fluids.

Peer review

The manuscript "Evaluation of leukocyte esterase and nitrite strip tests to detect spontaneous ascites infection in cirrhotic patiens" is very interesting since it evaluates a test for prompt diagnosis of a disastrous complication of cirrhosis with poor prognosis. In general terms, the study was well performed and the analysis was good.

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RAPID COMMUNICATION

Alterations in the function of circulating mononuclear cells derived from patients with Crohn's disease treated with mastic

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Abstract

AIM: To assess the effects of mastic administration on cytokine production of circulating mononuclear cells of patients with active Crohn's disease (CD).

METHODS: The study was conducted in patients with established mildly to moderately active CD, attending the outpatient clinics of the hospital, and in healthy controls. Recruited to a 4 wk treatment with mastic caps (6 caps/d, 0.37 g/cap) were 10 patients and 8 controls, all of who successfully completed the protocol. Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), macrophage migration inhibitory factor (MIF) and intracellular antioxidant glutathione (GSH) were evaluated in peripheral blood mononuclear cells (PBMC) before and after treatment.

RESULTS: Treating CD patients with mastic resulted in the reduction of TNF-α secretion (2.1 ± 0.9 ng/mL *vs* 0.5 ± 0.4 ng/mL, $P = 0.028$). MIF release was significantly increased (1.2 ± 0.4 ng/mL *vs* 2.5 ± 0.7 ng/mL, $P = 0.026$) meaning that random migration and chemotaxis of monocytes/macrophages was inhibited. No significant changes were observed in IL-6, MCP-1 and GSH concentrations.

CONCLUSION: This study shows that mastic acts as an immunomodulator on PBMC, acting as a TNF-α inhibitor and a MIF stimulator. Although further double-blind, placebo-controlled studies in a large number of patients is required to clarify the role of this natural product, this finding provides strong evidence that mastic might be an important regulator of immunity in CD.

INTRODUCTION

Crohn's disease (CD) is a chronic relapsing inflammatory condition of unknown cause[1]. Although the exact pathogenesis of CD is poorly understood, infection, environmental factors, heredity and immunological defects have been proposed as causes[2]. In one or another scenario, a variety of cytokines, such as tumor necrosis factor-alpha (TNF-α), are secreted at the site of inflammation by intestinal lamina propria and attract and activate effector cells[3-5]. Apart from the intestinal mucosa, TNF-α concentration is also raised in serum in patients with active Crohn's disease, compared to normal controls[6,7] and inactive disease[8]. The mechanism through which localized inflammation in the gastrointestinal tract in active CD is associated with systemic manifestations remains unconfirmed, but can involve activated mononuclear cells that migrate *via* the peripheral blood (PBMC) to other tissues. PBMC are highly activated in active CD and secret higher quantities of proinflammatory mediators[9-11]. Altered production of cytokines by PBMC is noted in patients with CD when compared to healthy subjects[12].

As the name implies, monocyte chemotactic protein 1 (MCP-1) acts as a potent chemoattractant and activator of monocytes/macrophages[13], as well as of NK cells, T cells, eosinophils, and basophils[14-17]. During acute inflammatory processes the expression of MCP-1 is increased. Once activated, cells produce an assortment of immunoregulatory cytokines that influence the course of the ensuing immune response. Interleukin 6 (IL-6) expressed among others by T cells and monocytes/

macrophages, stimulates T- and B-cell proliferation and differentiation[18]. The production of IL-6 in these various cells may be regulated by TNF. TNF-α is an important mediator of inflammation, found in substantial amount in the mucosa and stools of subjects with CD. It is a proinflammatory cytokine produced by monocytes, macrophages, and T cells that can affect proliferation, differentiation, and function of every cell type. Therefore, TNF inhibitors, which may be useful in the treatment of CD, have recently been developed[19]. Among these, infliximab has been developed as a therapeutic agent for TNF-α -mediated diseases[20]. Macrophage migration inhibitory factor (MIF) is a cytokine with dissimilar functions; inhibition of macrophage migration[21] or proinflammatory activity[22-25].

Nowadays, various immunosuppressive therapies such as azathioprine, mercaptopurine, and methotrexate, are available. Nevertheless, the treatment of patients with CD still remains a clinical challenge. Furthermore, in view of the increased number of CD patients, there is a considerable scientific and commercial interest in the discovery of novel classes of therapeutic compounds. In particular, plants represent a good source of novel molecules. *Pistacia lentiscus* var. *Chia* (*Anacardiaceae*) is an evergreen shrub widely distributed in the Mediterranean region. Mastic, the resinous exudate, has been reported to possess antioxidant[26] and antibacterial[27] activity, to be effective against peptic ulcers[28], to be hepatoprotective in tetrachloride-intoxicated rats[29] and to suppress the extent of iron-induced lipid peroxidation in rat liver homogenates[30]. We have previously shown that mastic administration resulted in the improvement of the clinical course and in the regulation of inflammatory biomarkers in plasma obtained from mildly to moderate active CD patients[7]. The aim of the present work was to investigate the immunomodulatory effect of mastic treatment on cytokine secretion. Additionally, because inflammation results in oxidative stress and endogenous antioxidants, such as glutathione (GSH), do not counteract it with subsequent mucosal damage, intracellular GSH production from PBMC obtained from patients with mildly to moderately active CD was also measured.

MATERIALS AND METHODS

Setting and participants

Ten consecutive patients with active Crohn's disease and eight healthy controls were included[7]. In brief, clinical evidence of mild to moderate Crohn's disease exacerbation was defined by a score of CD Activity Index (CDAI) 150 < CDAI < 400. Mean CDAI at baseline was 222.9 ± 18.7 (SE), while mean C-reactive protein (CRP) concentration was 40.3 ± 13.1 (SE) mg/mL. Exclusion criteria were elemental diet, parenteral nutrition or antioxidant/mineral supplementation and treatment with immunomodulators (biologic agents-infliximab) and/or corticosteroids. Controls were healthy volunteers, with normal concentrations of CRP [2.4 ± 0.7 (SE) mg/L] and albumin [42.1 ± 1.2 (SE) g/L], without chronic inflammatory disorder, BMI value < 30 (25.8 ± 3.3), none anti-inflammatory drug treatment or antioxidant vitamin/mineral supplementation. Informed consent was obtained from each subject included in the study. The Ethical Committees of both Harokopio University and Saint Panteleimon General State Hospital approved the protocol. Table 1 shows some demographic characteristics of patients and controls.

Table 1 Demographic characteristics and medications of patients with Crohn's Disease and controls

Characteristic	Patients	Controls
Age (yr)		
Mean	36.9	31.5
Range	18-73	25-45
Sex		
Female	5	4
Male	5	4
Duration of disease (yr)	6.4 (± 3.9)	-
Concomitant medication		-
None	3	
Mesalazine	3	-
Metronidazole	2	
Azathioprine	2	-
Location of Crohn's disease		
Small bowel	4	-
Small and large bowel	6	-
Fistulizing disease	3	-

Intervention

The trial protocol was carried out as previously described[7]. In short, participants were subjected to a 4-wk supplementation with mastic caps (0.37 g/cap, 2 × 3 caps/d, 2.2 g in total). Dietary instructions were given as to maintain consumption of foods rich in anti-inflammatory and antioxidant ingredients low as initially assessed by a food frequency questionnaire and 24 h recall interview and to refrain from mastic and mastic products. Blood samples were obtained prior and after the trial.

Cell cultures

PBMC were obtained from CD patients and controls as previously described[31]. Viability of peripheral blood mononuclear cells (PBMC) was determined by Trypan blue exclusion test. PBMC were resuspended in complete medium consisting of RPMI 1640 medium supplemented with 10% fetal calf serum, 1% L-glutamine and 1% penicillin/streptomycin. PBMC were added to each well of a 24-well plate at a density of 2×10^6 cells/mL and cultures were incubated at 37℃ in a humidified atmosphere containing 5% CO_2 for 18 h. At the end of incubation, conditioned media were collected and stored at -20℃ until assayed, while cells were harvested for GSH measurement. All cultures were run in duplicate.

Cytokine assays

Plasma cytokines from patients with CD and controls were assessed by quantitative, sandwich, enzyme-linked, immunosorbent assays (ELISA) (R&D Systems Abingdon, UK) according to the manufacturer's instructions. Sensitivity of TNF-α ELISA was less than 1.6 pg/mL, of

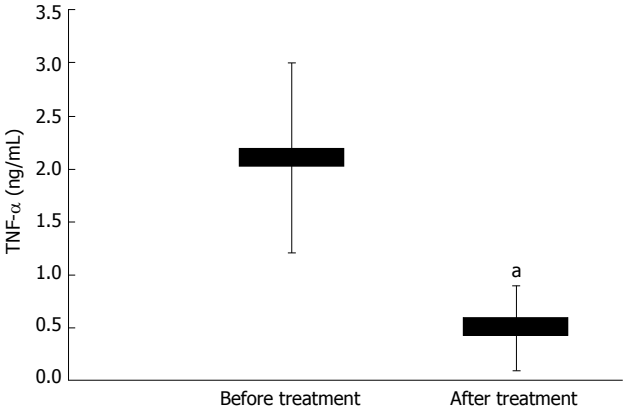

Figure 1 Secretion of tumor necrosis factor-alpha (TNF-α) was decreased in PBMC derived from patients with active Crohn's disease (n = 10) after 4-wk treatment with mastic caps ($^aP < 0.05$). Horizontal bars represent the mean ± SE.

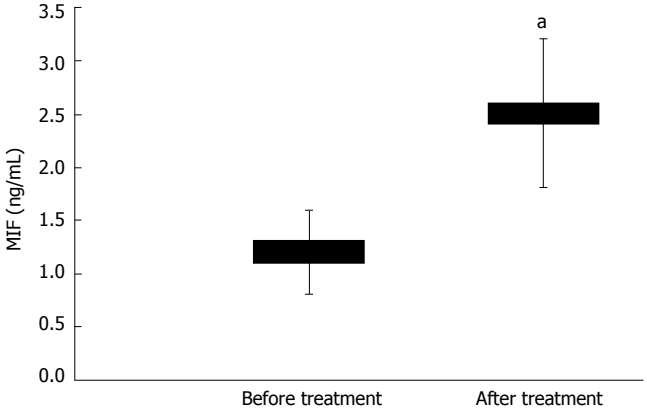

Figure 2 Secretion of macrophage migration inhibitory factor (MIF) was increased in PBMC derived from patients with active Crohn's disease (n = 10) after 4-wk treatment with mastic caps ($^aP < 0.05$). Horizontal bars represent the mean ± SE.

IL-6 was less than 0.70 pg/mL, of MIF less than 0.017 ng/mL and of MCP-1 less than 5.0 pg/mL.

Assay for GSH

At the end of incubation, cells were MPA-treated (5%) and then centrifuged at 2000 × g for 10 min. The resulting supernatant was separated and the GSH assay was performed using the Colorimetric Assay for Glutathione as indicated by the manufacturer (OxisResearch Inc., Portland, USA). GSH concentration was evaluated using a standard curve of Absorbance Units *vs* GSH concentrations and expressed as μmol/L.

Statistical analysis

Results are expressed as mean ± SE. The Mann-Whitney Test was used for comparing differences between patients and controls prior treatment. Differences reported primarily and at the end of the study within individual groups, were tested for significance by the Wilcoxon signed ranks test. A P value below 0.05 was regarded as limit of significance.

RESULTS

Secretion of TNF-α

Our data show that PBMC isolated from patients with active CD exhibited no significant difference in the production of TNF-α compared to controls prior to treatment (2.1 ± 0.9 ng/mL *vs* 1.2 ± 0.3 ng/mL, P = 0.200). TNF-α in controls administered mastic caps was not significantly changed (1.2 ± 0.3 ng/mL *vs* 0.5 ± 0.2 ng/mL, P = 0.173), while in patients it was significantly reduced (2.1 ± 0.9 ng/mL *vs* 0.5 ± 0.4 ng/mL, P = 0.028, Figure 1).

MIF production

MIF production was significantly increased in control group before mastic treatment compared to patients (4.7 ± 1.0 ng/mL *vs* 1.2 ± 0.4 ng/mL, P = 0.038). MIF production in controls administered mastic caps was not altered (4.7 ± 1.0 ng/mL *vs* 4.7 ± 0.7 ng/mL, P = 0.593), while, as shown in Figure 2, it was significantly increased in patients after mastic treatment (1.2 ± 0.4 ng/mL *vs* 2.5 ± 0.7 ng/mL, P = 0.026).

IL-6 and MCP-1

PBMC isolated from patients with active CD exhibited significantly elevated secretion of IL-6 compared to controls prior to therapy (622.5 ± 130.1 pg/mL *vs* 56.7 ± 20.2 pg/mL, P = 0.014). No significant difference was observed in controls before and after treatment (56.7 ± 20.2 pg/mL *vs* 19.9 ± 7.5 pg/mL, P = 0.285). In patients, a trend towards statistical significance was observed in IL-6, before and after treatment although, differences did not reach statistical significance (622.5 ± 130.1 pg/mL to 519.9 ± 176.5 pg/mL, P = 0.068).

In the case of MCP-1, no significant difference was observed between patients and controls prior to treatment (3.0 ± 1.1 ng/mL *vs* 0.7 ± 0.3 ng/mL, P = 0.302). MCP-1 secretion from controls' PBMC (0.7 ± 0.3 ng/mL *vs* 0.6 ± 0.3 ng/mL, P = 0.593) or from patients' PBMC (3.0 ± 1.1 ng/mL *vs* 1.7 ± 1.0 ng/mL, P = 0.463) before and after the trial was not significantly changed.

Intracellular GSH

No significant difference in intracellular GSH was detected between controls and CD patients before mastic treatment (66.4 ± 20.1 μmol/L *vs* 34.0 ± 15.8 μmol/L, P = 0.144). No significant difference was observed in GSH before and after treatment in controls (66.4 ± 20.1 μmol/L *vs* 55.4 ± 9.7 μmol/L, P = 0.285), whereas, even though not statistically significant, a trend towards statistical significance was observed in patients (34.0 ± 15.8 *vs* 56.6 ± 10.3 μmol/L, P = 0.075).

DISCUSSION

Four-week mastic administration has been previously shown to effectively regulate the clinical course, inflammation, and oxidative stress in CD patients. It statistically decreased CD activity index and plasma concentrations of C-reactive protein and IL-6, while it increased plasma total antioxidant potential. Also, nutrition risk index (NRI), one of the most useful measures of nutritional status that incorporates albumin level and body

weight, was improved[7]. In particular, the main element of NRI showing improvement was body weight gain, and since the daily energy intake was unchanged during the trial, increase in body weight and in NRI was attributed to decrease of liquid stools and consequent improvement in nutrient absorption. In two out of ten NRI was > 100 denoting adequate nutrient absorption and absence of nutritional risk. As a continuation of our research to evaluate the effect of mastic on CD and before conducting placebo-controlled studies in large cohorts, in the current study we demonstrated that mastic administration affects cytokine secretion from PBMC obtained from CD patients. Mastic acts as an immunomodulator (a) inhibiting the secretion of TNF-α and (b) inducing the secretion of MIF.

In the report of Grip and coworkers[32] TNF-α was significantly elevated in plasma obtained from CD patients, but not in PMBC, compared to healthy controls. Accordingly, the difference in TNF-α concentrations between patients and controls at baseline was significant in plasma[7], but insignificant in PBMC (present study). Interestingly though, secretion of TNF-α showed a significant decrease in CD patients subjected to mastic treatment (Figure 1). Even though the data reported about TNF-α is conflicting[33,34], the anti-TNF-α treatment in TNF-mediated diseases is developing. The mechanism of mastic's anti-TNF activity in CD may be related to specific blockade of TNF-α secretion. Because cells were always viable in all the experimental conditions, the mechanism including complement mediated lysis of cells expressing membrane bound TNF-α[35], is fairly excluded. A possible approach would be *via* the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. By blocking HMG-CoA reductase on human monocytes, cells reduce the production of TNF-α[32]. TNF-α is suggested to regulate MCP-1 secretion *via* the activation of nuclear factor-kappa B[36]. Yet, this is rather unlikely to be seen hereby, given that MCP-1 concentration was unaffected in CD patients administered with mastic. It is rather that the nuclear factor-kappa B pathway secondary to the decrease in TNF-α was not activated.

To shed more light on the mechanisms by which mastic might work, we also investigated the potency of mastic caps in affecting MIF secretion. Originally, MIF was described as an inhibitor of migration and chemotaxis of monocytes/macrophages[21]. Our findings of suppressed secretion of MIF in CD mononuclear cells compared to healthy prior treatment indicate that monocytes are sensitized to chemotaxis. Increased secretion after treatment (Figure 2) points to the inhibition of monocyte chemotaxis. The significance of this finding is that migration of chemokine or peptide or nonpeptide stimulated monocytes and differentiation to macrophages into the site of inflammation is limited and further trigger of inflammation is controlled. In some studies it is proposed to have proinflammatory properties and be the first cytokine appearing, followed by others[37]. However, since in our present and previous study[7] mastic administration was not followed by enhanced proinflammatory production, induction of MIF secretion should only be allied with inhibition of chemotaxis.

Even though insignificant, a marginal increment in intracellular GSH concentration ($P = 0.075$) was observed. GSH is the most abundant non-enzymatic antioxidant present in cells that plays an important role in the defence against oxidative-stress-induced cell injury[38]. During inflammatory processes, cells of the immune system are exposed to large amounts of reactive oxygen intermediates, and, thus, an efficient GSH system to neutralize free radicals that otherwise disturb immune functions is essential[39]. Mastic has been proven to induce GSH production in PBMC under oxidative conditions *in vitro*[26], while in CD patients to increase plasma total antioxidant potential *in vivo*[7].

IL-6 is thought to play a crucial role in the pathogenesis of CD[40]. We hereby demonstrated that IL-6 secretion in PBMC from CD patients was significantly elevated compared to healthy controls ($P = 0.014$), evident of the cytokine role in CD inflammation. While in plasma IL-6 was statistically decreased with mastic administration[7], in PBMC insignificant nevertheless decrease ($P = 0.068$) was reported, perhaps due to the small number of samples.

Cytokines play a central role in the modulation of the immune system and they have either proinflammatory, such as TNF-α, or antiinflammatory functions. In CD patients the imbalance between proinflammatory and antiinflammatory cytokines brings about the rationale for "anticytokine" treatment. It is however uncertain whether only one cytokine should be targeted or several pro- and antiinflammatory cytokines, or cytokine synthesis inhibitors, soluble receptors, receptor antagonists or receptor antibodies. In the case of mastic, the activity in CD shown previously[7] and hereby may well be extremely interesting. However, further studies -now in progress- are needed as to determine the target and whether there is one or a class or more than one class of compounds acting synergistically to obtain this effect. As a final point, mastic might serve well in the regulation of immunity in CD patients.

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We wish to thank the Chios Mastic Growers Association, especially Dr Christos Kartalis, for the production and kind donation of Chios mastic caps, exclusively for the needs of the trial.

COMMENTS

Background

Mastic administration improves the clinical course and regulates plasma inflammatory and antioxidative mediators of patients with mildly to moderately active Crohn's disease (CD). We aimed to assess the effects of mastic administration on cytokine production of circulating mononuclear cells of patients with active CD.

Research frontiers

The exact pathogenesis of Crohn's disease (CD) is poorly understood; infection, environmental factors, heredity and immunological defects have been proposed as causes. Peripheral blood mononuclear cells (PBMC) are highly activated in active CD and secret higher quantities of proinflammatory mediators. In view of the increased number of CD patients and of the severe side effects of the immunosuppressive therapies available there is a considerable scientific and commercial interest in the discovery of novel classes of therapeutic compounds.

Innovations and breakthroughs

This is the very first study regarding the effect of mastic administration on cytokine production of circulating mononuclear cells of patients with active Crohn's disease.

Applications

Although further double-blind, placebo-controlled studies in a large number of patients is required to clarify the role of this natural product, this finding provides strong evidence that mastic might be an important regulator of immunity in Crohn's disease.

Peer review

This study does provide some new information about a novel possible treatment for Crohn's disease.

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RAPID COMMUNICATION

Hepatitis B viral infection in maintenance hemodialysis patients: A three year follow-up

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Abstract

AIM: To observe the prevalence of hepatitis B virus (HBV) infection in maintenance hemodialysis patients.

METHODS: Eighty-eight hemodialysis patients who had been receiving hemodialysis regularly for an average of 39.45 ± 7.57 (range from 36 to 49) mo were enrolled in this study. HBV markers were measured in these patients before hemodialysis and in 100 healthy controls by the chemiluminescent microparticle immunoassay (CMI) method in order to compare the incidence of HBV infection in hemodialysis patients versus normal healthy people. All patients were then divided into two groups: patients positive for HBV markers (i.e. those positive for HBsAg, anti-HBc, HBeAg, anti-HBe, with or without positive anti-HBs) (n = 33), and patients negative for HBV markers (including those only positive anti-HBs) (n = 55). The following information was obtained for all patients: socio-demographic data, number of blood transfusions and some laboratory investigations. After 39.45 ± 7.57 mo follow-up, HBV markers were measured in these patients by CMI.

RESULTS: The incidence of HBV infection in maintenance hemodialysis patients was 37.5%, which was higher than in controls (9%). In the patients positive for HBV markers, there were 13 patients (39.4%) who had a history of blood transfusion, which was more than the number [12 (21.8%), P = 0.04] of patients negative for HBV markers. Eight of the 88 patients negative for HBV markers turned out to be positive, while three of the 33 patients positive for HBV markers turned out to be negative. There was no cirrhosis of the liver or hepatoma occurring in these patients.

CONCLUSION: Maintenance hemodialysis patients have a higher risk of HBV infection than the average population. The number of blood transfusions is associated with an increased prevalence of HBV. While it is hard for hemodialysis patients to eliminate HBV, the prognosis of patients with positive HBV markers is good.

Key words: Hepatitis B virus; Infection; Hemodialysis patients; Maintenance; Prevalence

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INTRODUCTION

Hepatitis B virus (HBV) infection remains a serious issue in the dialysis population[1-5]. Introduction of HBV vaccination, isolation of HBV positive patients, use of dedicated dialysis machines and regular surveillance for HBV infection has dramatically reduced the spread of HBV in this setting[6-10]. However, the frequency of HBV infection in patients undergoing maintenance dialysis in the industrialized world is low, but not negligible[11-14]. Persistent HBsAg seropositivity is much higher in less-developed countries[15-19], especially in China. The prevalence of HBV infection among hemodialysis patients varies between countries and between dialysis units within a single country. The present study was undertaken to investigate the prevalence of HBV infection among maintenance hemodialysis patients.

MATERIALS AND METHODS

Patients

Eighty-eight patients with ESRD on long-term hemodialysis in the center of blood purification of Beijing Chaoyang Hospital were recruited for this study. There were 42 male and 46 female patients. The median age was 55.46 ± 13.78 (range 25-76) years. The primary cause of ESRD was established in these patients: chronic glomerulonephritis in 35 patients (39.8%), diabetic nephropathy in 22 (25%), hypertension nephropathy in 19 (21.5%), glomerulopathy of unknown origin in 4 (4.5%), tubulointerstitial nephritis in 2 (2.3%), polycystic kidney

Table 1 Variations of HBV markers between hemodialysis patients and healthy controls

Hemodialysis patients	Volunteer controls	HBsAg	Anti-HBs	Anti-HBc	HBeAg	Anti-HBe
18	0			+		
15	2		+	+		
7	0		+	+		+
1	0			+		+
2	1	+		+	+	
0	6	+				

disease in 2 (2.3%), and renal cell carcinoma in 2 (2.3%). All patients had been on dialysis for 39.45 ± 7.57 (range from 36 to 49) mo. Hemodialysis was performed two to three times each week, 4-4.5 h per session, using single-use dialyzers with a membrane surface area of 1.3-1.7 m^2. Dialysis membranes were made of polysulfone (36.7%), cellulose acetate (25.4%), or polymethyl-metacrylate (37.9%).

The control group was made up of 100 healthy blood donors and hospital staff [52 males and 48 females, with a median age of 47.25 ± 10.10 (range 35-69) years], whose health status was assessed by periodical general check-ups. These were healthy persons without any infectious, hepatic or kidney diseases.

Methods

Before each patient entered into our blood purification unit, HBV markers were measured, including hepatitis B surface antigen (HBsAg), antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis B e antigen (HBeAg), and antibody to hepatitis B e antigen (anti-HBe). These markers were also measured in healthy controls. There were 33 (37.5%) patients positive for HBV markers, i.e. patients positive for HBsAg, anti-HBc, HBeAg, anti-HBe, with or without positive anti-HBs. We therefore divided all patients into two groups: patients positive for HBV markers (n = 33) and patients negative for HBV markers (including those only positive for anti-HBs) (n = 55).

Serum HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe were measured with chemiluminescent microparticle immunoassay (CMI) using an ARCHITECT immunoassay analyzer from the Diagnostics Division of Abbott Laboratories (Abbott Park, IL, United States). Biochemistry data were determined using an AU500 autoanalyzer for blood urea nitrogen (BUN), creatinine, serum albumin, alanine aminotransferase (ALT), aspirate aminotransferase (AST) and alkaline phosphatase (ALP).

RESULTS

Incidence of HBV in maintenance hemodialysis patients

There were thirty-three patients with positive HBV markers at the beginning of the study. The incidence of HBV in maintenance hemodialysis patients was 37.5%, which was significantly higher than that in healthy controls. Nine controls were positive for HBV markers. The incidence of HBV in controls was 9%. The variation of the two groups is shown in Table 1.

Table 2 Clinical characteristics and relevant laboratory data (mean ± SD)

	Positive HBV markers group n = 33	Negative HBV markers group n = 55	P value
Time on HD, months	38.45 ± 9.34	40.55 ± 8.53	0.245
Blood transfusion	13 (39.4%)	12 (21.8%)	0.042
Biochemical data			
BUN, mg/dL	59.53 ± 12.36	56.47 ± 11.43	0.755
Creatinine, mg/dL	59.53 ± 12.36	56.47 ± 11.43	0.755
Serum albumin, g/dL	3.6 ± 0.6	3.7 ± 0.5	0.206
AST, IU/L	26.3 ± 6.9	23.6 ± 7.4	0.052
ALT, IU/L	18.7 ± 8.3	16.5 ± 10.2	0.235
ALP, IU/L	76 ± 15.7	79 ± 13.6	0.072

BUN: Blood urea nitrogen, detected Pre-dialysis; AST: Asparate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase; HD: Hemodialysis.

Risk factors of HBV infection

The basic clinical characteristics of the patients are shown in Table 2. In the negative HBV marker group, the mean time on hemodialysis was longer than for the positive HBV marker group, but this difference was not statistically significant. The AST, ALT and ALP levels were, as expected, higher in the positive HBV marker group. The levels of BUN, creatinine, and serum albumin were similar in these two groups. In the positive HBV marker group, there were thirteen patients (39.4%) with a history of blood transfusion, more than the negative group [12 patients (21.8%), P = 0.04].

Turnover and prognosis of the two groups

There were twenty-eight patients positive for HBV markers at the beginning of the study. Three of these became negative, such that the rate was 5.4% (1.8% per year). There were 60 patients negative for HBV markers at baseline, eight of whom turned out to be positive, for a rate of 12.7% (4.2% per year). 39.45 ± 7.57 mo later, chronic cirrhosis and hepatoma had not occurred in these patients.

DISCUSSION

This work shows a high prevalence of HBV infection (37.5%) in maintenance hemodialysis patients (Table 1). The prevalence of HBV infection in hemodialysis patients was higher than that of normal controls (9%) and the general population (9.09%)[20]. This may be attributed to China's high endemic state for HBV infection. A potential contributor to this phenomenon is that significant cellular immune disturbances typically occur in hemodialysis patients. Sharing of supplies, instruments, or medications between hemodialysis patients and reuse of dialyzers would in theory increase the spread of HBV between patients. China, a country hyperendemic for HBV infection, has a higher rate of HBV infection than most industrialized nations. That is why the prevalence rate of HBV infection in our hemodialysis patients is higher than previous observations from western countries (0.9%-5.9%)[11-14] as well as from other developing nations (4%-17%)[15-19].

In order to study the risk of HBV infection, we examined the time on hemodialysis, the biochemical data and the history of blood transfusions of the two groups of patients. We conclude that there is an association between blood transfusions and the prevalence of HBV infection. However, there was no significant difference between time on hemodialysis and HBV infection. In our research, although AST, ALT and ALP levels were higher in the positive HBV marker group, this difference was not statistically significant. As for hemodialysis patients, Hung *et al*[21] revised cutoff values for AST (24 IU/L) and ALT (17 IU/L), which had better sensitivities. In the HBV infected group, the mean values of AST (26.3 IU/L) and ALT (18.7 IU/L) exceeded this criteria, which have important clinical implications in providing benefits of earlier detection and possible prevention of chronic hepatic deteriorations[22].

Because of cellular immune status disturbances, it is hard for hemodialysis patients to eliminate HBV[23-26]. In this study, three patients turned out to be negative, giving a rate of 5.4% (1.8% per year). As for the patients negative for HBV markers, eight turned out to be positive, with a rate of 12.7% (4.2% per year). HBV-related liver disease in patients on long-term dialysis often appears clinically mild, with only modest elevations in AST and ALT levels. Few studies have addressed the natural history of HBV in the dialysis population. Josselson *et al*[27] reported no significant differences in death rates, hospitalizations and hospitalized days between HBsAg-positive and -negative patients on maintenance hemodialysis in the US. However, different outcomes were noted in a retrospective study from India[28]. HBsAg positive patients had a significantly higher mortality rate than negative patients. In our study, the development of cirrhosis, hepatoma and decompensation of liver function is not observed in HBV infected hemodialysis patients. It has been suggested that the hemodialysis procedures lower HBV DNA levels by various mechanisms: the clearance of HBV DNA by the dialysate, the entrapment of HCV DNA particles onto the membrane surface of dialyzers, and the production of cytokines and other substances during hemodialysis sessions. Rampino *et al*[29] have measured a prolonged and marked production of hepatocyte growth factor (HGF) during hemodialysis sessions, and have suggested a beneficial effect of HGF through hepatocyte proliferation and accelerated liver repair. Badalamenti *et al*[30] observed that IFN-alpha levels markedly increase after dialysis using both cellulose and synthetic membranes. This increase in endogenous IFN could contribute to a reduction in viremia in HBsAg patients on maintenance dialysis.

In conclusion, the incidence of HBV in maintenance hemodialysis patients is significantly higher than the average population. The number of blood transfusions is associated with an increased prevalence of HBV. While it is hard for hemodialysis patients to eliminate HBV, the prognosis for patients with positive HBV markers is good.

COMMENTS

Background

Sharing of supplies, instruments, or medications between hemodialysis patients and reuse of dialyzers would in theory increase the spread of HBV between patients. Persistent HBsAg seropositivity is much higher in China than in other countries. In order to get the exact prevalence rate of hepatitis B virus (HBV) infection in maintenance hemodialysis patients, we investigate a dialysis unit in China.

Research frontiers

We studied eighty-eight hemodialysis patients who had been regularly receiving hemodialysis for an average of 39.45 ± 7.57 mo. We measured those patients' HBV markers before hemodialysis and after 39.45 ± 7.57 mo follow-up. We get the prevalence of HBV infection in maintenance hemodialysis patients.

Innovations and breakthroughs

Firstly, this work shows a high prevalence of HBV infection (37.5%) in maintenance hemodialysis patients. Secondly, it concludes that there is an association between blood transfusions and the prevalence of HBV infection. However, there was no significant difference between time on hemodialysis and HBV infection. Thirdly, the main difference from other related articles is that we find three positive HBV-infected patients turned out to be negative.

Applications

The actual application value: the prevalence of HBV infection in maintenance hemodialysis patients in China. The perspective of future application: the further study for the exact mechanisms as to how the hemodialysis patients eliminate HBV.

Terminology

Maintenance hemodialysis patients: The patients who suffer from end-stage renal disease have to receive regular hemodialysis.

Peer review

The authors have estimated the prevalence of HBV infection in a hemodialysis unit. It is concluded that maintenance hemodialysis patients have a higher risk of HBV infection than the average population. The number of blood transfusions is associated with an increased prevalence of HBV. While it is hard for hemodialysis patients to eliminate HBV, the prognosis of patients with positive HBV markers is good.

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RAPID COMMUNICATION

Decreased expression of serotonin in the jejunum and increased numbers of mast cells in the terminal ileum in patients with irritable bowel syndrome

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Abstract

AIM: To investigate if there are changes in serotonin (5-HT) levels, enterochromaffin (EC) cells and mast cells in small intestinal mucosa of patients with irritable bowel syndrome (IBS).

METHODS: Diarrhea-predominant (IBS-D, *n* = 20), or constipation-predominant (IBS-C, *n* = 18) IBS patients and healthy controls (*n* = 20) underwent colonoscopy and peroral small intestinal endoscopy, and mucosal samples were obtained at the descending part of the duodenum, proximal end of jejunum and terminal ileum. High-performance liquid chromatography-electrochemistry and immunohistochemical methods were used to detect 5-HT content, EC cells and mast cells.

RESULTS: (1) There were no differences in the number and distribution of EC cells between IBS patients and the normal group. (2) The mucosal 5-HT contents at the duodenum, jejunum and ileum in IBS-C patients were 182 ± 90, 122 ± 54, 61 ± 35 ng/mg protein, respectively, which were all lower than those in the normal group (256 ± 84, 188 ± 91, and 93 ± 45 ng/mg protein, respectively), with a significant difference at the jejunum ($P < 0.05$). There were no differences in the small intestinal mucosal 5-HT contents between IBS-D patients and the normal group. The mucosal 5-HT contents at the duodenum were significantly higher than those at the ileum in the three groups ($P < 0.001$). (3) The numbers of mast cells in patients with IBS-C and IBS-D at the ileum were 38.7 ± 9.4 and 35.8 ± 5.5/high power field (hpf), respectively, which were significantly more than that in the normal group (29.8 ± 4.4/hpf) ($P < 0.001$). There was no significant difference in the numbers of mast cells at the other two parts between IBS patients and the normal group. The numbers of mast cells in IBS-C, IBS-D, and normal groups were all significantly higher at the ileum (38.7 ± 9.4, 35.8 ± 5.5, 29.8 ± 4.4/hpf, respectively) than at the duodenum (19.6 ± 4.7, 18.5 ± 6.3, 19.2 ± 3.3/hpf, respectively, $P < 0.001$).

CONCLUSION: The changes in the 5-HT signaling pathway at the jejunum of IBS-C patients and the increase in mast cells in patients with IBS at the terminal ileum may offer evidence to explain the pathogenesis of IBS.

Key words: Enterochromaffin cell; Irritable bowel syndrome; Mast cell; Serotonin; Small intestinal mucosa

Wang SH, Dong L, Luo JY, Gong J, Li L, Lu XL, Han SP. Decreased expression of serotonin in the jejunum and increased numbers of mast cells in the terminal ileum in patients with irritable bowel syndrome. *World J Gastroenterol* 2007; 13(45): 6041-6047

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INTRODUCTION

Irritable bowel syndrome (IBS) has a very high global incidence nowadays[1,2]. Though it is not fatal, it exerts a strong influence on the quality of human life[3]. Preliminary epidemiological surveys in China had the same suggestions[4,5].

The pathogenesis of IBS is not clear. Studies have found that alterations of many elements in the gastrointestinal mucosa serotonin (5-HT) signaling pathway occurs in IBS patients[6], and this was one of the mechanisms for the disrupted visceral sensation and gastrointestinal motility in IBS patients. It has been shown that mast cells also play a role in the visceral hypersensitivity of IBS patients. Thus, 5-HT content in the mucosa, enterochromaffin cells (EC cells) and mast cells are the most important indicators. Thus far, many studies

on 5-HT content, EC cells and mast cells have been carried out based on samples taken from the ileocecum, colon and rectum. It is not clear whether the small intestinal mucosa 5-HT content, EC cells and mast cells contribute to the pathological and physiological mechanism in IBS patients. The purpose of this study was to compare 5-HT content, the distribution and number of EC cells and mast cells in small intestinal mucosa in order to determine if there are changes in the 5-HT signaling pathways and mast cells in IBS patients.

MATERIALS AND METHODS

Subjects

Following the Rome Ⅲ Criteria[7], 38 IBS patients were selected as subjects, all of whom were outpatients in Department of Gastroenterology, The Second Hospital of Xi'an Jiaotong University from July 2006 to February 2007. Among them, 20 patients (8 male, 12 female, aged 48.7 ± 16.6 years) had IBS-D and 18 IBS-C (9 male, 9 female, aged 41.5 ± 15.1 years). These patients all underwent testing and examinations of their blood, feces, liver function and fasting blood-glucose levels. A colonoscopy and other related procedures were also performed so as to exclude organic diseases. The patients were carefully interviewed about their situations before the onset of the disease to see if they had had any attacks of acute gastroenteritis infectiosa. Those who had were regarded as post-infection IBS (PI-IBS). In this study, the number of PI-IBS samples gathered were not sufficient so they were not included in the analysis and discussion. 20 healthy people were selected as the normal group (9 male, 11 female, aged 39.9 ± 19.5 years). 5 of them underwent examinations because they were suspected of developing malignant diseases caused by familial heredity. 10 of them came to the hospital for reexamination because of intestinal polypi. 5 were volunteers. All the people in the normal group were found to be clear of a history of chronic diseases or symptoms of gastrointestinal diseases such as abdominal pain, diarrhea and constipation. Written consent was obtained from each subject. The study was approved by the Ethics Committee of the Second Hospital of Xi'an Jiaotong University and conducted according to the principle of the Declaration of Helsinki in 1995.

Samples

All the subjects stopped administering any drugs or treatments which might affect the gut movement for at least a week prior to the examinations. The first examination they had was colonoscopy (Pentex EC 3830F) and 6 pieces of mucosa from the terminal ileum, 15 cm from the ileocecal valve, were taken at the same time. After 1 or 2 d for rest, they underwent peroral small intestinal endoscopy (Fujinon EN 450P) and 12 pieces of mucosa were taken, 6 from the descending part of the duodenum, 6 from the proximal end of the jejunum, 15 cm from the ligament of Traitz. For each kind of piece of mucosa, 2 pieces were immediately put into 40 g/L formaldehyde fixatives for use later in immunohistochemistry. Four pieces were put into the plastic tubes and preserved in a refrigerator at -80℃.

Immunohistochemical staining

The mucosa samples were paraffin-embedded in the typical manner and stained with immunohistochemical-SP. For EC cells and mast cells, rabbit anti-human 5-HT antibody (Zhongshan Jinqiao Company, Beijing, Product No. ZA-0231, dilution 1:100) and mouse anti-human tryptase antibody (Maixin_Bio Company, Fuzhou, Product No. MAB-0125, dilution 1:100) were used as primary antibodies. The secondary antibody staining kit was SP9000 of broad spectrum provided by the Zhongshan Jinqiao Company, Beijing. Next, the samples were observed under a powerful optical microscope (× 400). Each piece of mucosa was observed continuously from 6 non-overlapping fields of view and the numbers of positive immunoreactive cells were counted, with each number expressed with "mean ± SD".

Measurement of mucosal 5-HT content

300 μL 0.2 mol/L perchloric acid containing EDTA was added to the small intestinal mucosa samples prior to homogenation. 50 μL of centrifuged supernatant fluid was sent to the Xi'an Institute for Drug Control for determination of the 5-HT content with high-performance liquid chromatography-electrochemistry (HPLC-ECD), which was expressed in ng/mg protein.

Statistical analysis

One-way analysis of variance (ANOVA) was conducted with the SPSS 13.0 software to compare 5-HT content and the number of EC cells and mast cells between the three groups. $P < 0.05$ indicated significant differences.

RESULTS

EC cells

Under optical microscopy, EC cells were distributed in the intestinal gland cavity, mainly in intestinal crypts. They were next to the goblet cells and most of them were in the shape of a cone or rhombus-like. For each field of view under a powerful optical microscope (× 400), the number of EC cells at the descending part of the duodenum in patients with IBS-C and IBS-D was 9.4 ± 3.9/high power field (hpf) and 10.2 ± 3.7/hpf, respectively, 6.7 ± 2.6/hpf and 6.2 ± 2.4/hpf at the proximal end of the jejunum, 2.7 ± 1.4/hpf, 3.2 ± 1.9/hpf at the terminal ileum. Compared with those in the normal group (10.5 ± 3.4/hpf, 6.6 ± 3.4/hpf and 3.1 ± 1.7/hpf, respectively), there were no significant differences ($P > 0.05$, Figure 1). The distribution of EC cells in the small intestinal mucosa in patients with IBS-C and IBS-D was similar to that in the normal group, proportionately decreasing from the descending part of the duodenum to the terminal ileum. In all three groups, the number of EC cells at the descending part of the duodenum was significantly different from that at the terminal ileum ($P < 0.001$). The number of EC cells at the proximal end of the jejunum was significantly less than that at the descending part of the duodenum ($P < 0.05$) and significantly more than that at the terminal ileum ($P < 0.01$). Figure 2 shows the EC cells after staining under a powerful microscope.

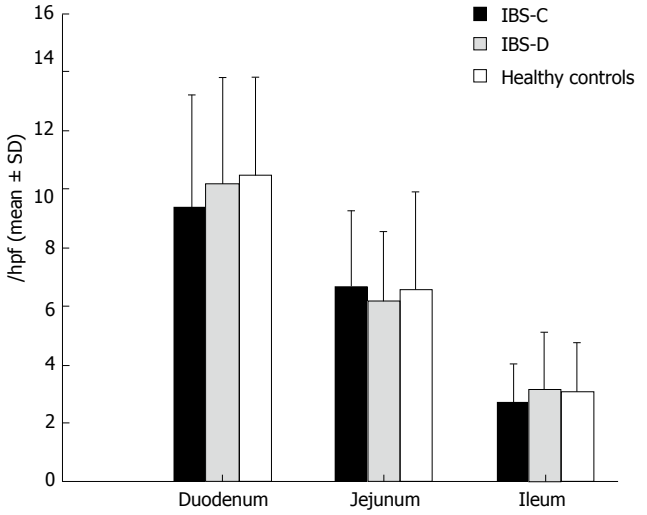

Figure 1 The number of small intestinal mucosal EC cells in IBS patients and the healthy controls. The number and distribution of EC cells in IBS patients are similar to those in the healthy controls. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS; hpf; High power field.

5-HT content

The 5-HT content in IBS-C patients at the descending part of the duodenum, proximal end of the jejunum and terminal ileum were 182 ± 90 ng/mg protein, 122 ± 54 ng/mg protein and 61 ± 35 ng/mg protein, respectively, which were all lower than that in the normal group (256 ± 84 ng/mg protein, 188 ± 91 ng/mg protein and 93 ± 45 ng/mg protein, respectively). In addition, the 5-HT content at the proximal end of the jejunum had a significant difference compared with that of the normal group ($P < 0.05$). The P value at the descending part of the duodenum was 0.058, and at the terminal ileum, 0.063. The 5-HT contents in IBS-D patients at the descending part of the duodenum, proximal end of the jejunum and terminal ileum were 220 ± 96 ng/mg protein, 167 ± 58 ng/mg protein and 70 ± 41 ng/mg protein, respectively, which were all lower than those in the normal group, but they did not show statistical significance ($P > 0.05$). Of all the three groups, the 5-HT content at the descending part of the duodenum was significantly higher than that at the terminal ileum ($P < 0.001$), with the 5-HT content at the jejunum falling between them (Figure 3).

Mast cells

Under optical microscopy, mast cells were distributed in the lamina propria, in the shape of an egg or ellipse. They were scattered between the mucous glands with brown cytoplasm. For each field of view under a powerful optical microscope (× 400), the number of mast cells in patients with IBS-C and IBS-D at the terminal ileum were 38.7 ± 9.4/hpf and 35.8 ± 5.5/hpf, respectively, which were significantly more than those in the normal group (29.8 ± 4.4/hpf) ($P < 0.001$). However, the numbers of mast cells in patients with IBS-C and IBS-D did not show significant differences ($P > 0.05$). The number of mast cells at the descending part of the duodenum in patients with IBS-C and IBS-D were 19.6 ± 4.7/hpf and 18.5 ± 6.3/hpf, respectively, and 18.8 ± 5.8/hpf and 19.7 ± 4.8/hpf at the proximal end of the jejunum. Compared with those in the normal group (19.2 ± 3.3/hpf and 20.0 ± 6.9/hpf, respectively), they did not have significant differences ($P > 0.05$, Figure 4). The number of mast cells in the three groups at the ileum were all significantly more than those at the duodenum ($P < 0.001$). However, the numbers of mast cells at the jejunum and at the duodenum did not show significant differences ($P > 0.05$). Figure 5 shows the mast cells after staining under a powerful microscope.

DISCUSSION

The pathological mechanism of IBS is not clear[8]. It is thought that it is associated with alterations in mentality, GI motility, visceral sensitivity, *etc*. The abnormality in the 5-HT content is one of the reasons for the visceral hypersensitivity and gastrointestinal dysmotility of IBS patients[9], and the mast cells have something to do with the visceral hypersensitivity of IBS patients[10]. Because of the difficulty and trouble in taking samples from intestines, many studies on IBS so far have been based on research in mucosa from the ileocecum, colon and rectum, rather than the whole small intestine. To our knowledge, this study is a pioneering one that aims to determine the 5-HT content and number of EC cells and mast cells in the small intestinal mucosa, esp. duodenum and jejunum, in IBS patients. It is also a first study selecting subjects by following Rome Ⅲ Criteria. In order to better understand the mechanism of IBS, patients were divided into two groups, i.e. patients without any attacks of acute gastroenteritis infectiosa before the onset of the disease, and those with PI-IBS, although the number was not sufficient for the analysis.

5-HT is a very important neurotransmitter of the digestive tract; it is essential to the brain-gut connection and related to gastrointestinal motility and visceral sensation. Most of the 5-HT in the digestive tract is stored in EC cells. When EC cells are stimulated, they release 5-HT, which will act upon 5-HT receptors in intestinal nerve fibers and smooth muscle, initiate peristaltic, secretory, vasodilatory, vagal, and nociceptive reflexes, or regulate sensory function by way of vagal spinal afferent nerves. The serotonin-selective reuptake transporter (SERT) terminates the physiological function of 5-HT by taking it back up in the mucosa[11]. When studying the 5-HT signaling pathway, important elements to examine include the number of EC cells, 5-HT content, tryptophan hydroxylase level, 5-hydroxyindoleacedic acid (HIAA), plasma 5-HT concentration and SERT expression[12]. Studies on animals and humans have measured the above elements in gastric and small intestinal mucosa and drawn some meaningful conclusions with regard to the mucosa 5-HT signaling pathway[13-15]. In this study, two important indicators, EC cells and 5-HT content, were used to study the small intestinal 5-HT signaling pathway in IBS patients.

The clinical symptoms of IBS patients include abdominal pain, diarrhea and constipation. 5-HT is closely related to gastrointestinal motility and visceral sensation. Therefore, abnormalities in the 5-HT signaling pathway are regarded as the cause of visceral hypersensitivity, gastrointestinal dysmotility and parasecretion in IBS

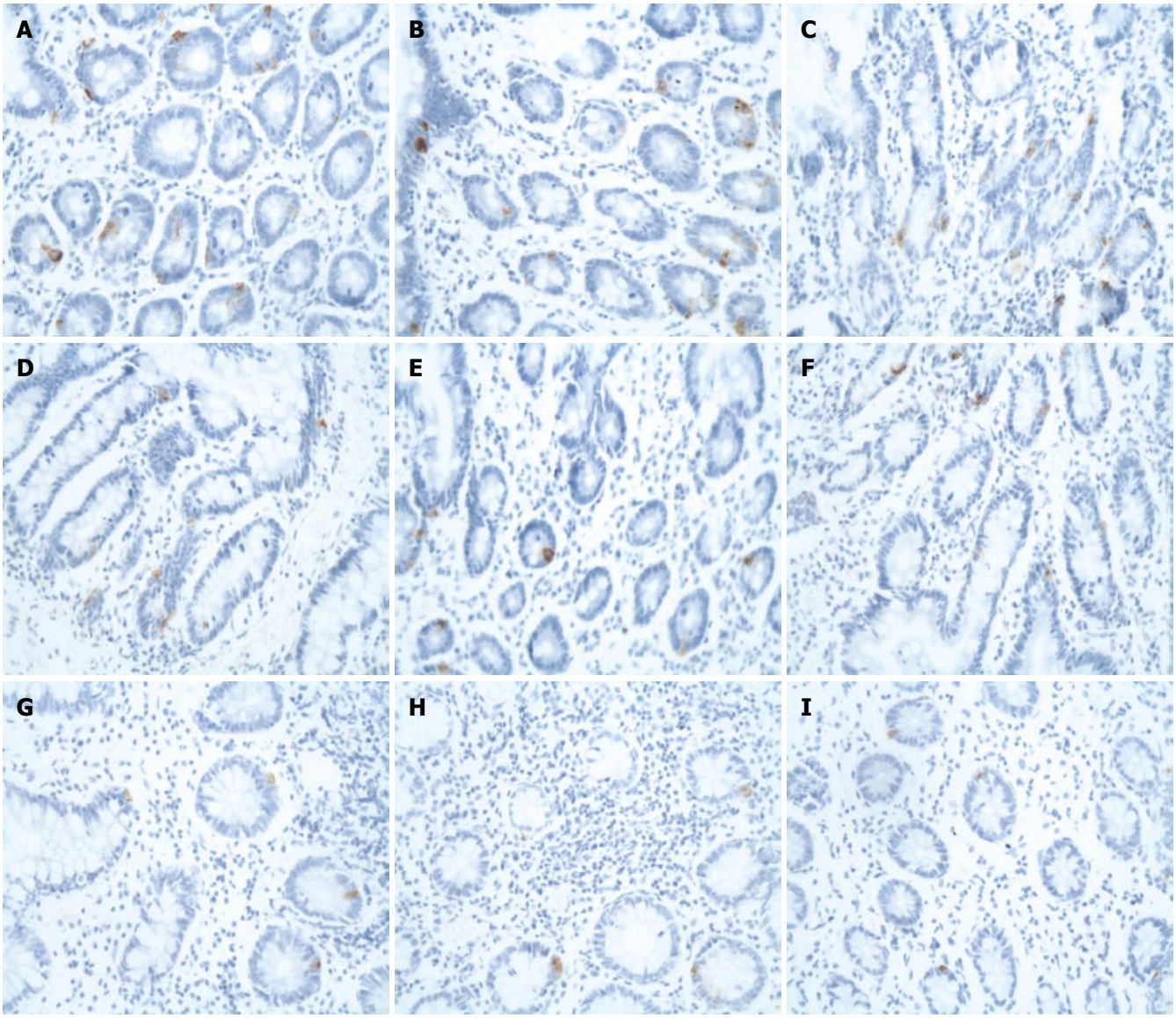

Figure 2 EC cells after being stained by rabbit anti-human 5-HT antibody under a powerful microscope (× 400). **A-C**: EC cells at the descending part of duodenum in healthy controls, IBS-C patients and IBS-D patients, respectively; **D-F**: EC cells at the proximal end of the jejunum in healthy controls, IBS-C patients and IBS-D patients, respectively; **G-I**: EC cells at the terminal ileum in healthy controls, IBS-C patients and IBS-D patients, respectively. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS.

patients. A study performed by Coates *et al*[12] suggested that the 5-HT signaling pathway in the mucosa of the rectum in patients with IBS-C and IBS-D had a molecular defect. However, thus far, there have been no studies on 5-HT in the whole small intestine mucosa in IBS patients.

Previous studies on 5-HT content in the colonic mucosa in IBS patients resulted in different conclusions. Coates *et al*[12] thought that the 5-HT content in the rectal mucosa in patients with IBS-C and IBS-D was significantly lower than that of the normal group. A study by Wang *et al*[16] in China suggested that although the 5-HT content in the colonic mucosa in IBS patients was lower than that of the normal group, it did not have statistical significance. In this study, the 5-HT content in various parts of the small intestinal mucosa in IBS-C patients were all decreased, and the 5-HT content at the proximal end of the jejunum was statistically different from that in the normal group ($P < 0.05$). Based on other previous studies, we propose that the decrease in 5-HT results in the weakening of various reflexes, decreasing of secretion and constipation. The fact that there was not much change in 5-HT content in the small intestinal mucosa in IBS-D patients suggested the existence of an abnormality in other 5-HT signaling pathways. Other studies draw different conclusions. Miwa *et al*[17] thought that the increased 5-HT content in colonic mucosa in IBS-C patients relative to normal controls and IBS-D patients suggested that the synthesis of 5-HT was normal, but the release of 5-HT was changed after EC cells were stimulated.

Other studies on EC cells in IBS patients came to different conclusions. The change in EC cells in the intestinal tract in IBS patients was first found in PI-IBS patients. The increase in EC cells was regarded as a characteristic change in PI-IBS patients[18-20], but it was not applied to IBS patients who had had no infection before the onset of the disease. Studies made by Dunlop

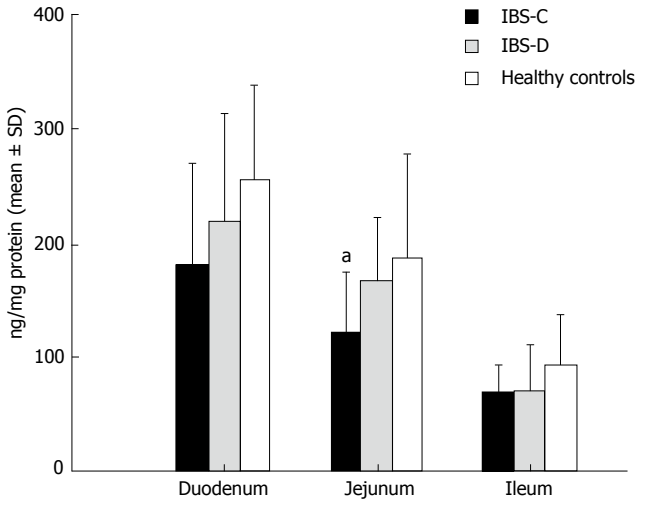

Figure 3 The content of small intestinal mucosal 5-HT in IBS patients and the healthy controls. For each part, the 5-HT content in the IBS-C patients is lower than that in the healthy controls. The 5-HT content at the proximal end of jejunum shows a significant difference compared with that in the normal group ($P < 0.05$). The 5-HT content in the three groups at the descending part of duodenum are all significantly higher than that at the terminal ileum ($P < 0.001$). $^aP < 0.05$ *vs* healthy controls. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS.

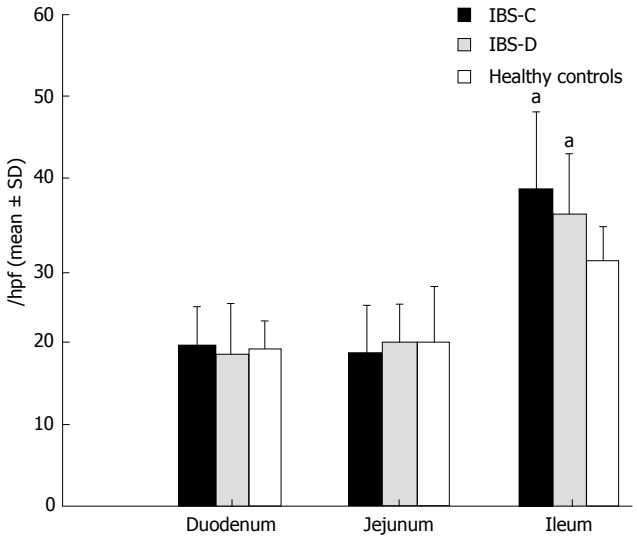

Figure 4 The number of small intestinal mucosal mast cells in IBS patients and the healthy controls. The number of mast cells at the terminal ileum in patients with IBS is significantly different compared with that in the healthy controls ($P < 0.001$). The numbers of mast cells in patients with IBS-C and IBS-D at the other two parts of the intestine do not have significant differences, compared with those in the healthy controls ($P > 0.05$). $^aP < 0.05$ *vs* healthy controls. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS; hpf: High power field.

et al[18,19] and Spiller *et al*[20] found no increase in EC cells in IBS patients with no infection before the onset of the disease. Coates *et al*[12] thought that there was no change in the number of EC cells in the rectal mucosa in patients with IBS-C and IBS-D. Li *et al*[21] and Jiang *et al*[22] found that the number of EC cells at the junction of the rectum and sigmoid colon in patients with IBS-C and IBS-D significantly increased, but in the ileocecum it did not. In this study, the number and distribution of EC cells in the small intestinal mucosa in IBS patients did not show significant differences compared with those in the normal group, which suggested there were no obvious pathological changes in the EC cells in the small intestinal mucosa in IBS patients.

Looking at the small intestinal mucosa 5-HT content and the distribution and number of EC cells in IBS patients, we found that the 5-HT content decreased but the number of EC cells remained unchanged compared with that in the normal group, which suggested that the amount of 5-HT released by EC cells in the small intestinal mucosa in IBS-C patients was less than that in the normal group. In IBS-D patients, the 5-HT content and number of EC cells remained the same as those in the normal group, suggesting a difference in 5-HT signaling pathways in IBS-C and IBS-D patients.

Studies have shown that mast cells have something to do with the visceral hypersensitivity of IBS patients[23]. Many agents released by mucosa mast cells can affect intestinal nerve and smooth muscle. Experiments on animals and studies in human beings all proved that mast cells and intestinal nerves are closely connected[24], and in IBS patients, the connection was even closer[25]. The increase of mast cells in IBS patients results in more agents being released by mast cells. All these, together with the close connection between the mast cells and nerve fibers, contribute to the seriousness of abdominal pain.

Many studies have demonstrated that the increase of mast cells in the ileocecum is a characteristic change of IBS[23,26,27]. Park *et al*[23], Dong *et al*[26] and Chen *et al*[27] found an increased number of mast cells in the ileocecum mucosa in IBS patients. Meanwhile, studies suggested the ileocecum might be the site of origin for abdominal pain, bloating, and changes in bowel habits, showing more sensitivity when a balloon dilates[28]. In this study, the number of mast cells at the terminal ileum in IBS patients increased significantly compared with that of the normal group, which is in line with the conclusions drawn from previous studies. It is indicated that the change of mast cell number in the terminal ileum in IBS patients is the characteristic pathological change. Studies on mast cells in other parts of the colonic mucosa (except the cecum) in IBS patients have produced different conclusions. Some found an increase in the number of mast cells[18,23,26], while others did not[19,26]. So far, in China, there have been no studies on mast cells in the duodenum and jejunum in IBS patients. In this study, the numbers of mast cells in the duodenum and jejunum in patients with IBS-C and IBS-D were almost the same as that in the normal group. This study also revealed that the distribution of mast cells in small intestinal mucosa in IBS patients was the same as that in the normal group, i.e. gradually increasing from the duodenum to the ileum.

In conclusion, this study reveals, for the first time, a change in the 5-HT signaling pathway in the jejunum in patients with IBS-C. It also suggests that the increase of mast cells in the ileocecum is the characteristic pathological change in IBS patients. These changes in the mucosa of gastrointestinal tract cause IBS-related symptoms. This

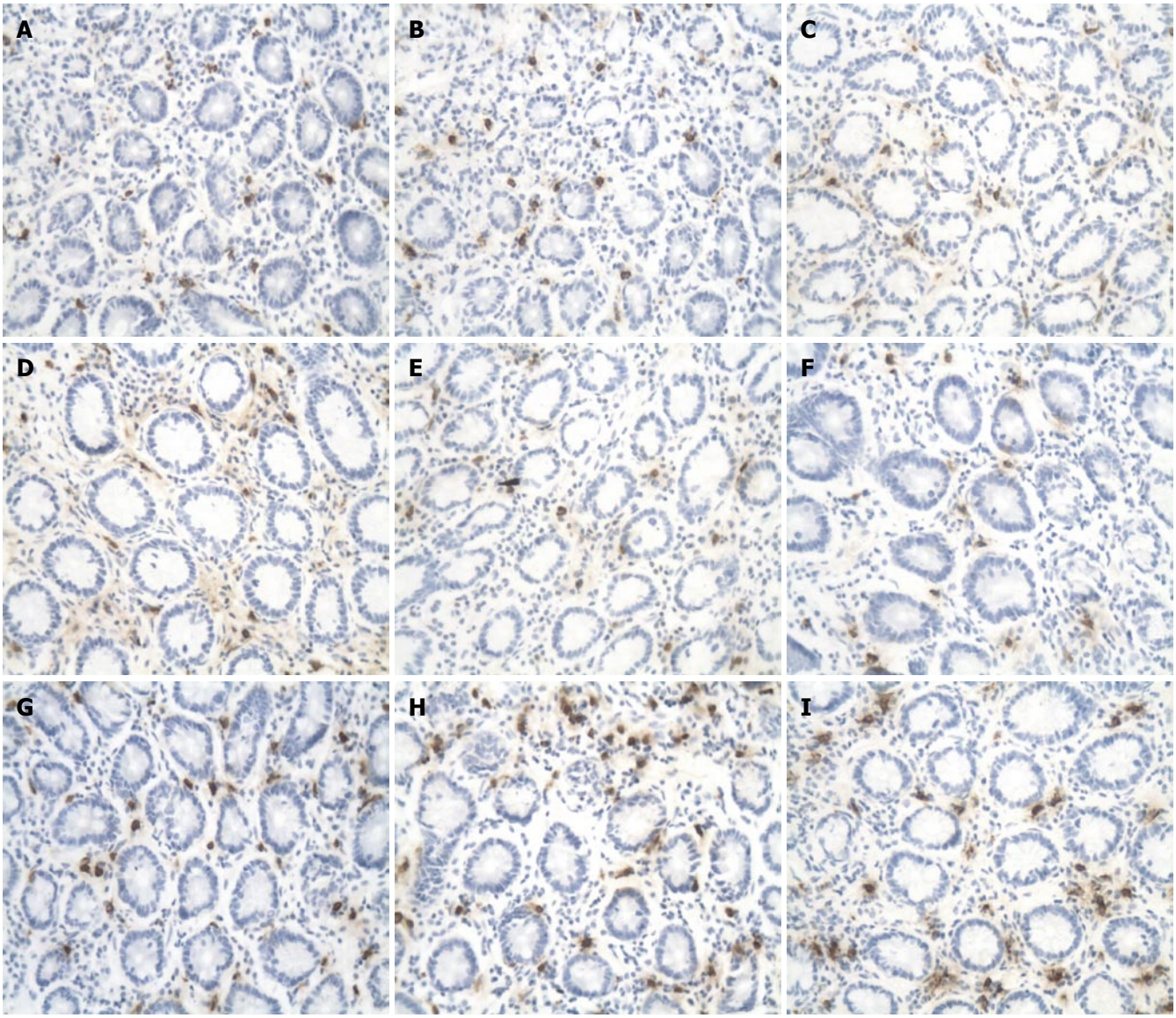

Figure 5 Mast cells after staining by mouse anti-human tryptase antibody under a powerful microscope (× 400). **A-C**: Mast cells at the descending part of the duodenum in healthy controls, IBS-C patients and IBS-D patients, respectively; **D-F**: Mast cells at the proximal end of jejunum in healthy controls, IBS-C patients and IBS-D patients, respectively; **G-I**: Mast cells at the terminal ileum in healthy controls, IBS-C patients and IBS-D patients, respectively. EC cell: Enterochromaffin cells; 5-HT: Serotonin; IBS: Irritable bowel syndrome; IBS-C: Constipation predominant IBS; IBS-D: Diarrhea predominant IBS.

study offers new insight that may be useful for further research into the pathogenesis of IBS.

COMMENTS

Background

Studies have found that abnormal serotonin (5-HT) levels and mast cells are two of the reasons for the disturbance of visceral sensation and gastrointestinal motility in patients with irritable bowel syndrome (IBS). Many studies on 5-HT contents, enterochromaffin (EC) cells and mast cells in IBS have been carried out based on samples taken from mucosa from the ileocecum, colon and rectum, rather than in the whole small intestine.

Research frontiers

The purpose of this study was to compare 5-HT content, the distribution and number of EC cells and mast cells in small intestinal mucosa in order to determine if there were changes in 5-HT signaling pathways and mast cells in small intestinal mucosa in IBS patients.

Innovations and breakthroughs

(1) This study is the first one, to our knowledge, to determine the 5-HT contents, EC cells and mast cells in the small intestinal mucosa, especially the duodenum and jejunum, in IBS patients. (2) It is also the first study on the selection of subjects following Rome Ⅲ Criteria. (3) In order to better understand the mechanism of IBS, patients were divided into two groups; i.e. patients without any attack of acute gastroenteritis infectiosa before the onset of IBS and patients with previous gastrointestinal (GI) infections (post-infection IBS or PI-IBS) although, they could not be used as a group for the analysis due to the small number of cases.

Applications

These changes of 5-HT signaling pathway and mast cells in mucosa of the GI tract will cause IBS-related symptoms. This study offers new insight towards further research into the pathogenesis of IBS.

Terminology

Visceral hypersensitivity: When the GI tract is stimulated by luminal distention and other stimuli, perception of abdominal pain or discomfort is increased. This is widely regarded as the reason for the development of functional gastrointestinal diseases, including functional dyspepsia and irritable bowel syndrome. 5-HT signaling pathways: This refers to the whole process including 5-HT release in the GI tract and central nervous system, binding to its receptors, re-uptake and degradation. Elements involved in the study of the 5-HT signaling pathway include determination of the number of EC cells, 5-HT content, tryptophan hydroxylase

level, 5-hydroxyindoleacedic acid (HIAA), plasma 5-HT concentration and serotonin-selective reuptake transporter (SERT) expression.

Peer review

The study is of interest and permits a better understanding of IBS. It shows that the changes of 5-HT signaling pathway at the jejunum of IBS-C patients and the increase of mast cells in patients with IBS at ileocecum may offer evidence to explain the pathogenesis of IBS.

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RAPID COMMUNICATION

Prognostic value of lateral lymph node metastasis for advanced low rectal cancer

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Abstract

AIM: To evaluate the risk factors for lateral lymph node metastasis in patients with advanced low rectal cancer, in order to make the effective selection of patients who could benefit from lateral lymph node dissection, as well as the relationship of lateral lymph node metastasis with local recurrence and survival of patients with advanced low rectal cancer.

METHODS: A total of 96 consecutive patients who underwent curative surgery with lateral pelvic lymphadenectomy for advanced lower rectal cancer were retrospectively analyzed. The relation of lateral lymph node metastasis with clinicopathologic characteristics, local recurrence and survival of patients was identified.

RESULTS: Lateral lymph node metastasis was observed in 14.6% (14/96) of patients with advanced low rectal cancer. Lateral lymph node metastasis was detected in 10 (25.0%) of 40 patients with tumor diameter $\geqslant$ 5 cm and in 4 (7.1%) of 56 patients with tumor diameter < 5 cm. The difference between the two groups was statistically significant (χ^2 = 5.973, P = 0.015). Lateral lymph node metastasis was more frequent in patients with 4/4 diameter of tumor infiltration (7 of 10 cases, 70.0%), compared with patients with 3/4, 2/4 and 1/4 diameter of tumor infiltration (3 of 25 cases, 12.0%; 3 of 45 cases, 6.7%; 1 of 16 cases, 6.3%) (χ^2 = 27.944, P = 0.0001). The lateral lymph node metastasis rate was 30.0% (9 of 30 cases), 9.1% (4 of 44 cases) and 4.5% (1 of 22 cases) for poorly, moderately and well-differentiated carcinoma, respectively. The difference between the three groups was statistically significant (χ^2 = 8.569, P = 0.014). Local recurrence was 18.8% (18 of 96 cases), 64.3% (9 of 14 cases), and 11.0% (9 of 82 cases) in patients with advanced low rectal cancer, in those with and without lateral lymph node metastasis, respectively. The difference between the two groups was statistically significant (χ^2 = 22.308, P = 0.0001). Kaplan-Meier survival analysis showed significant improvements in median survival (80.9 ± 2.1 m, 95% CI: 76.7-85.1 m *vs* 38 ± 6.7 m, 95% CI: 24.8-51.2 m) of patients without lateral lymph node metastasis compared with those with lateral lymph node metastasis (log-rank, P = 0.0001).

CONCLUSION: Tumor diameter, infiltration and differentiation are significant risk factors for lateral lymph node metastasis. Lateral pelvic lymphadenectomy should be performed following surgery for patients with tumor diameter $\geqslant$ 5 cm. Lateral lymph node metastasis is an important predictor for local recurrence and survival in patients with advanced low rectal cancer.

Key words: Low rectal cancer; Lateral lymph node metastasis; Local recurrence; Prognosis

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INTRODUCTION

It is well known that rectal carcinoma is one of the most common carcinomas in China. Since total mesorectal excision was adopted as the standard treatment of patients with rectal carcinoma, improvements have been made in decreasing its local recurrence and prolonging survival of patients[1-8]. However, even having undergone radical resection with total mesorectal excision, about 5%-40% of patients with rectal carcinoma have local recurrence[9-14]. The survival of patients with advanced low rectal cancer

still remains poor. It was reported that lateral lymph node metastasis may be the most important factor for local recurrence and poor prognosis of advanced low rectal cancer[15-18]. In the current study, the data on 96 consecutive patients who underwent curative surgery with total mesorectal excision and lateral lymph node dissection for advanced low rectal cancer at the Department of General Surgery of Guangdong Provincial People's Hospital were retrospectively analyzed. The relationship of lateral lymph node metastasis with local recurrence was identified. The prognostic value of lateral lymph node metastasis for advanced low rectal cancer was also evaluated. Moreover, the risk factors for lateral lymph node metastasis and indications of lateral lymph node dissection remain unclear. Therefore, this study was to explore the risk factors for lateral lymph node metastasis in order to make effective selection of patients who could benefit from lateral pelvic lymphadenectomy. The relation of lateral lymph node metastasis with clinicopathologic characteristics of advanced low rectal cancer was analyzed.

MATERIALS AND METHODS

Patients and methods

A total of 96 consecutive patients who underwent curative surgery with total mesorectal excision and lateral lymph node dissection for advanced low rectal cancer at the Department of General Surgery of Guangdong Provincial People's Hospital were retrospectively analyzed. There were 46 men and 50 women, ranging in age from 25 to 86 years, with a mean age of 65 years. None of these patients received preoperative chemotherapy or radiotherapy. Twenty-one patients (21.9%) had a family history, 40 patients (41.6%) had a high cancer embryonic antigen (CEA) level and a tumor diameter $\geqslant$ 5 cm, 56 had a tumor diameter < 5 cm. According to the Ming's criteria, 42 tumors were classified as expansive type carcinoma, 54 tumors as infiltrative type carcinoma. Fifty-six patients (58.3%) had positive lymph node metastases and 36 patients (37.5%) had positive vessel cancerous emboli. Thirty patients had a poorly differentiated carcinoma, 44 patients had a moderately differentiated carcinoma, 22 patients had a well-differentiated carcinoma. Low anterior resection was performed in 68 patients and abdominal perineal resection in 28 patients. A total of 1776 lymph nodes were dissected from these 96 patients (average 18.5 lymph nodes per patient). Two pathologists who were blinded to the clinicopathological data observed the specimens independently.

Statistical analysis

Statistical analysis was performed by chi-square test to examine the association of lateral lymph node metastasis with clinicopathologic characteristics and local recurrence of advanced low rectal cancer. The relationship between lateral lymph node metastasis and survival in patients with advanced low rectal cancer was evaluated by Kaplan-Meier survival analysis and log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

Correlation between lateral lymph node metastasis and clinicopathologic characteristics of advanced low rectal cancer

Lateral lymph node metastasis was observed in 14.6 (14/96) of patients with advanced low rectal cancer. Lateral lymph node metastasis was detected in 10 (25.0%) of 40 patients with tumor diameter $\geqslant$ 5 cm and in 4 (7.1%) of 56 patients with tumor diameter < 5 cm. The difference between the two groups was statistically significant (χ^2 = 5.973, P = 0.015). Lateral lymph node metastasis was more frequent in patients with 4/4 diameter of tumor infiltration (7 of 10 cases, 70.0%), compared with patients with 3/4, 2/4 and 1/4 diameter of tumor infiltration (3 of 25 cases, 12.0%; 3 of 45 cases, 6.7%; 1 of 16 cases, 6.3%) (χ^2 = 27.944, P = 0.000). The lateral lymph node metastasis rate for poorly, moderately and well differentiated carcinoma was 30.0% (9 of 30 cases), 9.1% (4 of 44 cases) and 4.5% (1 of 22 cases), respectively. The difference between the three groups was statistically significant (χ^2 = 8.569, P = 0.014). No significant correlation was found between lateral lymph node metastasis and other variables such as gender (χ^2 = 0.168, P = 0.682), age (χ^2 = 0.103, P = 0.749), family history (χ^2 = 0.430, P = 0.512), high CEA level (χ^2 = 0.468, P = 0.494), Ming's classification (χ^2 = 0.430, P = 0.512), lymph node metastases (χ^2 = 0.239, P = 0.625) and vessel cancerous emboli (χ^2 = 0.201, P = 0.654) (Table 1).

Correlation between lateral lymph node metastasis and local recurrence of advanced low rectal cancer

Local recurrence was 18.8% (18 of 96 cases), 64.3% (9 of 14 cases), and 11.0% (9 of 82 cases) in patients with advanced low rectal cancer and those with and without lateral lymph node metastasis The difference between the two groups was statistically significant (χ^2 = 22.308, P = 0.0001).

Correlation between lateral lymph node metastasis and survival in patients with advanced low rectal cancer

In a median follow-up period of 73 (range 16-90) mo, Kaplan-Meier survival analysis showed a significantly improved median survival (80.9 $\pm$ 2.1 m, 95% CI: 76.7-85.1 m *vs* 38 $\pm$ 6.7 m, 95% CI: 24.8-51.2 m) in patients without lateral lymph node metastasis compared with those with lateral lymph node metastasis. The difference between the two groups was statistically significant (log-rank, P = 0.0001) (Figure 1).

DISCUSSION

Lateral pelvic lymphadenectomy for advanced low rectal cancer is controversial[19-25]. In Japan, lateral pelvic lymphadenectomy is routinely performed for patients with advanced low rectal cancer, whereas it is not frequently performed in the Western countries[26-28]. In the current study, a retrospective analysis was performed in 96 patients with advanced low rectal cancer who underwent curative surgery with lateral lymph node dissection. The relations of lateral lymph node metastasis with clinicopathologic

Table 1 Relations between lateral lymph node metastasis and clinicopathologic characteristics of patients with advanced low rectal cancer

Variable	*n*	Lateral lymph node metastasis		
		Positive (%)	Negative (%)	χ^2/*P* value
Gender				
Male	46	6 (13.0)	40 (87.0)	
Female	50	8 (16.0)	42 (84.0)	$\chi^2 = 0.168/P = 0.682$
Age (yr)				
< 60	38	5 (13.2)	33 (86.8)	
⩾ 60	58	9 (15.5)	49 (84.5)	$\chi^2 = 0.103/P = 0.749$
Family history				
Yes	21	4 (19.0)	17 (81.0)	
No	75	10 (13.3)	65 (86.7)	$\chi^2 = 0.430/P = 0.512$
CEA level				
High	40	7 (17.5)	33 (82.5)	
Normal	56	7 (14.6)	49 (85.4)	$\chi^2 = 0.468/P = 0.494$
Superficial diameter (cm)				
< 5	56	4 (7.1)	52 (92.9)	
⩾ 5	40	10 (25.0)	30 (75.0)	$\chi^2 = 5.973/P = 0.015$
Diameter of infiltration				
1/4	16	1 (6.3)	15 (93.7)	
1/2	45	3 (6.7)	42 (93.3)	
3/4	25	3 (12.0)	22 (88.0)	
4/4	10	7 (70.0)	3 (30.0)	$\chi^2 = 27.944/P = 0.000$
Ming's classification				
Expansive	42	5 (11.9)	37 (88.1)	
Infiltrative	54	9 (16.7)	45 (83.3)	$\chi^2 = 0.430/P = 0.512$
Histologic differentiation				
Well	22	1 (4.5)	21 (95.5)	
Moderate	44	4 (9.1)	40 (90.9)	
Poorly	30	9 (30.0)	21 (70.0)	$\chi^2 = 8.569/P = 0.014$
Lymph node metastasis				
Positive	56	9 (16.1)	47 (83.9)	
Negative	40	5 (12.5)	35 (87.5)	$\chi^2 = 0.239/P = 0.625$
Vessel cancerous emboli				
Positive	36	6 (16.7)	30 (83.3)	
Negative	60	8 (13.3)	52 (86.7)	$\chi^2 = 0.201/P = 0.654$

CEA: Carcinoma embryonic antigen.

characteristics, local recurrence and survival of advanced low rectal cancer were analyzed.

In our study, lateral lymph node metastasis was observed in 14.6% (14/96) of patients with advanced low rectal cancer, showing a significant correlation with tumor diameter, infiltration and differentiation. Lateral lymph node metastasis was found in 10 (25.0%) of 40 patients with tumor diameter ⩾ 5 cm and in 4 (7.1%) of 56 patients with tumor diameter < 5 cm ($\chi^2 = 5.973$, $P = 0.015$). Lateral lymph node metastasis was more frequent in patients with 4/4 diameter of tumor infiltration (7 of 10 cases, 70.0%) than in patients with 3/4, 2/4 and 1/4 diameter of tumor infiltration (3 of 25 cases, 12.0%; 3 of 45 cases, 6.7%; 1 of 16 cases, 6.3%) ($\chi^2 = 27.944$, $P = 0.000$). The lateral lymph node metastasis rate of poorly, moderately and well differentiated carcinoma was 30.0% (9 of 30 cases), 9.1% (4 of 44 cases) and 4.5% (1 of 22 cases), respectively ($\chi^2 = 8.569$, $P = 0.014$), indicating that tumor diameter ⩾ 5 cm, tumor infiltration and differentiation are risk factors for lateral lymph node metastasis of advanced low rectal cancer. Therefore, lateral pelvic lymphadenectomy should be performed following the management of patients with tumor diameter ⩾ 5 cm, tumor infiltration or differentiation.

It is well known that local recurrence is the most important prognostic factor for rectal carcinoma[29-31]. It was reported that local recurrence can be found in 4%-50% of patients with rectal carcinoma after curative resection, and lateral lymph node metastasis may be the important factor for local recurrence[15,16]. Ueno *et al*[15] reported that patients with lateral node metastases have an increased risk for local recurrence (44% *vs* 11.7%; $P < 0.001$) compared with those without lateral node metastases. Sugihara *et al*[16] also reported that positive lateral lymph nodes are significantly associated with increased local recurrence of rectal cancer. Similarly in this study, lateral lymph node metastasis was significantly correlated with local recurrence of advanced low rectal cancer. The local recurrence rate of advanced low rectal cancer was 64.3% (9 of 14 cases) and 11.0% (9 of 82 cases) in patients with and without lateral lymph node metastasis ($\chi^2 = 22.308$, $P = 0.000$), respectively, indicating that lateral pelvic lymphadenectomy can significantly reduce local recurrence of advanced low rectal cancer.

In the present study, patients without lateral lymph node metastasis had significant improvements in median survival (80.9 ± 2.1 m, 95% CI: 76.7-85.1 m *vs* 38 ± 6.7 m, 95% CI: 24.8-51.2 m) compared to those with lateral

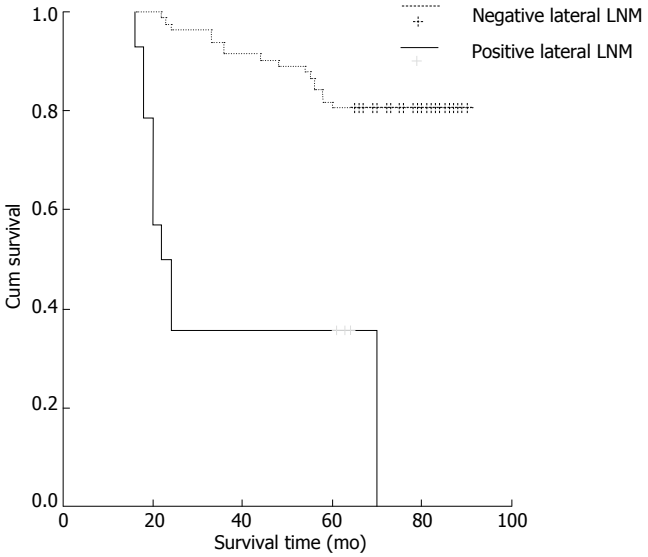

Figure 1 Relations between lateral lymph node metastasis and survival of patients with advanced low rectal cancer (Kaplan-Meier survival analysis). LNM: Lymph node metastasis.

lymph node metastasis. The difference between the two groups was statistically significant (log-rank, $P = 0.0001$), supporting that lateral lymph node metastasis has a significant prognostic value for advanced low rectal cancer. Lateral pelvic lymphadenectomy may effectively improve the survival of patients with advanced low rectal cancer. Ueno *et al*[32] also reported that advanced low rectal cancer patients having lymph node involvement in the lateral pelvic area are likely to benefit from lymphadenectomy.

COMMENTS

Background

Even having undergone radical resection with total mesorectal excision, about 5%-40% of patients with rectal cancer have local recurrence. The survival of patients with advanced low rectal cancer remains poor. Whether patients with advanced low rectal cancer could benefit from lateral lymph node dissection is still controversial.

Research frontiers

At present, lateral lymphadenectomy for advanced low rectal cancer is controversial. However, lateral lymph node metastasis is significantly associated with local recurrence and poor prognosis of advanced low rectal cancer.

Innovations and breakthroughs

The results of this study indicate that tumor diameter $\geqslant$ 5 cm, tumor infiltration and differentiation are risk factors for lateral lymph node metastasis of advanced low rectal cancer. Lateral lymph node metastasis is significantly correlated with local recurrence and prognosis of advanced low rectal cancer.

Applications

Lateral pelvic lymphadenectomy may effectively reduce local recurrence and improve the survival in patients with advanced low rectal cancer.

Peer review

The present study investigated the relations of lateral lymph node metastasis with local recurrence and survival in patients with advanced low rectal cancer. The study design is good and data analysis is extensive. The manuscript is well written.

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RAPID COMMUNICATION

Effect of Bcl-2 and Bax on survival of side population cells from hepatocellular carcinoma cells

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Abstract

AIM: To understand the role and significance of side population (SP) cells from hepatocellular carcinoma (HCC) in hepatocarcinogenesis, development, relapse and metastasis, we simulated the denutrition conditions that cancer cells experience in clinical therapy, observed the different anti-apoptosis ability of SP cells and non-SP cells under such conditions, and established the possible effects of P53, Bcl-2 and Bax on survival of SP cells.

METHODS: We used flow cytometry to analyze and sort the SP and non-SP cells in established HCC lines MHCC97 and hHCC. We evaluated cell proliferation by methyl thiazolyl tetrazolium (MTT) assay and investigated the expression of p53, bcl-2 and bax genes during denutrition, by RT-PCR and immunofluorescence staining.

RESULTS: The percentage of SP cells in the two established HCC lines was 0.25% and 0.5%, respectively. SP cells had greater anti-apoptosis and proliferation ability than non-SP cells. Expression of Bcl-2 and Bax in SP and non-SP cells differed during denutrition. The former was up-regulated in SP cells, and the latter was up-regulated in non-SP cells.

CONCLUSION: It may be that different upstream molecules acted and led to different expression levels of Bcl-2 and Bax in these two cell lines. There was a direct relationship between up-regulation of Bcl-2 and down-regulation of Bax and higher anti-apoptosis ability in SP cells. It may be that the existence and activity of SP cells are partly responsible for some of the clinical phenomena which are seen in HCC, such as relapse or metastasis. Further research on SP cells may have potential applications in the field of anticancer therapy.

Key words: Side population; Hepatocellular carcinoma; Bax; Bcl-2; Apoptosis

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INTRODUCTION

It is believed that cancer is unicellular in origin[1], although cancer cells from a lot of tumors generally exhibit functional heterogeneity in experimental and clinical settings[2]. There are two theories[3,4]. One is the stochastic model, which figures that cancer is composed of a comparatively homogeneous population; only a few cells undergo stochastic events, so that they have the potential to proliferate extensively and form new tumors. The other is the hierarchy model, which suggests that there is some kind of pyramid scale in cancer cells. In this model, the subpopulation cells, which are on acme in the pyramid scale, comprise cancer stem cells (CSCs) that self-renew, generate downstream descendants, and initiate new tumors.

Recently, the latter hypothesis has gained significant recognition. The possible existence of CSCs has been shown in leukemia and some solid tumors, including breast cancer and brain tumors[5-9]. These cells are detected by their own ability to efflux Hoechst 33342 dye through an ATP-binding cassette (ABC) membrane transporter. They are also named side population (SP) cells for their location on flow cytometry charts.

Hepatocellular carcinoma (HCC) is a common malignancy and still has a high mortality rate[10]. Clinical operations and chemotherapy can lead most such cancer cells to death or proliferation inhibition through denutrition. However, there are always some cells that can survive and result in relapse or metastasis, which often leads to therapeutic failure and poor prognosis. The CSC hypothesis offers an explanation for these clinical phenomena. In some studies, it has been found that SP cells are easier to initiate tumors than non-SP cells are in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice[11]. Under conditions of denutrition

that are similar to those after clinical treatment, do SP cells survive more easily and have higher anti-apoptosis ability than non-SP cells?

p53, the most frequently mutated gene in human malignancies, is found inactivated in about 50% of tumors in any location and of any histological type. It has been named the "guardian of the genome"[12]. bcl-2 was the first example of an oncogene that inhibits cell death rather than promotes proliferation. According to differences in structure, the Bcl-2 family can be divided into two subgroups. Bax belongs to one subfamily that can oligomerize and integrate into the outer mitochondrial membrane to initiate events during apoptosis. Normally, Bcl-2 inhibits Bax activation. But, when cells are under stress (either oncogenic or genotoxic stress), Bcl-2 is inactivated by P53 as its downstream molecule. As a consequence, apoptotic cell death continues[13,14]. Under denutrition conditions, how are these three genes expressed in SP cells? What is their relationship with the anti-apoptosis ability of SP cells?

Taking clinical and experimental data together, we consider that the understanding of biologic characteristics of SP cells from HCC cells may serve to elucidate the mechanism of hepatocarcinogenesis and lead to novel therapeutic approaches.

In this study we therefore analyzed and sorted SP cells from established HCC cell lines. We observed the anti-apoptosis ability of two cell lines under denutrition conditions by methyl thiazolyl tetrazolium (MTT) assay. We further estimated the expression level of P53, Bcl-2 and Bax in SP cells and non-SP cells by RT-PCR and immunofluorescence during denutrition.

MATERIALS AND METHODS

Cell culture

The human liver cancer cell line MHCC97 was obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China), the hHCC cell line was from the Department of Biochemistry and Molecular Biology of Fourth Military Medical University (Xi'an, China). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Invitrogen), and incubated at 37℃ in an atmosphere containing 5% CO_2.

SP cell analysis and purification using flow cytometry

Cells were detached from the dishes with trypsin-EDTA (Invitrogen) and suspended at 1×10^6 cells/mL in Hank's balanced salt solution (HBSS) supplemented with 3% fetal calf serum and 10 mmol/L HEPES. These cells were then incubated at 37℃ for 90 min with 20 μg/mL Hoechst 33342 (Sigma, St Louis, MO, USA), either alone or in the presence of 50 μmol/L verapamil (Sigma), which is an inhibitor of verapamil-sensitive ABC transporter. After 90 min incubation, the cells were centrifuged immediately for 5 min at 300 × *g*, 4℃ and resuspended in ice-cold HBSS. The cells were kept on ice to inhibit efflux of Hoechst dye. Then, 1 μg/mL propidium iodide (PI; BD Pharmingen, San Diego, CA, USA) was added to discriminate dead cells. Finally, these cells were filtered through a 40-μm cell strainer (BD Falcon; BD Pharmingen, San Diego, CA, USA) to obtain single suspension cells. Cell dual-wavelength analysis and purification were performed using dual-laser cytometry (FACSVantage; BD Biosciences, Franklin Lakes, NJ. Hoechst 33342 solution was excited at 355 nm UV light; blue fluorescence was collected with a 450/20 band-pass (BP) filter and red fluorescence with a 675-nm edge filter long-pass (EFLP). A 610-nm dichroic mirror short-pass was used to separate the emission wavelengths. PI-positive dead cells were excluded from the analysis.

MTT assay

All cells were maintained in PBS for 3, 6 and 9 h to induce denutrition. Then, cell proliferation was evaluated by MTT assay. MTT solution in PBS was added to a final concentration of 0.5 mg/mL, and cells incubated for 4 h at 37℃. Supernatant was removed and cells were resuspended in 150 μL DMSO for 10 min, and absorbance was measured at 490 nm using a microplate reader (Bio-rad, Japan).

Semi-quantitative RT-PCR

After all cells were induced in PBS for their respective number of hours, total RNA was extracted from SP and non-SP cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and was reverse-transcribed by using a First-Strand cDNA Synthesis Kit (Fermentas, Lithuania), as described in the instructions. RT-PCR was carried out in a 50 μL reaction mixture that contained 1 μL cDNA as template, 1 μmol/L specific oligonucleotide primer pair, and 25 μL Taq mixture that contained 0.5 U Taq DNA polymerase (Tangen, Beijing, China). Cycle parameters for p53, bcl-2, bax and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNAs were 15 s at 95℃, 30 s at 55℃, and 60 s at 72℃ for 33, 32, 30 and 20 cycles, respectively. Primer sequences were as follows: p53, 5'-GTTTCCGTCT GGGCTTCTTG-3' and 5'-CCTGGGCATCCTTGA GTTCC-3'; bcl-2, 5'-ACACTGTTAAGCATGTGCC G-3' and 5'-CCAGCTCATCTCACCTCACA-3'; bax, 5'-GGATGCGTCCACCAAGAA-3' and 5'-ACTCCCG CCACAAAGATG-3'; and G3PDH, 5'-ACCACAGTCC ATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCT GTA-3'.

Immunofluorescence staining

Cells were cultured on coverslips and received the same treatment of induced denutrition in PBS as described above. Cells were fixed in methanol at -20℃ for 20 min and washed in PBS containing 0.1% Tween 20 (Sanland Chemicals, Xiamen, China). After blocking with 10% goat normal serum for 1 h, fixed cells were incubated with primary antibodies, rabbit anti-Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-Bax (Santa Cruz Biotechnology), in a moist chamber at 4℃ overnight. Cells were washed in PBS containing 0.1% Tween 20, blocked again for 30 min, and treated

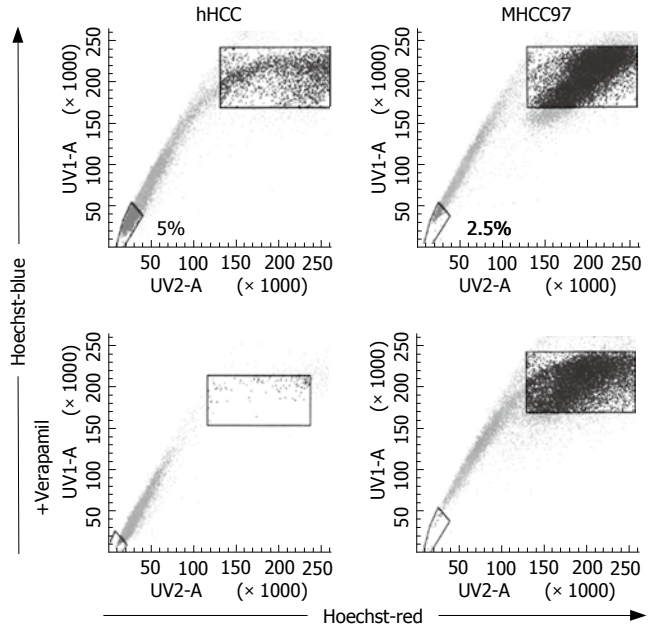

Figure 1 SP cells were detected in 0.25 and 0.5% of MHCC97 and hHCC cells, respectively. The SP cells disappeared with Hoechst 33342 and verapamil co-treatment.

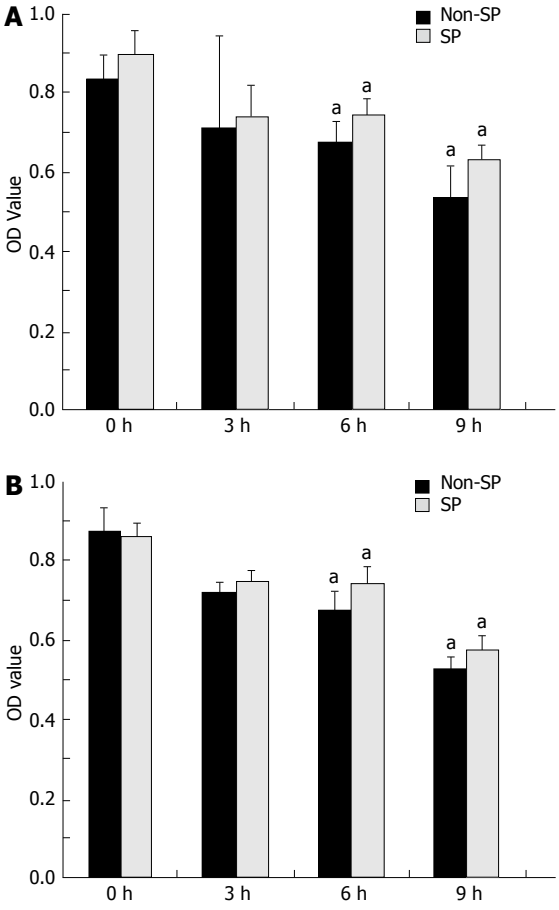

Figure 2 Proliferation of MHCC97 and hHCC cells at 3, 6 and 9 h during denutrition, as observed by MTT assay. SP cells purified from MHCC97 (**A**) and hHCC (**B**) cell lines demonstrated greater viability than the corresponding non-SP cells. $^{a}P < 0.05$.

with TRITC (Tetramethyl Rhodamine Isothiocyanate)-conjugated goat anti-rabbit IgG (Santa Cruz) and FITC (Fluorescein Isothiocyanate)-conjugated goat anti-mouse IgG (Santa Cruz) at 37℃ for 1 h. After washing in PBS, the coverslips were covered on slips with 30% glycerol phosphate buffer and examined under an Olympus IX70 microscope (Olympus, Japan).

Statistical analysis

Bands from RT-PCR were quantified by Smart View Bio-electrophoresis Image Analysis System software (Furi Science & Technology, Shanghai, China). Relative mRNA levels were calculated by referring them to the amount of G3PDH. Numerical data from the MTT assay were presented as the mean ± SEM. The difference between means was measured with Student's *t* test. All statistical analyses were performed using SPSS11.0 software (Chicago, IL, USA). $P < 0.05$ was considered as statistically significant.

RESULTS

Detection of SPs in HCC cells

Flow cytometry analysis with Hoechst 33342 staining demonstrated that MHCC97 and hHCC cells included 0.25% and 0.5% SP cells, respectively. The number of these SP cells was diminished in the presence of Hoechst 33342 and verapamil, a calcium channel blocker. The SP and non-SP cells in MHCC97 and hHCC cells were sorted separately and used for further experiments (Figure 1).

MTT assay

MTT assay was used to determine the proliferation of SP and non-SP cells under denutrition conditions. The results illustrated that denutrition inhibited proliferation of SP and non-SP cells in both cell lines in a time-dependent manner. The data showed that there was a difference between SP and non-SP cells at 6 and 9 h, and it seemed that SP cells had a greater proliferation ability than non-SP cells under denutrition (Figure 2).

RT-PCR

RT-PCR was used to detect mRNA expression levels in SP and non-SP cells in two cancer cell lines. The p53 gene was expressed weakly but steadily during the whole experiment. The bcl-2 gene was up-regulated, while the bax gene was down-regulated in SP cells under denutrition. Interestingly, the regulation of the two genes was reversed in non-SP cells. It may be that the upstream molecule which adjusted these two genes' expression was not P53 (Figure 3).

Immunofluorescence staining

Expression levels of Bcl-2 and Bax proteins were examined by immunofluorescence staining in hHCC and MHCC97 cell lines (Figure 4). It was clear that the expression of Bcl-2 increased in a time-dependent manner in SP cells, in contrast with non-SP cells. Conversely, the expression of Bax increased in non-SP cells in the same manner and decreased in SP cells.

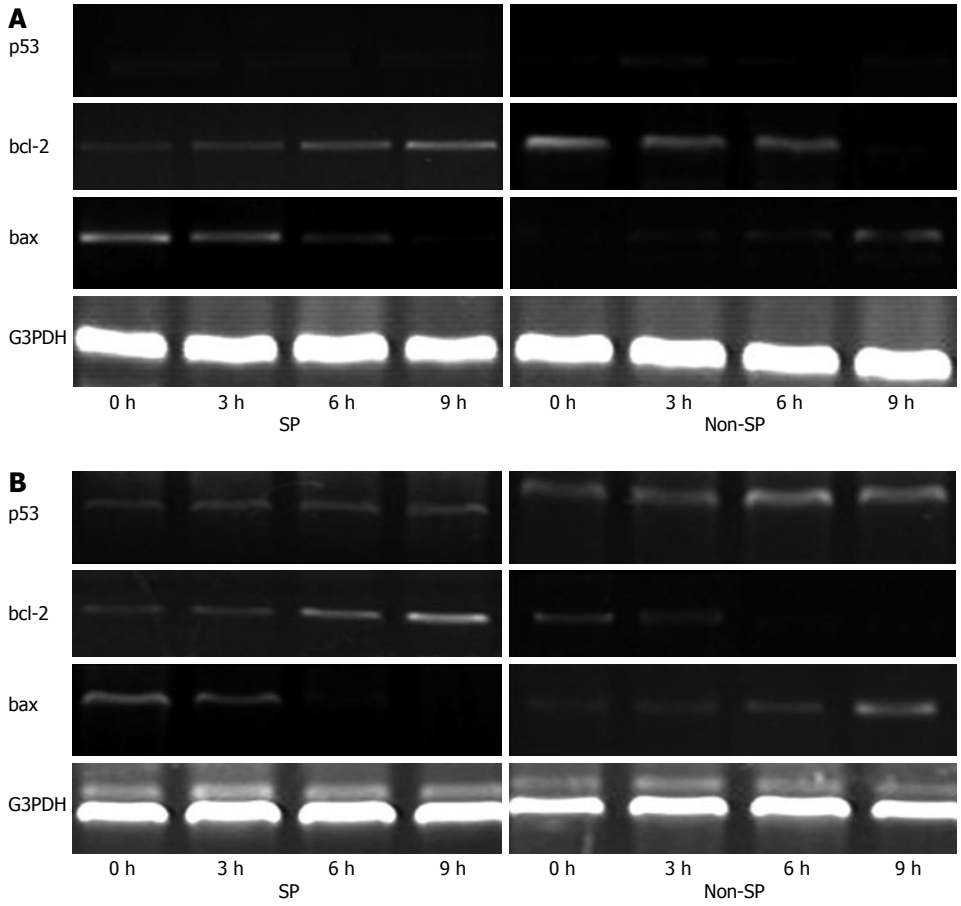

Figure 3 p53, bcl-2 and bax mRNA levels were evaluated by RT-PCR. bcl-2 levels were up-regulated in SP cells from MHCC97 (**A**) and hHCC (**B**) cell lines, and bax levels were down-regulated in non-SP cells during denutrition.

DISCUSSION

HCC ranks among the most common cancers in many countries. A recent estimate indicates that HCC represents the fifth most common cancer in males, and the eighth most common in females, with a total of 560 000 new cases each year, 83% of which occur in developing countries, and more than one-half in China alone. Moreover, because of its very poor prognosis, HCC represents the third leading cause of cancer death worldwide[10].

From the clinical standpoint, there are some conspicuous biological characteristics in HCC, such as anti-apoptosis, chemotherapy resistance, extensive proliferation, and even early metastasis. These characteristics are particularly prevalent in cases of relapse and metastasis.

Recently, it has been reported that CSCs are seen in many kinds of tumors and established cancer cell lines[15-18]. Using the previously described methods, we analyzed and sorted some of these CSCs, namely SP cells, in established HCC cell lines. The proportion of SP cells in the two cell lines was 0.25% and 0.5%, respectively. The MHCC97 (Metastatic Human Hepatocellular cancer 97) cell line was established from a highly metastatic case of HCC in 1997, and the hHCC (human Hepatocellular cancer) cell line was cultured from a case with a high level of chemotherapy resistance, with the proportion of SP cells being a little higher than those reported previously.

In clinic, whatever surgery or chemotherapy, it will bring denutrition directly or indirectly so as to inhibit the proliferation of cancer cells, beside destroy the structure of tumors or kill them. In clinics, surgery, radiotherapy and chemotherapy are used to destroy the structure of tumors, induce denutrition, kill cancer cells directly, and inhibit cancer cell proliferation. However, there are still some cells that can survive in denutrition conditions, and these may lead to relapse and metastasis. What difference is there between these and other cells? What mechanism is behind these phenomena? In our experiment, we simulated the denutrition conditions and observed the anti-apoptosis or proliferation ability of SP cells.

By MTT assay, we found that SP cells had better resistance to denutrition than non-SP cells. Using RT-PCR and immunofluorescence staining, we found that P53 may not be the key molecule that is responsible for the anti-apoptosis ability of SP cells. p53 was one of the most important genes in stabilizing the cell genome. It regulated the expression of numerous pro-apoptotic genes, such as bcl-2 and bax. Our results showed that the normal activity of P53 in the two cell lines may have been inhibited. The expression levels of two members of the Bcl-2 family were clearly altered between SP and non-SP cells; specifically, the expression of Bax was inhibited in SP and activated in non-SP cells, but the expression of Bcl-2 was reversed. Bax was a cytosolic monomer in viable cells but during apoptosis, it changed its conformation, integrated into the outer mitochondrial membrane, and was oligomerized. It provoked the permeabilization of the outer mitochondrial membrane (PT) and contributed to the release of pro-apoptotic factors into the cytosol, such as cytochrome C, which led to formation of the apoptosome and activation of the caspase cascade. However, the anti-apoptotic guardian Bcl-2 could bind Bax strongly, and this interaction

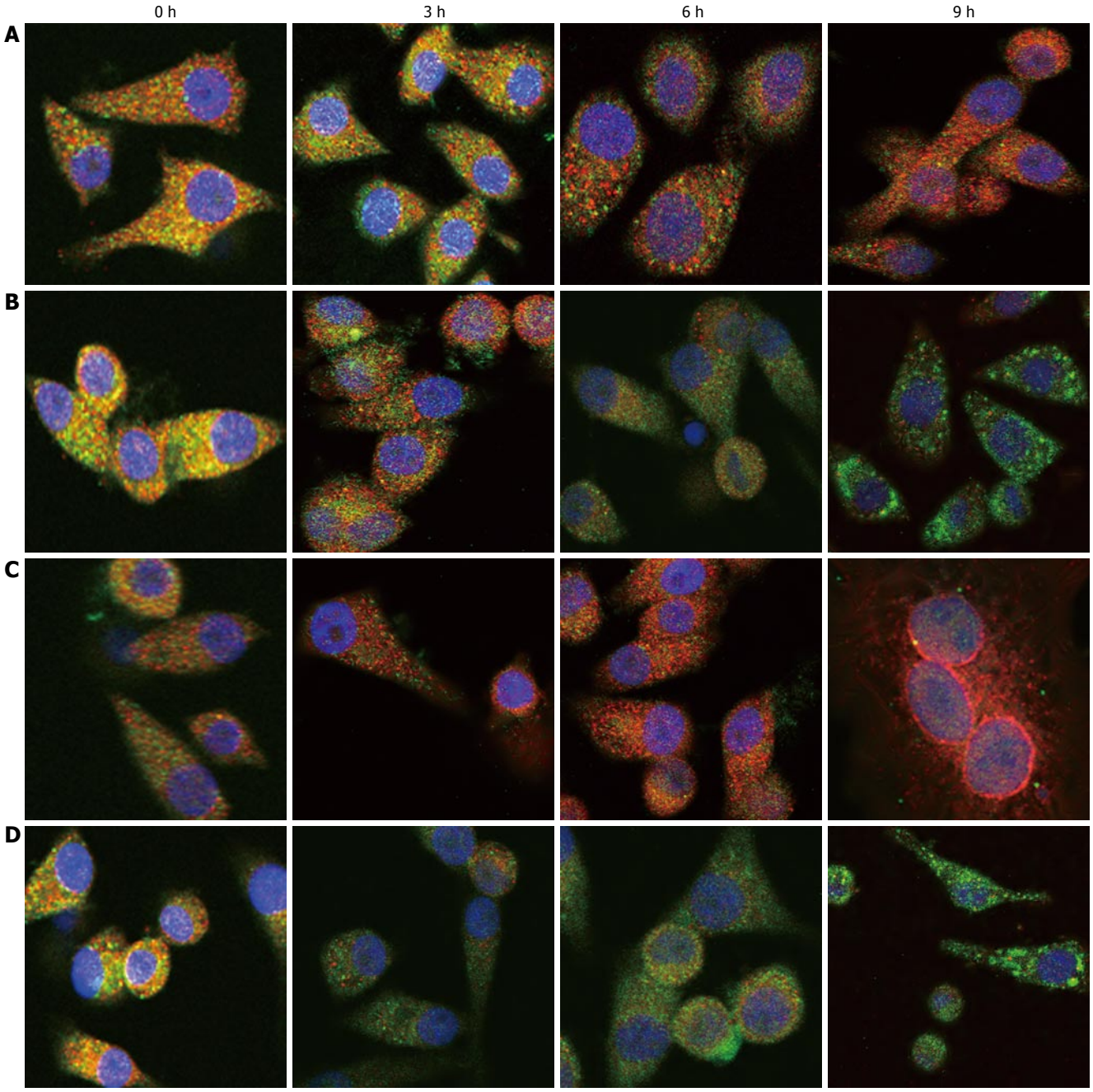

Figure 4 Bcl-2 (red) and Bax (green) expression was examined in SP (**A**) and non-SP (**B**) cells of MHCC97, and SP (**C**) and non-SP (**D**) cells of hHCC cells. The expression of Bcl-2 in SP cells was up-regulated, while Bax was down-regulated.

inhibited Bcl-2 activation, which sustained cell survival. In our study, the up-regulation of Bcl-2 and down-regulation of Bax were effective during the anti-apoptosis in SP cells. In other words, in MHCC97 and hHCC cell lines, SP cells had greater anti-apoptosis or proliferation ability than non-SP cells had. Expression of Bcl-2 and Bax had a pivotal role in the anti-apoptosis procedure during denutrition.

We have previously found that the expression level of alpha-fetoprotein (AFP) in SP cells is significantly higher than in non-SP cells in established HCC cell lines, e.g. MHCC97[19]. AFP is one of the most useful markers, and has been used in clinical diagnosis of HCC. AFP is synthesized in large quantities by the fetal yolk sac and the liver during embryonic development[20,21]. According to clinical experience, if a high level of AFP is found in the serum, the first thought is that the patient has HCC. If this appears after surgery or chemotherapy, it indicates a poor prognosis, such as recurrence or metastasis[22-27]. The ABC transporter for discharging Hoechst 33 342, which is called the breast cancer resistance protein, has a high efflux capacity with a wide substrate range, including mitoxantrone and methotrexate[28]. Further, the higher expression level of ABC transporter on SP cells indicates a possible relationship between them and clinical chemotherapy resistance.

Taking the experimental results and clinical experiences together, we found that the characteristics displayed in SP cells, such as high expression level of AFP and Bcl-2 and

drug efflux capacity, are consistent with the characteristics displayed in tumors, such as high expression level of AFP, high anti-apoptosis ability and chemotherapy resistance. We conclude that perhaps the existence and activity of SP cells are responsible for these clinical phenomena shown in HCC; moreover, it is reasonable to recognize SP cells as CSCs.

ACKNOWLEDGMENTS

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COMMENTS

Background

The theory of CSCs is one of the most significant theory in tumor research. According to the theory, there is some kind of pyramid cell structure in tumor cells, and CSCs are at its apex. They have self-renewal and differentiation abilities, and other cells are their descendents. CSCs have many biological activities, such as anti-drug and greater ability to proliferate. CSC theory also explains some clinic phenomena such as chemotherapy resistance and relapse. Therefore, research on the characteristics of CSCs may be the foundation of future clinical therapy.

Research frontiers

In 1996, Goodell sorted a certain kind of cells from mouse whole bone cells by cytometry, and he found that those cells expressed different biological characteristics, such as higher proliferation and multi-differentiation. Many workers have reported the existence of such cells in different tissues, and they have been collectively named SP cells. SP cells from tumors or tumor cell lines have greater proliferation and tumor formation ability, they resist drugs, and they maintain themselves in whole cells at a rate of a few percent.

Innovations and breakthroughs

The observation of SP cells backs up the theory of CSCs, which was introduced many years ago. Using Hoechst 33342 dye, it is possible to sort the cells by cytometry. SP cells have been found in HCC, brain tumor, prostate cancer and leukemia. By analyzing their biological abilities, it seemed that side population cells existed between the tip and bottom of the pyramid cell structure, and that they had intersection with real CSCs. Thus, though we can not be certain that SP cells are CSCs, it seems that that SP cells have a typical CSC phenotype.

Applications

In clinical cases, there have heretofore been many failures caused by tumor chemotherapy resistance or metastisis. CSC theory and the discovery of SP cells bring new hope for tumor therapy in the future. How to locate these cells in tumors, how to remove them completely by surgery, and how to inhibit or kill them by chemotherapy, are just a few of the clinical questions that need to be answered. Even a small advancement in this research field may result in a new breakthrough in tumor therapy.

Terminology

SP cells were first discovered by Goodell when he analyzed mouse bone cells by cytometry using Hoechst 33342. They were named after their location in 2-D cytometry charts. They display low fluorescence, and are located at the edge of the chart, away from other cells.

Peer review

SP cells from tumors or tumor cell lines are one of the hottest topics in tumor research. This study confirmed their existence and numbers in two human HCC cell lines.

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RAPID COMMUNICATION

Effect of fluoxetine on depression-induced changes in the expression of vasoactive intestinal polypeptide and corticotrophin releasing factor in rat duodenum

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Abstract

AIM: To investigate changes in vasoactive intestinal polypeptide (VIP) and corticotrophin releasing factor (CRF) in the plasma and duodenum of chronic stress-induced depressed rats and the effects of fluoxetine hydrochloride (fluoxetine) treatment on depression-induced changes in VIP and CRF.

METHODS: A Sprague-Dawley rat model of chronic stress-induced depression was produced. Thirty experimental rats were randomly divided into the following groups: control group, saline-treated depressed group, and fluoxetine-treated depressed group. Open-field testing was performed to assess the rats' behavior. VIP and CRF levels in plasma were measured by ELISA. Immunofluorescence techniques combined with laser scanning confocal microscopy (LSCM) were used to investigate VIP and CRF expression in the duodenum.

RESULTS: The open-field behavior, both crossing and rearing, of depression model rats, decreased significantly compared with those of normal control rats over 5 min. Defecation times increased significantly. Compared to the control group, FITC fluorescence of duodenal CRF expression and plasma CRF levels in the depressed rats increased significantly (fluorescence intensity of duodenal CRF: 11.82 ± 2.54 *vs* 25.17 ± 4.63; plasma CRF: 11.82 ± 2.54 ng/L *vs* 25.17 ± 4.63 ng/L, $P < 0.01$), whereas duodenal VIP expression and plasma VIP levels decreased significantly (fluorescence intensity of duodenal VIP: 67.37 ± 18.90 *vs* 44.51 ± 16.37; plasma VIP: 67.37 ± 18.90 ng/L *vs* 44.51 ± 16.37 ng/L, $P < 0.01$). Fluoxetine improved depressed behavior, increased VIP expression and decreased CRF expression in plasma and the duodenal tissue of depressed rats.

CONCLUSION: Chronic stress can induce injury to the duodenum, accompanied by increasing CRF and decreasing VIP in the plasma and duodenum. Treatment with fluoxetine can ameliorate pathological changes in the duodenum of depressed rats, which suggests that antidepressants are an effective therapeutic agent for some duodenal diseases caused by chronic stress. VIP is a potential therapeutic strategy.

Key words: Depression; Plasma; Duodenum; Rat; Vasoactive intestinal polypeptide; Corticotrophin releasing factor; Fluoxetine hydrochloride

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INTRODUCTION

In clinical studies, it has become clear that psychological factors, especially anxiety and depression, play an important role in gastrointestinal diseases by precipitating exacerbation of symptoms[1,2]. Several studies have shown that the prevalence of chronic stress disorders in patients with gastrointestinal symptoms is about 60%-85%[3,4]. Stress often worsens the symptoms of gastrointestinal diseases, which might be explained by altered neuroendocrine and visceral sensory responses to stress[5].

In recent years, along with extensive research on the enteric nerve system (ENS), increasing evidence shows that peptidergic neurotransmitters could regulate gastrointestinal diseases. Vasoactive intestinal peptide (VIP), a 28-amino acid peptide, was first discovered, isolated, and purified from porcine intestinal extracts[6]. It was also found in submucous and myenteric plexuses,

as well as the central and peripheral nervous systems[7]. It is now recognized as a major neuropeptide in the brain and gut, with functions ranging from neurotransmission to neuromodulation with neurotrophic properties. Corticotrophin releasing factor (CRF) is a 41 amino-acid peptide which stimulates adrenocorticotropic hormone (ACTH) secretion. Some data strongly suggest that CRF plays an important role in the pathophysiology of gastrointestinal diseases and electrophysiological properties of the brain during visceral perception[8]. Patients with gastrointestinal diseases may have a higher tone of corticotropin-releasing hormone (CRH) in the brain. In common, both central and peripheral nervous pathways are involved in the release of gastrointestinal hormones due to psychological stress, thus modulating gastrointestinal motility[9]. A large body of evidence derived from experiments suggests that CRF can accelerate small intestine transit, while VIP can inhibit it[10].

Fluoxetine is a SSRI (selective serotonin re-uptake inhibitor), which are a class of antidepressants used in the treatment of depression and anxiety disorders. SSRIs increase the extracellular expression of the neurotransmitter serotonin by inhibiting its re-uptake into the presynaptic cell. Serotonin is also involved in the regulation of carbohydrate metabolism. Few analyses of the role of SSRIs in treating depression have covered the effects on carbohydrate metabolism from intervening in serotonin handling by the body. Studies have suggested that SSRIs may promote the growth of new neural pathways or neurogenesis[11]. Also, SSRIs may protect against neurotoxicity caused by other compounds as well as from depression itself. Recent studies have shown that pro-inflammatory cytokine processes occur during depression in addition to somatic disease, and it is possible that symptoms manifested in these psychiatric illnesses are being attenuated by the pharmacological effects of antidepressants on the immune system[12]. SSRIs have been shown to be immunomodulatory and anti-inflammatory against pro-inflammatory cytokine processes[13,14].

However, there has been no report so far concerning the changes in VIP and CRF aroused by depression in plasma and duodenal tissue, and the effect of antidepressants on the duodenum. Therefore, we devised a rat depression model and observed the levels of VIP and CRF in the plasma and duodenum, and the effect of fluoxetine on the duodenum of depressed rats.

MATERIALS AND METHODS

Animals

Forty healthy male Sprague-Dawley rats, weighing 250 ± 30 g, from the Animal Center, Academy of Hubei Preventive Medical Sciences, were employed in the present study. The animals were fed standard rat chow, allowed access to tap water and were acclimated to their surroundings for 1 wk prior to the experiments. After this period, 30 rats were selected according to their open-field behavior.

Reagents

FITC (Fluorescein isothiocyanate)-conjugated goat anti-rabbit IgG, VIP and CRF rabbit anti- mouse antibodies were purchased from Sigma Co., USA. Fluoxetine hydrochloride capsules were purchased from Lilly Co. Ltd., and ELISA kits were purchased from Beijing SUNBIO Biological Technology Co. Ltd. Other reagents used in the study were all of analytical grade.

Experimental protocols (preparation of the rat depression model treated with saline or fluoxetine)

A rat model of chronic stress-induced depression was established[15-17]. The rats received a variety of stressors for 21 d, including tail nip for 1 min, cold water swimming at 4℃ for 5 min, heat stress at 45℃ for 5 min, water deprivation for 24 h, food deprivation for 24 h, 12-h inverted light/dark cycle (7:00 a.m. lights off, 7:00 p.m. lights on), paw electric shock (electric current 1.0 mA 10 s, every 1 min, lasting for 10 s, 30 times), *etc.* Stressors were administered throughout the experiment, could occur at any time of day (or night), and were each applied for a period of between 8 and 24 h. Their sequence was at random in order to be completely unpredictable to the animal. The animals were randomly divided into three groups (10 rats per group): model control, saline + chronic stress-induced, fluoxetine + chronic stress-induced therapy group. The depressed animals were treated with normal saline or fluoxetine (10 mg/kg) by stomach, (once a day, from the 24 h after the depressed model was established until the end of the experiment). A normal control group of rats (10 rats) without receiving any stress was included and housed in a separate room; food and water were freely available in their home cage.

Open field test (OFT)

The open-field test was designed to measure the reaction of rats to a novel environment. In this test, rats were individually placed in the center of a square, wooden, white-colored open-field box with 36 squares measuring 10 cm × 10 cm each. Their activity was assessed for 5 min. The number of squares from which rats crawled out was the total number of crossings. The number of occasions on which the animals stood on their hind legs was the total number of rearings. Defecation times were counted every 5 min. Each rat was housed in one cage and fasted before sucrose intake testing, after which 10 g/L sucrose solution consumption in 24 h was examined.

Assessment of duodenal histological damage

Duodenal tissue was sampled for a variety of determinations after the rats were anesthetized with 200 d/L urethane. Duodenal tissue was fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, sectioned in 4 μm thick sections, and stained with haematoxylin and eosin. The criteria of the histological score used was a previously validated scoring system from 0 to 4 that depends on the number and size of ulcers as well as the presence or absence of adhesions[18,19]: (1) the infiltration of acute inflammatory cells: 0 = no, 1 = mild increasing, 2 = severe increasing; (2) the infiltration of chronic inflammatory cells: 0 = no, 1 = mild increasing, 2 = severe increasing; (3) the deposition of fibrotin protein: 0 = negative, 1 = positive; (4) submucosa edema: 0 =

Table 1 Open-field activities and sucrose intake test of rats (n = 10, mean ± SD)

Group	Crossing/5 min	Rearing/5 min	Defecation/5 min	Sucrose intake mL/24 h
Control	133.00 ± 11.309	28.53 ± 10.22	3.90 ± 0.57	36.18 ± 10.24
Saline + depressed	51.80 ± 7.441[b]	11.10 ± 5.18[b]	6.80 ± 0.57[b]	8.95 ± 7.39[b]
FH + depressed	76.60 ± 3.534[d]	15.58 ± 7.367[d]	5.10 ± 0.43[d]	18.10 ± 6.43[d]

FH: Fluoxetine hydrochloride. [b]$P < 0.01$ *vs* control group; [d]$P < 0.01$ *vs* saline-treated depressed group.

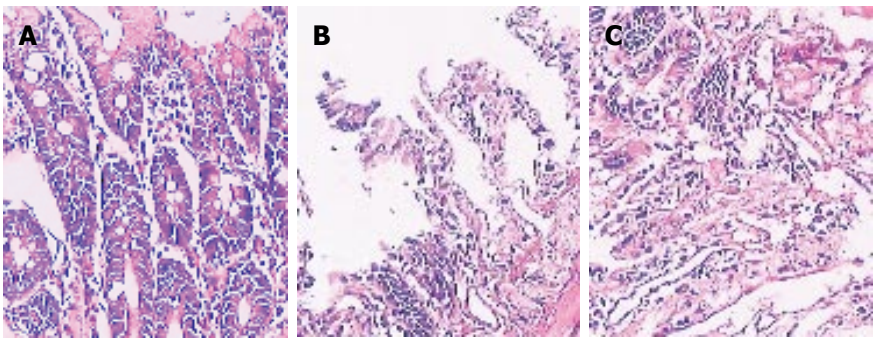

Figure 1 Haematoxylin and eosin staining of duodenum tissues. **A:** No damage in the normal control group (HE, × 200); **B:** Histological changes in the FH + depressed group (HE, × 200); **C:** Histological changes in the saline + depressed group (HE, × 200).

none, 1 = patchy-like, 2 = fusion-like; (5) epithelial necrosis: 0 = no, 1 = limiting, 2 = widening; (6) epithelial ulcers: 0 = negative, 1 = positive. The ulceration, inflammation, lesion and fibrosis were scored and put together as a result ranging between the minimum of 0 and maximum of 10.

Measurement of plasma VIP and CRF

Immediately after the rats were sacrificed, blood samples were collected into chilled tubes containing 0.3 μL ethylenediamine tetraacetic acid (EDTA) and 1000 KIU aprotinin. Blood samples were immediately centrifuged at 3500 r/min at 4℃ for 10 min. The supernatant was aspirated and stored at -70℃ until analysis. VIP and CRF were determined by enzyme linked immunosorbent assays (ELISA) according to the manufacturer's instructions.

Detection of duodenal VIP, CRF expression

Duodenal tissue was fixed in 4% paraformaldehyde for 4 h. One hundred micron sections from the primary tissue were employed in the fluorescent immunohistochemical analysis, which used rabbit anti-rat VIP/CRF antibody, diluted 1:500 in phosphate-buffered saline (PBS). The staining procedure was as follows: (1) the sections were washed in PBS, then pretreated with 0.25% Triton X-100 for 30 min at 37℃ and rinsed in PBS; (2) incubation for 12 h at 4℃ in a 1:500 dilution of the primary antibody of VIP /CRF in PBS; (3) incubation with 1:100 diluted secondary antibodies (FITC -conjugated goat anti-rabbit IgG) in PBS for 30 min at 37℃. The sections were washed three times for 10 min after incubation steps 1 to 3, respectively, and were finally mounted in 50 g/L glycerin.

Detection was carried out according to the kit instructions (Leica SP2 TCS AOBS made in Germany). The specimens were excited with a laser beam at a wavelength of 488 nm (FITC). Five visual fields in three sections of each tissue were randomly selected and observed under a laser scanning confocal microscope (LSCM) and analyzed with a Leica Q500IW image analysis system in terms of FITC fluorescent intensity. This study recorded the relative value of fluorescence intensity for the expression of VIP and CRF.

Statistical analysis

The data were expressed as the mean ± SD and analyzed with SPSS 11.5 statistic software. Statistical analysis was performed by using one-way ANOVA and Student-Newman-Keuls test for multiple comparisons. A P value less than 0.05 was considered statistically significant.

RESULTS

Open-field behavior of depression model rats (both crossing and rearing), was significantly decreased compared with that of the normal control rats (Table 1, $P < 0.01$). Defecation times significantly increased. The consumption of 10 g/L sucrose solution significantly decreased compared with that of the normal control (Table 1, $P < 0.01$). Treatment with fluoxetine hydrochloride (FH) significantly attenuated these effects.

Histological evaluation of the duodenum

No histological damage was seen in the normal control group. Rats with chronic stress-induced duodenitis showed neutrophil, macrophage, lymphocyte and eosinophil infiltration in the mucosa and submucosa. Ulceration and mucosal damage was obvious. Treatment with fluoxetine significantly attenuated the extent and severity of the histological signs. Damage scores of duodenum tissues were as follows: control: 0.39 ± 0.51; saline plus depressed: 7.46 ± 2.14; and FH plus depressed: 4.81 ± 1.37 (Figure 1).

VIP and CRF concentrations in plasma

Plasma VIP levels showed a significant difference among the three groups (Table 2, $P < 0.01$). VIP levels were higher in control and fluoxetine plus depression groups; however, they decreased significantly in the depressed group.

Plasma CRF levels showed a significant difference

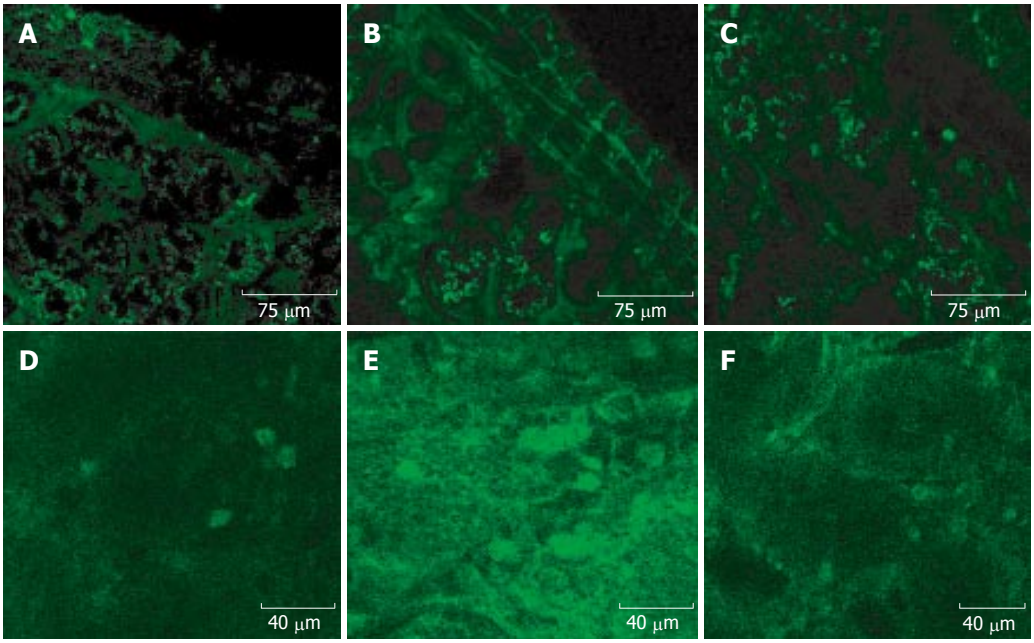

Figure 2 Duodenal tissue staining procedure included: (1) pretreatment with 0.25% Triton X-100; (2) incubation in the primary rabbit anti-rat antibody of VIP/CRF; (3) incubation with secondary antibodies (FITC -conjugated goat anti-rabbit IgG). Expression of FITC-labeled VIP/CRF (green). **A:** Expression of VIP in the control group; **B:** Expression of VIP in the depressed group; **C:** Expression of VIP in the FH + depressed group; **D:** Expression of CRF in the control group; **E:** Expression of CRF in the depressed group; **F:** Expression of CRF in the FH + depressed group.

Table 2 Changes of levels of VIP/CRF in plasma (n = 10, mean ± SD)

Group	VIP in Plasma (ng/L)	CRF in Plasma (ng/L)
Control	67.37 ± 18.90	11.82 ± 2.54
Saline + depressed	44.51 ± 16.37[b]	25.17 ± 4.63[b]
FH + depressed	60.86 ± 19.27[d]	17.05 ± 3.69[d]

[b]P < 0.01 *vs* control group; [d]P < 0.01 *vs* saline-treated depressed group.

among the three groups (Table 2, P < 0.01). CRF levels were lower in control and fluoxetine plus depression groups, but increased significantly in the depressed group.

VIP and CRF alterations and the effects of fluoxetine on the content of VIP and CRF in duodenum tissue of depression model rats

Compared with that of normal control rats, the average fluorescence intensity of duodenal CRF increased significantly, while the average fluorescence intensity of duodenal VIP decreased significantly in chronic stress-induced depressed rats (Table 3, P < 0.01). Furthermore, a significant improvement of the elevated duodenal VIP content and the significant reduction of duodenal CRF content were observed in animals treated with fluoxetine (Figure 2, Table 3, P < 0.01).

DISCUSSION

We found that experimental rats had almost all demonstrable symptoms of depression, consistent with the classic and mature model of depression[16,20]. Our results showed that both crossing and rearing behavior of depressed rats over five minutes significantly decreased compared with that of the normal control rats (Table 1, P < 0.01). Defecation times significantly increased. The consumption of 10 g/L sucrose solution significantly decreased compared with that of the normal control.

Table 3 The average fluorescence intensity analysis of VIP and CRF alterations in duodenum of depression model rats (n = 10, mean ± SD)

Group	Fluorescence intensity of VIP	Fluorescence intensity of CRF
Control	36.28 ± 17.16	10.87 ± 9.28
Saline + depressed	19.07 ± 13.84[b]	50.83 ± 24.66[b]
FH + depressed	28.29 ± 15.02[d]	29.18 ± 17.34[d]

FH: Fluoxetine hydrochloride; VIP: Vasoactive intestinal polypeptide; CRF: Corticotrophin releasing factor. [b]P < 0.01 *vs* control group; [d]P < 0.01 *vs* saline-treated depressed group.

Crossing reflected the degree of animal activity, rearing reflected the degree of curiosity to the novel surroundings, defecation times responded to intestinal function, and sucrose intake tests reflected the animal's response to rewards[21,22]. The chronic stressors caused a generalized decrease in action and responsiveness to rewards, and a functional gastrointestinal disorder. The behavioral changes of the depressed rat were reversed by chronic treatment with fluoxetine hydrochloride (a type of antidepressant).

On the other hand, rats with chronic stress-induced depression showed significant histological damage from duodenitis. For example, a number of neutrophils, macrophages, lymphocytes and eosinophils were found in the mucosa and submucosa; ulceration and mucosal damage could also be observed. No change was seen in the normal control group, and treatment with fluoxetine hydrochloride significantly attenuated the extent and severity of the histological signs. The antidepressant fluoxetine hydrochloride inhibited the extent of inflammation, prevented mucosa injury, minimized the ulceration area, and alleviated the duodenitis seen in the depressed animals.

In this study, we also found that there were different changes in VIP and CRF in the depressed rats' duodenum and plasma. The average fluorescence intensity of duodenal CRF expression of the depression model rats

increased significantly compared with that of the normal control rats, whereas that of duodenal VIP expression decreased significantly.

Brain gut peptides (BGPs) are distributed extensively in the brain and the gastrointestinal tract. Studies have demonstrated that some BGPs including VIP and CRF participate in gastrointestinal motility, secretion and absorption[23]. Several investigations have found that basal CRF levels have increased significantly during stress in patients. Because the gut and the brain are highly integrated and communicate in a bidirectional fashion largely through the ANS and HPA axis, patients also responded with higher expression of ACTH during stress and had higher basal expression of noradrenalin than the normal group. Stress induced exaggeration of the neuroendocrine response and visceral perceptual alterations occur during and after stress by CRF[6]. On the other hand, some studies have indicated that VIP participated in the modulatory effect of drugs on gastrointestinal motility and played an important role in gastrointestinal disorders caused by psychological stress[24]. It had been demonstrated that stress-induced plasma VIP expression decreased gastrointestinal transit disorder beyond a certain intensity range of stress. VIP had potent protective activity against sepsis and increased the survival rate of septic animals[25]. In this study, the significant increase in the expression of CRF possibly suggests that depression could induce an inflammatory response of the duodenum by releasing CRF in rats. The effect of VIP on inflammatory cells could be an additional important mechanism of its potent protective activity on chronic stress-induced duodenitis. The results of our studies suggest that gastrointestinal motility disorders during psychological stress may be partially mediated by release of VIP and CRF.

Our results also show that an antidepressant plays an important role in decreasing symptoms in depressed rats. The behavioral changes of depressed rats were reversed by chronic treatment with fluoxetine. Treatment with fluoxetine significantly attenuated the extent and severity of the histological signs. Fluoxetine at the therapeutic dose of 10 mg/kg was effective in decreasing the expression of CRF and increasing the expression of VIP in the duodenum of depressed rats. Some data has shown that antidepressants may adjust other brain gut peptides or unknown factors, and thus ameliorate the damage of chronic stress-induced gastrointestinal disorders[26-28]. Future serotegenic antidepressants may be made to specifically target the immune system by either blocking the actions of pro-inflammatory cytokines or increasing the production of anti-inflammatory cytokines[29,30].

In summary, brain-gut interaction and psychological factors altered not only the pathology of brain tissue, but also duodenal tissue. The results of our study show that depression can induce injury to the duodenum accompanied by increasing CRF and decreasing VIP. Treatment with fluoxetine can ameliorate pathological changes in the duodenum in depressed rats, suggesting that SSRIs are an effective therapeutic agent for some duodenum diseases caused by psychological factors. We suggest that VIP prevention of inflammatory cell reactivity could be a potential therapeutic strategy for chronic stress-induced gastrointestinal disorders.

COMMENTS

Background

In clinical studies, it has become clear that depression plays an important role in gastrointestinal diseases by precipitating exacerbation of symptoms. Stress often worsens the symptoms of gastrointestinal diseases.

Research frontiers

Some data strongly suggested that corticotrophin releasing factor(CRF) and vasoactive intestinal peptide(VIP) played important roles in pathophysiology of gastrointestinal diseases. Selective serotonin reuptake inhibitors (SSRI's) have been shown to be immunomodulatory and anti-inflammatory against pro-inflammatory cytokine processes.

Innovations and breakthroughs

We set up a rat depression model, and observed the level of VIP and CRF of plasma and duodenum and the effect of fluoxetine on duodenum of the depressed rats.

Applications

The authors of the present study showed that chronic stress can induce injury to the duodenum. This was accompanied by an increase in immunofluorescence staining for CRF and a decrease in immunofluorescence staining for VIP. Treatment with the serotonin reuptake inhibitor, fluoxetine, reversed these changes and in addition reversed the behavioral changes of depressed rats.

Terminology

Vasoactive intestinal peptide (VIP) was found in submucous and myenteric plexus, central and peripheral nervous systems. It is now recognized as a major neuropeptide in the brain and gut. Corticotrophin releasing factor (CRF) stimulates adrenocorticotropic hormone (ACTH) secretion and plays an important role in the pathophysiology of gastrointestinal diseases.

Peer review

The authors of the present study showed that chronic stress can induce injury to the duodenum. This was accompanied by an increase in immunofluorescence staining for CRF and a decrease in immunofluorescence staining for VIP. Treatment with the serotonin reuptake inhibitor, fluoxetine, reversed these changes but in addition reversed the behavioral changes of depressed rats.

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RAPID COMMUNICATION

Protective effect of inducible nitric oxide synthase inhibitor on pancreas transplantation in rats

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Abstract

AIM: To investigate the effect of inducible nitric oxide synthase inhibitor, aminoguanidine, on pancreas transplantation in rats.

METHODS: A model of pancreas transplantation was established in rats. Streptozotocin-induced diabetic male Wistar rats were randomly assigned to sham-operation control group (n = 6), transplant control group (n = 6), and aminoguanidine (AG) treatment group (n = 18). In the AG group, aminoguanidine was added to intravascular infusion as the onset of reperfusion at the dose of 60 mg/kg, 80 mg/kg, 100 mg/kg body weight, respectively. Serum nitric oxide (NO) level, blood sugar and amylase activity were detected. Nitric oxide synthase (NOS) test kit was used to detect the pancreas cNOS and inducible NOS (iNOS) activity. Pancreas sections stained with HE and immunohistochemistry were evaluated under a light microscope.

RESULTS: As compared with the transplant control group, the serum NO level and amylase activity decreased obviously and the evidence for pancreas injury was much less in the AG group. The AG (80 mg/kg body weight) group showed the most significant difference in NO and amylase (NO: 66.0 ± 16.6 *vs* 192.3 ± 60.0, P < 0.01 and amylase: 1426 ± 177 *vs* 4477 ± 630, P < 0.01). The expression and activity of tissue iNOS, and blood sugar in the AG (80 mg/kg body weight) group were much lower than those in the transplant control group (iNOS: 2.01 ± 0.23 *vs* 26.59 ± 5.78, P < 0.01 and blood sugar: 14.2 ± 0.9 *vs* 16.8 ± 1.1, P < 0.01).

CONCLUSION: Selective iNOS inhibitor, aminoguanidine as a free radical, has a protective effect on pancreas transplantation in rats by inhibiting NO and reducing its toxicity.

INTRODUCTION

Pancreas transplantation is frequently complicated by acute pancreatitis, largely due to ischemia/reperfusion injury secondary to cold preservation[1,2]. During the reperfusion period, oxygen-derived free radicals can lead to a severe impairment. Nitric oxide (NO) is a free radical with a strong reactivity, and has a fierce cytotoxicity. However, NO can significantly dilate blood vessels and remit vasospasm of grafts. Therefore, NO plays an ambivalent role in ischemia/reperfusion during pancreas transplantation. In this study, we established a model of pancreas transplantation in rats to investigate the expression of nitric oxide synthase (NOS) isoforms, and the effect of inducible nitric oxide synthase (iNOS) inhibitor (aminoguanidine) on pancreas transplantation.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing 250-300 g (Experimental Animal Center, China Medical University, China) were used as donors and recipients. The animals were kept in standard conditions with free access to water and rodent chow. Diabetes was induced by intravenous injection of streptozotocin at a single dose of 55 mg/kg body weight. Only rats with non-fasting plasma glucose levels of more than 22 mmol/L were used as recipients. We performed recipient transplantation surgery on days 14 and 15 after the injection of streptozocin. A total of 30 recipient animals were randomly assigned to the sham-operation group (n = 6) in which animals underwent midline laparotomy only, transplant control group (n = 6) in which animals underwent transplantation and received a bolus injection of saline instead of aminoguanidine, and aminoguanidine treatment group (n = 18) in which animals

Table 1 Serum NO level and amylase activity 4 h after transplantation (mean ± SD)

Group	*n*	NO (μmol/L)	Amylase (U/dL)
Sham-operation group	6	30.0 ± 3.5	342 ± 73
Transplant control group	6	192.3 ± 60.0[b]	4477 ± 630[f]
AG-60 mg/kg body weight	6	137.3 ± 21.1	2848 ± 354
AG-80 mg/kg body weight	6	67.9 ± 19.5[d]	1494 ± 263[h]
AG-100 mg/kg body weight	6	66.0 ± 16.6	1426 ± 177

[b]$P < 0.01$, [f]$P < 0.01$ *vs* sham-operation group; [d]$P < 0.01$, [h]$P < 0.01$ *vs* transplant control group.

underwent transplantation. Before reperfusion, a bolus injection of aminoguanidine (60 mg/kg, 80 mg/kg or 100 mg/kg body weight) was given *via* the vena dorsalis penis.

Transplantation and collection of specimens

Synergetic pancreaticoduodenal transplantation was performed in diabetic recipients to assess islet cell functions. After overnight fasting with free access to water, the rats were anesthetized and underwent heterotopic pancreaticoduodenal transplantation as previously described[3] with certain modifications. After shaving and disinfecting the abdomen with 75% alcohol, a midline incision was made. The donor pancreas was isolated on an aortic segment branching off the celiac axis and the superior mesenteric artery. The venous outflow was provided by the portal vein. Pancreas grafts were flushed with and stored in cold (4℃) heparinized lactate Ringer's solution. Heterotopic intra-abdominal transplantation was performed by end-to-side anastomosis of the aortic segment of the graft and the recipient infrarenal aorta. The graft portal vein was anastomosed to the recipient vena cava using the same technique. Enteric diversion of exocrine graft secretion was accomplished by end-to-side duodenojejunostomy. The abdomen was closed in two layers with 2-0 silk suture. After a single intramuscular injection of 5 mg cefamandole post-operation, the rats were kept under warming lamps until they became active. The warming and cooling ischemic time was less than 15 min and 25 min, respectively. The animals were killed after 4 h of reperfusion. The pancreas was harvested and divided into two segments with one fixed in 10% PBS formalin and the other preserved at -70℃. The blood was withdrawn without anticoagulant and centrifuged at 2000 r/min for 10 min. The serum was preserved at -20℃.

Determination of serum NO and NOS levels

Nitrate reductase was used to detect the serum NO level and NOS test kit was used to detect the cNOS and iNOS activity in the pancreas.

Determination of serum blood and amylase levels

Serum glucose concentration was measured with an Exac Tech blood glucose meter in samples collected from the cut tip of the tail. Serum amylase concentration was measured with a multianalyzer (Clinilizer, CL-7150, Nippon Denshi, Tokyo, Japan).

Histopathology examination

One pancreas segment was fixed in 10% PBS formalin, dehydrated through a grade ethanol series, washed in xylene and embedded in paraffin. The segment was cut into 4 μm-thick sections. The sections were stained with haematoxylin and eosin and evaluated using light microscope.

Immunohistology

Primary antibody and anti-iNOS polyclonal antibody were produced in rabbits. Strept avidin-biotin complex immunoperoxidase staining system was used, and the positive staining was reddish-brown in color.

Statistical analysis

The data were presented as mean ± SD. All statistical analyses were performed using the SPSS 10.0 software. Differences in groups were tested by analysis of variance (ANVOA). $P < 0.05$ was considered statistically significant.

RESULTS

Serum NO level

The NO level increased significantly in transplant control group and deceased in the sham-operation group ($P < 0.01$) 4 h after reperfusion. After administration of aminoguanidine (AG), a selective iNOS inhibitor, NO level decreased significantly ($P < 0.01$). The effect of AG (80 mg/kg body weight) was obviously better than that of AG (60 mg/kg body weight) ($P < 0.01$). However, the effect of AG (100 mg/kg body weight) was not better than that of AG (80 mg/kg body weight) ($P > 0.05$) (Table 1).

Serum amylase activity

The amylase activity was higher in the transplant control group than in sham-operation group ($P < 0.01$). After administration of AG, the amylase activity decreased markedly, and the effect of AG (80 mg/kg body weight) was better ($P < 0.01$) (Table 1).

Blood sugar level

The blood sugar level decreased after pancreas transplantation, and was the lowest in the AG (80 mg/kg body weight) group ($P < 0.01$) (Table 2).

Activity of NOS isoforms in pancreas tissue

Four hours after reperfusion, the iNOS activity in pancreas tissue increased significantly ($P < 0.01$), but the cNOS activity had no change ($P > 0.05$). After administration of AG (80 mg/kg body weight), the iNOS activity decreased obviously ($P < 0.01$) while the cNOS activity remained normal (Table 3).

Histology

The pancreas was enlarged and swollen in the transplant control group, and appeared relatively normal in all animals of the sham-operation and AG (80 mg/kg body weight) groups. The microscopic pancreatic injury, as indicated by intracytoplasmic vacuoles, interstitial oedema, polymorphonuclear cell infiltrate, venous congestion, and

Table 2 Blood sugar level 4 h after transplantation (mean ± SD)

Group	*n*	Pretransplantation (mmol/L)	Posttransplantation (mmol/L)
Sham-operated control	6	19.6 ± 1.4[a]	-
Transplant control group	6	20.1 ± 2.0[a]	16.9 ± 2.0
AG-60 mg/kg body weight	6	19.9 ± 1.5[a]	16.8 ± 1.1
AG-80 mg/kg body weight	6	19.8 ± 1.7[a]	14.2 ± 0.9[b]
AG-100 mg/kg body weight	6	20.5 ± 1.6[a]	15.1 ± 1.8[c]

[a]$P > 0.05$, [b]$P < 0.01$ *vs* AG-60 mg/kg body weight post transplantation; [c]$P > 0.05$ *vs* AG-80 mg/kg body weight post transplantation.

Table 3 Activity of NOS isoforms in pancreatic tissue 4 h after transplantation (mean ± SD)

Group	*n*	cNOS (U/mL)	iNOS (U/mL)
Sham-operation group	6	5.35 ± 1.01[a]	1.87 ± 0.19
Transplant control group	6	5.91 ± 0.71[a]	26.59 ± 5.78[b]
(AG-80 mg/kg body weight)	6	5.64 ± 0.97[a]	2.01 ± 0.23[d]

[a]$P > 0.05$, [b]$P < 0.01$ *vs* sham-operation group; [d]$P < 0.01$ *vs* transplant control group.

local tissue hemorrhage and necrosis occurred 4 h after transplantation (Figure 1A and B). However, none of the samples from the AG (80 mg/kg body weight) group revealed histological evidence of pancreatic injury (Figure 1C and D).

Immunohistochemistry

Four hours after reperfusion, heavily stained specimens from transplant control group were positive for anti-iNOS, while iNOS staining was mainly localized on the endothelium, vascular smooth muscle, and islet cells (Figure 1E and F). No stained anti-iNOS antibody was detected in all specimens from the AG (80 mg/kg body weight) group (Figure 1G and H).

DISCUSSION

A model of pancreas transplantation in rats was established. Four hours after pancreas graft reperfusion, the expression and activity of iNOS on pancreas increased significantly, serum NO level and amylase activity, leading to severe pancreatitis, whereas cNOS remained normal. After administration of AG, the iNOS activity and NO concentration decreased, the toxicity of NO as free radicals was reduced, and the amylase activity decreased markedly. The severity of ischemia/reperfusion injury and postgraft pancreatitis was reduced, protecting the pancreas graft against ischemia/reperfusion injury.

Ischemia/reperfusion injury remains a major problem in pancreas transplantation. During the reperfusion period, endothelial dysfunction, activation of endogenous enzymes, leucocyte recruitment and activation all lead to generation of oxygen-derived free radicals, promote lipid peroxidation and deplete glutathione and other antioxidation compounds, leading to pancreatitis[4]. Contradictory results about the role of NO in pancreatic ischemia/reperfusion have been reported[5]. NO may lose an electron to form nitrosonium cation (NO^+), which can combine with the superoxide radicals to form peroxynitrite ($ONOO^-$), a highly active free radical with fierce cytotoxicity[5]. In pathological conditions, significant activation of iNOS by the release of inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), can increase NO concentration[6]. It was reported that endogenous NO is involved in formation of pancreatic edema in L-arginine-induced acute pancreatitis by increasing the vascular permeability and protein extravasation[7]. Treatment with L-NAME significantly reduces amylase activity and edema formation in the pancreas. A study about severe acute pancreatitis has shown a positive correlation between serum NO level and the number of adherent leucocytes[8]. The expression of iNOS is correlated to changes in the pancreatic histomorphology[9-12]. The expression of iNOS during reperfusion following pancreatic ischaemia contributes significantly to the development of acute pancreatitis[13]. Vasoactive mediators, such as bradykinin, platelet activating factor, endothelin and NO, participate in the development of pancreatic microcirculatory failure. Recently, in drug-induced pancreatitis models, some researchers found that there is a correlation among NF-kappaB activation, serum amylase, reactive oxygen species level and tissue damage, suggesting that NF-kappaB and iNOS play a key role in the pathogenesis of acute pancreatitis[14-16]. After treatment with antioxidants or NOS inhibitors, the levels of myeloperoxidase, serum amylase and NO, as well as iNOS activities are decreased significantly, and the pancreatic inflammation is improved[14,15]. Ma *et al*[16] found that the expression of NF-kappaB and iNOS in peritoneal macrophages is significantly higher in rats with severe acute pancreatitis, and anti-inflammatory agents decrease the expression of TNF-alpha, IL-1 and NO in peritoneal macrophages, reducing the severity of pancreatitis. In Folch-Puy's experiment, infusion of a contrast medium into the pancreatic duct could result in an inflammatory process characterized by increased lipase levels in plasma and edema as well as increased myeloperoxidase activity in pancreas, suggesting that activation of NF-kappaB is correlated with iNOS expression in pancreatic cells[17]. It was reported that ischemia/reperfusion provokes severe acute necrotizing pancreatitis with a high mortality rate and leads to systemic inflammatory reaction due to the activation of cytokine cascade and iNOS, indicating that NO overproduction by iNOS corresponds with the apoptotic process in the pancreas and the lung[18,19]. In a study on ischemia/reperfusion injury, Duchen found that calcium overload is associated with NO generation, and their combination leads

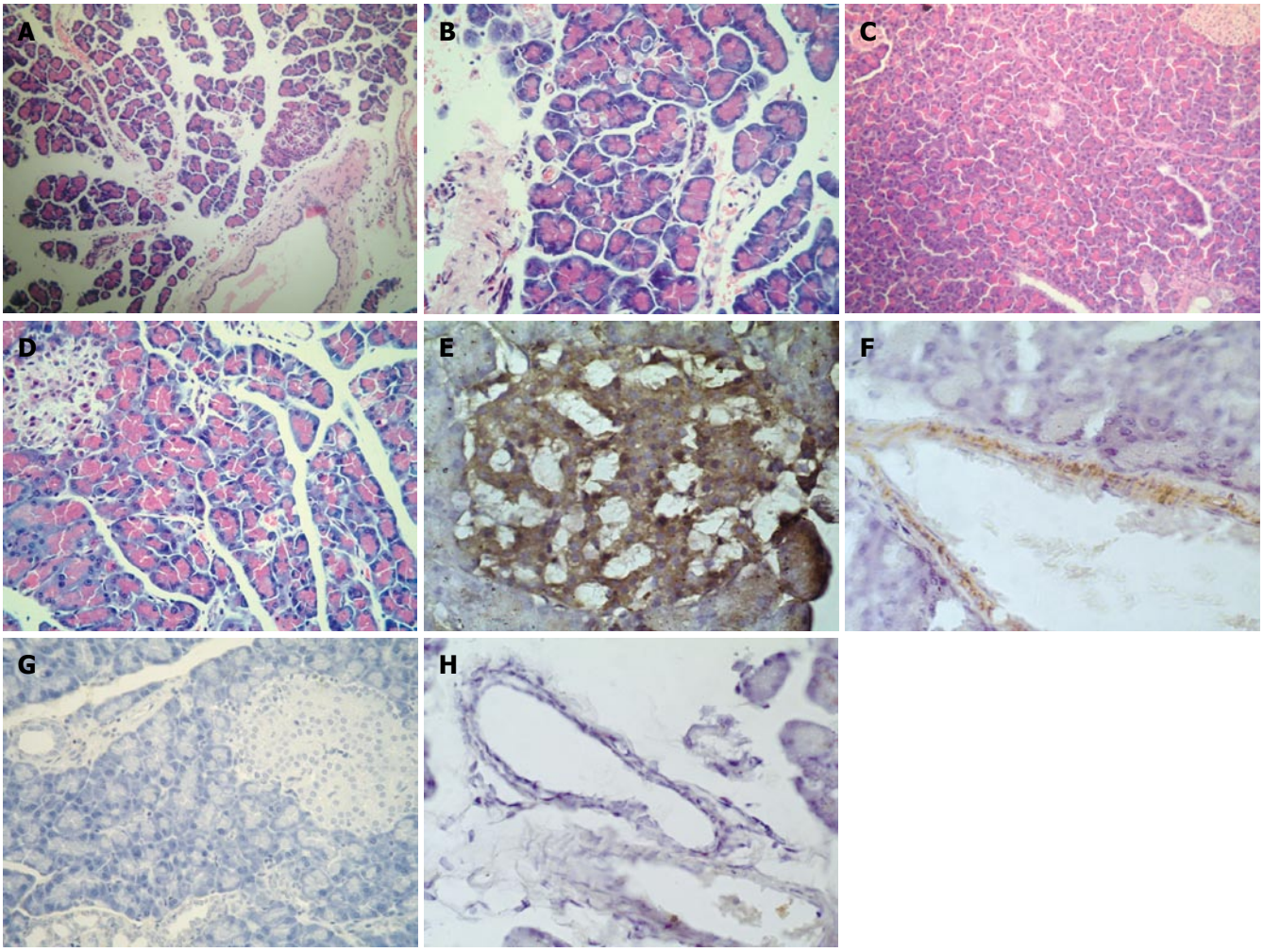

Figure 1 Histology displaying pancreatic injury (**A**, **B**) and no pancreatic injury (**C**, **D**), while immunohistochemistry showing positive anti-iNOS (**E**, **F**) and no stained anti-iNOS (**G**, **H**) in different groups.

to collapse of mitochondrial membrane potential followed by cell death[20]. It was reported that after administration of selective iNOS inhibitors, the iNOS activity and NO concentration are decreased significantly and the severity of pancreatitis is reduced[21]. However, some experiments indicate that NO could activate guanylate cyclase, reduce the activity of platelets and inflammatory cells, relax smooth muscle, and dilate blood vessels[22,23]. Therefore, NO can remit the vasospasm of grafts and decrease the occurrence of vascular crisis. Supplement of NO for donors during reperfusion of pancreatic isografts seems to prevent organ injury because NO attenuates leukocyte-dependent tissue injury[26]. Thus, it remains debatable whether the increased production of NO due to pancreas transplantation is beneficial or detrimental to the tissue.

Based on the findings of this study and present reports, it is very likely that NO plays a dual role in ischemia/reperfusion injury of pancreas. During the early reperfusion period, NO, under the charge of cNOS, can improve postischemic reperfusion. With the prolongation of reperfusion time, NO depletion results in failure of microcirculation, during which supplement of NO or NOS substrate could protect microcirculation against failure[24]. When reperfusion is prolonged (more than 4 h), activation and excessive expression of iNOS due to the release of inflammatory agents such as TNF-α, IL1-β, result in a considerable increase in NO concentration, and the toxic effect of NO as a free radical leads to the development of graft pancreatitis[25]. Therefore, administration of selective iNOS inhibitors can not only reduce the toxicity of NO as a free radical, but also retain vasodilatation effect, and protect the graft against pancreatitis. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of covalent modification of the iNOS protein and heme residue at the active site[26].

At present, some experiments demonstrated that in the transplanted islets, iNOS and toxic NO are produced due to infiltration of inflammatory cells into islets and production of proinflammatory cytokines (such as TNF-α, IL1-β), and an excessive production of NO is deleterious to pancreas β-cells[27-29].

In conclusion, selective iNOS inhibitor, aminoguanidine as a free radical, has a protective effect on pancreas transplantation in rats by inhibiting NO and reducing toxicity.

COMMENTS

Background

Pancreas transplantation is frequently complicated by acute pancreatitis, largely due to ischemia/reperfusion injury. During the reperfusion period, nitric oxide (NO) may form peroxynitrite ($ONOO^-$), a highly active free radical, and has a fierce cytotoxicity. However, NO can significantly dilate blood vessels and remit the vasospasm of grafts, protecting pancreas graft from thrombosis due to transplantation. Therefore, NO plays an ambivalent role in ischemia/reperfusion injury during pancreas transplantation. However, contradictory results about the role of NO in pancreatic ischemia/reperfusion injury have been reported. It remains debatable whether the increased production of NO due to pancreas transplantation is beneficial or detrimental to the tissue.

Research frontiers

Based on the findings of this study and recent reports, it is very likely that NO plays a dual role in ischemia/reperfusion injury of pancreas. During the early reperfusion period, NO under the charge of cNOS, could improve pancreas perfusion. With the prolongation of reperfusion time, NO depletion could result in failure of microcirculation, during which supplement of NO or NOS substrate can protect microcirculation against failure. When reperfusion is prolonged, activation of iNOS due to the release of inflammatory agents, such as tumor necrosis factor α and interleukin-1β, can increase NO concentration. The toxic effect of NO as a free radical can lead to graft pancreatitis. Hence, administration of selective iNOS inhibitors can reduce the toxicity of NO, and protect graft against pancreatitis.

Innovations and breakthroughs

We established a model of pancreas transplantation in rats. Administration of selective iNOS inhibitors could not only reduce the toxicity of NO as a free radical, but also retain vasodilatation effect, and protect graft against pancreatitis. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of the iNOS protein and heme residue at the active site. Our data also suggest that blood sugar level in AG group was much lower than that in transplant control group, indicating that the selective iNOS inhibitor, AG, has a protective effect on pancreas transplantation.

Applications

Pancreas transplantation can give IDDM additional pancreas to take the place of its own, which has lost the function of insulin secreting. Pancreas regulates insulin secretion, and maintains the blood glucose level. At present, nothing else could achieve this object. Factors influencing pancreas functions following transplantation include graft pancreatitis and rejection which are difficult to treat with a poor prognosis. In this study, after administration of selective iNOS inhibitor AG, the iNOS and amylase activity and NO concentration were decreased, the toxicity of NO as a free radical was reduced. The severity of ischemia/reperfusion injury and postgraft pancreatitis was reduced, protecting the graft against pancreastitis.

Terminology

Inducible nitric oxide synthase (iNOS): NO is synthesized from L-arginine by nitric oxide synthase (NOS). iNOS does not express at normal conditions, and produces NO several orders greater than cNOS and may have a more important pathological role. Aminoguanidine (AG) is a mechanism-based inactivator of NOS isoforms and exhibits a marked specificity for the inactivation of its inducible isoform, which proceeds through multiple pathways of the iNOS protein and heme residue at the active site.

Peer review

This study investigated the effect of inducible nitric oxide synthase inhibitor, aminoguanidine, on pancreas transplantation, showing its scientific and clinical values.

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RAPID COMMUNICATION

Polypropylene mesh-reinforced pancreaticojejunostomy for periampullar neoplasm

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Abstract

AIM: To evaluate the effect of polypropylene mesh-reinforced pancreatojejunostomy on pancreatic leakage.

METHODS: Seventeen consecutive patients with paraampullar malignancy received polyprolene mesh-reinforced pancreatodudeonectomy and the Child's method was used to rebuild the alimentary tract.

RESULTS: The mean time of polyprolene mesh-reinforced pancreatojejunostomy was 22 min. Anastomosis could endure 30-500 cm H_2O pressure during operation. All patients recovered without pancreatic leakage.

CONCLUSION: Polyprolene mesh-reinforced pancreato-jejunostomy is a feasible and reliable procedure to prevent pancreatic leakage.

Key words: Pancreatic leakage; Pancreatojejunostomy; Anastomosis; Pancreatodudenectomy

Huang DY, Wang XF, Zhou W, Xin Y, Mou YP, Cai XJ. Polypropylene mesh-reinforced pancreaticojejunostomy for periampullar neoplasm. *World J Gastroenterol* 2007; 13(45): 6072-6075

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INTRODUCTION

Pancreatic anastomotic leakage is a common lethal complication of pancreaticojejunostomy. The incidence of pancreatic anastomosis leakage depends on multiple factors, among which the anastomotic method is the key factor. Different anastomotic methods in pancreaticojejunostomy have been reported in the literature, but none of them is able to prevent pancreatic leakage. Recently, we designed a new anastomotic method: polypropylene mesh-reinforced pancreaticojejunostomy (MRP), by which the sheath of jejunum is bound to the pancreatic remnant wrapped by a strip of mesh. We have applied this method in 17 consecutive periampullar neoplasm patients, and none of them developed pancreatic leakage.

MATERIALS AND METHODS

Clinical data

Nine male and eight female patients with periampullar malignancy, aged 38-72 years, were included in this study, including 6 patients with carcinoma of the panceatic head, 6 with distal common bile duct cancer, 3 with ampullar carcinoma and 2 with duodenal carcinoma. All the patients received polypropylene mesh-reinforced pancreatoduodenectomy and the Child's method was used to rebuild the alimentary tract. Initial polypropylene MRP was performed on 16 patients, the other patient received end-to-end inviganation anastomosis during the first operation but developed pancreatic leakage after operation. On the 8th postoperative day, the patient developed massive intraabdominal bleeding and received the second laparotomy along with polypropylene MRP.

Technique

The pancreas was transected with a scalpel on the scheduled line. Hemostasis was secured by suture ligatures with 4-0 polyprolene stitches (Ethicon, Somerville, NJ) or electrocautery. A 3.0 cm cut end of the pancreatic remnant was isolated. A 1.0 cm wide polypropylene mesh (Ethicon, Somerville, NJ) strip was tightly wrapped over the pancreatic stump about 1.0 cm from the cut margin with a few stitches. The mesh was fixed if it could not be moved with force. If the main pancreatic duct was identified, a stent tube was inserted into the main pancreatic duct and fixed with suturing thread (Figure 1A). Then, the pancreatic stump and the free margin of jejunum were brought together. A posterior row of continuous running sutures using a 3-0 polyprolene stitch was placed between the inner edge of the mesh and jejunum. The sutures were passed carefully through the

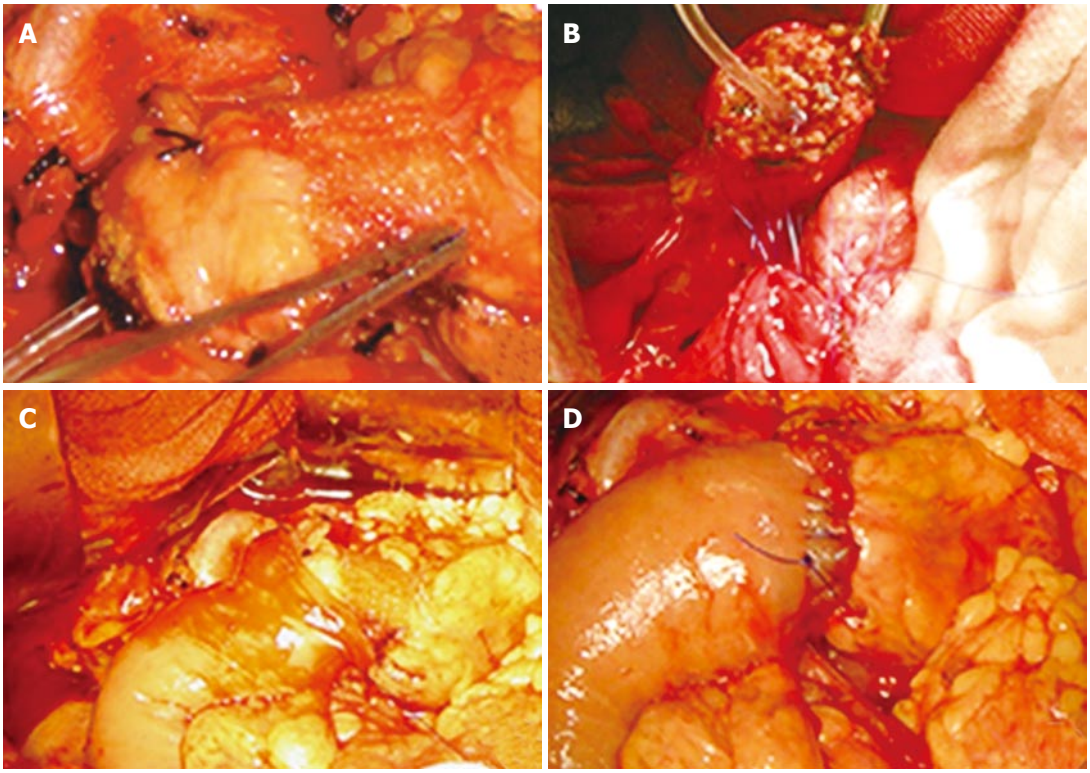

Figure 1 Fixation of mesh around the pancreatic stump (**A**); running suture on the posterior wall of pancreas with a stitch placed between inner edges of the mesh and jejunum (**B**); invagination of the pancreatic stump into the jejunum (**C**); running suture on the anterior wall of the pancreas with a stitch placed between inner edges of the mesh and jejunum (**D**).

mesh, pancreatic capsule and full thickness of jejunum. We began at the farthest point on the cranial side of the pancreatic stump and the caudal side was ligated with six to eight sutures (Figure 1B). After the posterior sutures were completed, they were gently pulled to invaginate the pancreatic stump into the jejunum (Figure 1C). The opposite end of the pancreatic duct stent tube was traversed through sites where bilioenteric anastomosis was performed. Finally, the continuous sutures were extended anteriorly using the same stitch. The sutures were tied after the tightness of the suture line was confirmed. As a result, the jejunal stump invaginated the pancreatic stump of about 2.0 cm (Figure 1D). Care was taken to cover the entire mesh in the jejunal lumen. A urinary catheter was inserted into the jejunum, and saline solution was injected to test for a watertight closure. The seromuscular surface of jejunum 1.0 cm from the margin was anchored to the superior and inferior peritoneal attachments of the pancreatic body to minimize the tension. Biliary anastomosis was constructed in an end-to-side fashion 15 cm distal from the pancreaticojejunostomy. Then a Jackson-Pratt drainage tube was placed near the anastomosis. Daily output and amylase content of abdominal drainage were measured after operation. Pancreatic leakage was defined as the persistent amylase-rich (more than three times the serum concentration) drainage output excess 50 mL/d[1,2]. Prophylactic octreotide was not used.

RESULTS

The mean operative time for polypropylene MRP was 22 min (ranged 10-25 min). No pancreatic leakage was observed under the pressure of 20 cmH_2O during operation. The abdominal drainage output was less than 50 mL in 14 patients, and the amylase content in drainage was within normal range (ranged 46-98 IU/L). The abdominal drainage was removed 4-5 d after the operation, and the patients recovered well and were discharged on the 10th postoperative day. Two patients had a high volume of abdominal drainage during the first 3 postoperative days, but the amylase content in the abdominal drainage was within normal range. The abdominal drainage was removed on the 7th postoperative day and the patients recovered well. A 55-year old male patient received pancreaticojejunectomy for distal common bile duct with an end-to-end invagination anastomosis. The pancreas was soft and fragile. The abdominal drainage output was > 100 mL/d after operation and the amylase content was > 2000 IU/L. Pancreatic anastomotic leakage was identified. The patient had no fever or abdominal pain and received conservative therapy initially. On the 8th postoperative day, fresh blood was drained from the abdominal drainage tube and the patient received the second laparotomy. During the operation, laceration of pancreatic tissue was found on the suture of anastomosis with massive fluid collection. The bleeding came from the splenic artery involved in the fluid collection. After the bleeding was stopped, the pancreas was found to be edematic and fragile. In order to make a direct suture on the pancreas, we decided to perform polypropylene MRP. The patient recovered well after the second operation without pancreatic leakage. All patients were followed up in the outpatient clinic and no adverse events occurred.

DISCUSSION

Since Whipple introduced pancreaticoduodenectomy, it has become a standard procedure for malignant and

benign disorders of the pancreatic head and periampullary region[3-5]. Although the mortality from the surgical procedure has come down considerably during the past three decades[6], it is still higher than that of other radical procedures for abdominal malignancy[7-9]. Pancreatic anastomotic leakage is still the most important determinant of its morbidity, with an incidence of 2%-14%[10,11] and mortality of 28%[12,13]. The etiology of pancreatic anastomotic leakage covers several aspects, including quality of pancreatic tissue, size of major pancreatic duct, exocrinal status of pancreas, general condition and nutritional status of the patient, skill of the surgeon and method of pancreaticojejunostomy[6,14-16]. Among them, method of pancreaticojejunostomy seems to be the key factor[17]. Different methods for pancreaticojejunostomy have been reported in the literature[19], including end-to-side anastomosis, duct-to-mucosa anastomosis, or end-to-end or end-to-side invagination anastomosis. The suturing techniques for anastomosis include running or interrupted suture, single layer or double layers suture[18-20]. But all these methods cannot absolutely prevent the leakage, the incidence of leakage of the most widely applied end-to-side invagination anastomosis is still as high as 11%[21]. No consensus on the choice of anastomotic technique has been reached, and currently each technique finds its application among different groups of surgeons[22].

It is well known that the incidence of pancreatic leakage is higher in patients with a soft and normal pancreatic parenchyma because it is prone to develop parenchymal laceration from shear forces applied during tying of the sutures, especially while performing suturing on the posterior wall of the pancreas. In patients with normal pancreatic parenchyma, the incidence of leakage is 12% to 28%, compared with 5% to 9% in those considered to have pancreatic fibrosis[23]. The efferent loop filled with bile and pancreatic juice also increases the shear force[24]. Some retrospective or prospective studies also suggested that technical modifications may reduce the leakage rate[25-27], suggesting that if the pancreas is soft with a narrow duct, it would be most secure when the pancreaticojejunal anatomosis is intraluminal into the jejunum by invaginating the pancreatic stump.

Polyprolene mesh-reinforced pancreaticojejunostomy was developed based on the binding pancreaticojejunostomy described by Peng *et al*[28,29], who reported a widely invaginated end-to-end anastomosis with ablated jejunal mucosa. Our procedure with single layer continuous sutures is less complicated than the binding pancreaticojejunostomy. The success of this technique may be due to the following four aspects. First, the mesh forms a safe "clothing" around the remnant pancreas for anchoring sutures, thus preventing the possibility of parenchymal laceration and bleeding from the sutures in soft pancreatic parenchyma caused by the suture. We identified the advantages of a new technique in the case where a secondary polypropylene MRP was received for leakage from the first operation site. During the second operation, the pancreatic parenchyma was severely edematic and fragile, making the direct suture impossible. However, it was easy and convenient to perform polypropylene MRP on this patient, and no leakage occurred postoperatively. Second, sine the shape of the pancreatic stump can be modified and reduced by the mesh, it is more convenient to make an invagination. Third, the posterior single layer continuous sutures are simple and require less time. Fourth, it is very convenient to perform polypropylene MRP under different conditions and the time required is less than single end-to-end invagination. The mesh in the anastomosis can promote fibroblast attachment and enhance the anastomotic healing process[30,31]. However, it still needs further confirmation.

An ideal pancreaticojejunal anastomosis should be safe and convenient. Moreover, laparoscopy is more and more widely applied by general surgeons, and the convenience of a surgical procedure should be considered. The use of polypropylene MRP ensures a tight seal for any type of pancreatic stump regardless of the pancreas consistency, thus a more secure and reliable anastomosis can be obtained. The preliminary results are very encouraging. However, an appropriate prospective study in randomized patients is needed. Up to date, we have not observed any adverse effect of polypropylene MRP, but a long following-up time is needed to confirm it.

COMMENTS

Background

Pancreaticoduodenectomy is the standard procedure for malignant and benign disorders of the pancreatic head and periampullary region. Mortality and morbidity of this procedure are still higher than other radical procedures for abdominal malignancy. Pancreatic anastomotic leakage is still the most important determinant of its morbidity.

Research frontiers

The method of pancreaticojejunostomy is the key factor for pancreatic anastomotic leakage. We designed a new technique of polypropylene mesh-reinforced pancreaticojejunostomy to prevent pancreatic leakage.

Innovations and breakthroughs

Up to date, all the existing methods of pancreaticojejunostomy cannot perish anastomotic leakage. Our new method is effective in preventing pancreatic leakage.

Applications

This technique is safe and can be applied in pancreaticoduodenectomy under all conditions even though the pancreas is very fragile. If its advantage and disadvantage can be proved by large clinical trials, it can be used as one of the standard procedures.

Terminology

Pancreaticoduodenectomy: excision of the pancreatic head and the encircling loop of the duodenum to which it is connected. Pancreaticojejunostomy: surgical anastomosis of the pancreatic duct or the divided end of transected pancreas with the jejunum.

Peer review

This article seems to be a challenging artifice to reduce postoperative complication in pancreatic surgery. The results are encouraging and the procedure can be used as a standard method of pancreaticojejunostomy.

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RAPID COMMUNICATION

Correlation of matrix metalloproteinase suppressor genes RECK, VEGF, and CD105 with angiogenesis and biological behavior in esophageal squamous cell carcinoma

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Abstract

AIM: To explore the expression of reversion inducing cysteine-rich protein with Kazal motifs (RECK), vascular endothelial growth factor (VEGF) and endoglin (CD105) protein and its correlation with occurrence, development, invasion and metastasis in esophageal squamous cell carcinoma (ESCC).

METHODS: Streptavidin-peroxidase (SP) immunohistochemistry was used to detect expression of RECK and VEGF in 62 cases of ESCC, 31 cases of adjacent atypical hyperplastic epithelium and 62 cases of normal esophageal epithelium. CD105 Mb was used to assess microvessel density (MVD).

RESULTS: The expression of RECK was closely correlated with histological grade, infiltrative depth and lymphatic metastasis in ESCC ($P < 0.05$). The expression of RECK decreased during cancer development: normal esophageal epithelium (85.5%, 53/62), adjacent atypical hyperplastic epithelium (71.0%, 22/31), and carcinoma (59.7%, 37/62). There was a significant difference among the groups ($P < 0.05$). The expression of VEGF protein was closely correlated with infiltrative depth and lymphatic metastasis in ESCC ($P < 0.05$). The expression of VEGF protein increased during cancer development: normal esophageal epithelium (29.0%, 18/62), adjacent atypical hyperplastic epithelium (54.8%, 17/31), and carcinoma (67.7%, 42/62). There was a significant difference among the groups ($P < 0.05$). MVDCD105 increased in accordance with histological grade, but there was no significant difference (grade Ⅰ, 36.92 ± 10.85; grade Ⅱ, 37.65 ± 9.50; and grade Ⅲ, 38.06 ± 12.19). The MVDCD105 was closely correlated with infiltration and lymphatic metastasis in ESCC ($P < 0.05$). The expression of RECK was inversely correlated with the expression of VEGF and CD105.

CONCLUSION: RECK, VEGF and CD105 play important roles in the infiltration, metastasis and carcinogenesis in esophageal carcinoma. Angiogenesis in ESCC may be promoted by over-expression of CD105.

Key words: Reversion inducing cysteine rich protein with Kazal motifs; Vascular endothelial growth factor; CD105; Esophageal squamous cell carcinoma; Immunohistochemistry; Microvessel density

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INTRODUCTION

Reversion inducing cysteine-rich protein with Kazal motifs (RECK) is a recently discovered tumor suppressor gene with a special function of inhibiting matrix metalloproteinase (MMP) expression and activity, which serves as an MMP inhibitor[1]. Expression of the RECK gene is closely related to tumor invasion and metastasis and angiogenesis. Previous studies indicate that the level of RECK gene expression is inversely correlated to tumor invasiveness in liver cancer, pancreatic cancer, mammary cancer and pulmonary carcinoma, and for patients with higher RECK gene expression, the prognosis is sometimes apparently better than that of patients with low expression[2-5]. No studies have been published in China or abroad on the correlation of the RECK gene with invasion and metastasis of esophageal cancer, and the relationship between RECK, vascular endothelial growth factor (VEGF)

and endoglin (CD105) expression. The streptavidin–peroxidase (SP) immunohistochemistry method was used to perform a combined test on expression of RECK, VEGF and CD105 gene in tissues from 62 cases of esophageal squamous cell carcinoma (ESCC), 31 cases of para-carcinoma atypical hyperplasia, and 62 specimens of normal esophageal mucous membrane, to establish the role of RECK, VEGF and CD105 in the generation and development of esophageal cancer, so as to ascertain the molecular index for early diagnosis and prognosis judgment.

MATERIALS AND METHODS

Materials

Resection specimens from 62 cases of esophageal cancer were collected from the Municipal Cancer Hospital of Anyang, Henan Province, China from 26 February to 16 March, 2006, which is one of the most epidemic regions for esophageal cancer. No patients had a history of chemotherapy, radiotherapy or immunotherapy. The specimens were taken from 36 male and 26 female patients aged 38-75 years (average 60.6 ± 9.5), who were all verified to have ESCC by histopathological examination. The histological grading included Class Ⅰ (15 cases), Class Ⅱ (25 cases) and Class Ⅲ (22 cases); 20 cases were accompanied with lymphatic metastasis, and 42 cases had no lymphatic metastasis. The depth of invasion was divided into two groups that consisted of seven cases with invasion of the superficial muscularis, and 55 with invasion of the deep muscularis or fibrous membrane. All the samples were taken from within 3 cm of the tumor focus, as well as from three areas of distal normal mucous membrane, and were fixed with 40 g/L paraformaldehyde solution, normally dehydrated, embedded in paraffin, and serial sections were cut to a thickness of 4-6 μm, and used for hematoxylin and eosin and immunohistochemistry staining. Mouse anti-human RECK monoclonal antibody (mAb) and anti-human VEGF mAb were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and mouse anti-human CD105 single clone antibody and the SP immunohistochemistry kit were purchased from Beijing Zhongshan Golden Bridge Biotech Development (China).

Methods

The SP immunohistochemistry SP method was employed. RECK, VEGF and CD105 mAbs were diluted 1:100, stained with diaminobenzidine, and counterstained with hematoxylin, strictly in accordance with the instructions, and PBS solution was used as a negative control replacing primary antibody. RECK- and VEGF-positive signals all show brown granule-like materials that are located in the cytoplasm. Under a high-power magnifying glass, five fields of vision (FOVs) were randomly selected (for each FOV, there were no fewer than 200 cells), and the results were interpreted in accordance with the percentage of cells and depth of stain[6]. (1) Scored in accordance with depth of staining of cells in a section: 0, no cell coloration; 1, light yellow; 2, brown; 3, tan. (2) Scored in accordance with the percentage of positive cells in like-kind cells: 1, < 30%; 2, 30%-70%; 3 > 70%. The product of (1) and (2) was used as the total score, where 0-1 indicates a negative score (-), 2-3 a weak positive score (+), and ⩾ 4 a positive score (++). Tumor MVD measurement was done using the methods reported by Weidner[7], i.e. any brown endothelial cell or cell cluster is used as a vessel. We first observed all the FOVs for a given section under a low-power magnifying glass to find the highest density area of tumor vessels, and then we counted the number of microvessels in three FOVs under a high-power magnifying glass; the average values were then used as the MVD.

Statistical analysis

SPSS 10.0 statistical software was used and the χ^2 test, single factor analysis of variance, *t* test were applied, correlation test was applied, Spearman correlation analysis. The test level was $\alpha = 0.05$.

RESULTS

RECK expression in ESCC tissues and correlation with clinical and biological behavior

RECK expression was mainly located in the cytoplasm of tumor cells, and appeared as light to dark yellow (Figure 1A). RECK expression increased sequentially as ESCC developed: normal tissue (59.7%, 37/62), para-carcinoma atypical hyperplastic tissue (71.0%, 22/31), and ESCC (85.5%, 53/62), and comparison between the groups indicated a significant difference ($\chi^2 = 10.331$, $P < 0.01$) (Table 1). RECK expression was related to histological grading, invasion depth and lymphatic metastasis (the respective values of χ^2 were 10.422, 8.550 and 4.751; average $P < 0.05$) (Table 2).

VEGF expression in ESCC tissues and correlation with clinical and biological behavior

VEGF staining was located in the cytoplasm, and appeared as light to dark yellow (Figure 1B). VEGF expression decreased sequentially as ESCC developed: normal mucous membrane (67.7%, 42/62), para-carcinoma atypical hyperplastic tissue (54.8, 17/31), and carcinoma (29.0% 18/62); and comparison between the groups indicated a significant difference ($\chi^2 = 18.994$, $P < 0.05$) (Table 1). VEGF expression was not related to histological grading ($P > 0.05$), but was related to depth of invasion and lymphatic metastasis (the respective values of χ^2 were 10.319 and 6.693; average $P < 0.05$) (Table 2).

Correlation of MVDCD105 with differentiation and metastasis in ESCC

CD105 expression was mainly located in the cytoplasm of vascular endothelial cells of tumor stroma, and appeared as light to dark yellow granules (Figure 1C). In grade Ⅰ, Ⅱ and Ⅲ ESCC tissues, MVDCD105 tended to increase as the degree of cancer tissue differentiation decreased: grade Ⅰ, 37.87 ± 3.60; grade Ⅱ, 37.44 ± 3.99; and grade Ⅲ, 39.00 ± 4.47), but there was no significant difference between the results ($F = 0.885$, $P > 0.05$) (Table 1). In cancer tissues with lymphatic metastasis, MVD (41.00 ±

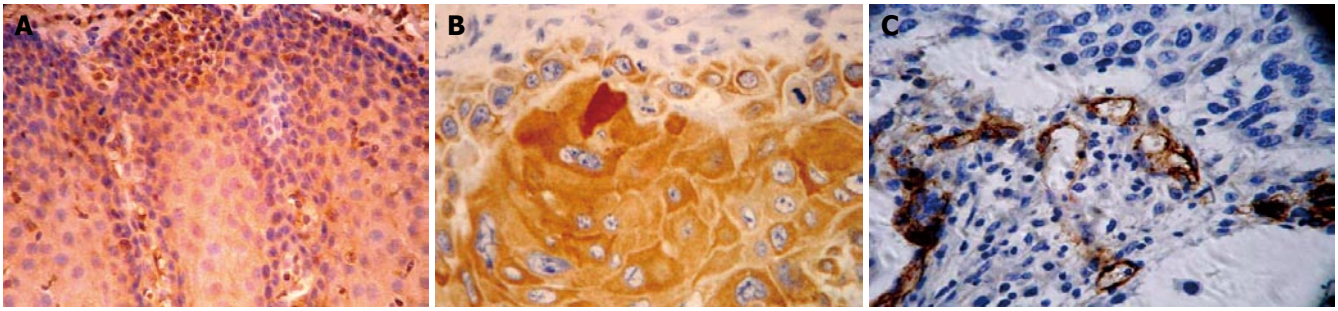

Figure 1 **A**: Expression of RECK in the normal esophageal epithelium; **B**: Expression of VEGF in ESCC; **C**: Expression of CD105 in ESCC (SP, × 400).

Table 1 RECK and VEGF expression in ESCC, atypical hyperplasia and normal mucous membrane tissues

		RECK			χ^2	*P*	VEGF			χ^2	*P*
	n	-	+	Positive ratio (%)			-	+	Positive ratio (%)		
Normal mucous membrane tissues	62	9	53	85.5			44	18	29		
Atypical hyperplasia	31	9	22	71.0	10.331	0.006	14	17	54.8	18.994	0.000
ESCC	62	25	37	59.7			20	42	67.7		

Table 2 Correlation of RECK and VEGF expression with clinical and biological behavior of ESCC

	n	RECK			χ^2	*P*	VEGF			χ^2	*P*
		-	+	Positive ratio (%)			-	+	Positive ratio (%)		
Histology grading											
I	15	1	14	93.3			8	7	46.7		
II	25	11	14	56	10.422	0.005	7	18	72.0	4.014	0.134
III	22	13	9	40.9			5	17	77.3		
Invasion depth											
Superficial muscularis	7	0	7	100			6	1	14.3		
Deep muscularis	55	25	30	54.5	8.550	0.021	14	41	74.5	10.319	0.001
Lymph metastasis											
N	42	13	29	69	4.751	0.029	18	24	57.1	6.693	0.010
Y	20	12	8	40			2	18	90.0		

3.26) was higher than that without metastasis (36.33 ± 3.76). MVD (38.80 ± 3.60) of cancer tissues with invasion of the deep muscularis was higher than that of the superficial muscularis (32.57 ± 3.46), and the difference was significant (Table 3).

Correlation of RECK, VEGF and CD105 expression in ESCC

RECK expression was inversely correlated to that of VEGF. In tissues with positive RECK expression, VEGF expression rate was 51.4% (19/37), and in tissues negative for RECK expression, VEGF expression rate was 92.0% (23/25). Comparison between the groups indicated a significant difference (γ_s = -0.427, $P < 0.01$). In tissues with positive RECK expression, MVDCD105 was 35.76 ± 9.42, and tissues negative for RECK expression, MVDCD105 was 41.59 ± 10.80. Comparison between the groups indicated a significant difference (t = -2.969, $P < 0.01$) (Table 4).

DISCUSSION

The RECK gene were discovered by Takahashi *et al*[1] in NIH3T3 cell lines transfected by the v-Ki-Ras gene, which is located on chromosome 9p13-p12 and encodes a membrane-anchored glucose protein with a relative molecular mass of 110 000. RECK gene has high expression in normal tissues, but no expression in various tumor cell lines and cells affected by cancer genes such as Ras. Many cancer genes such as ras, fos and myc can all decrease the expression of the RECK gene[8,9], which indicates that the RECK gene may be a negatively adjusted target jointly acted upon by cancer genes, and the proper expression of the RECK gene can inhibit angiogenesis[10-12]. It has been shown that the RECK gene is closely related to the prognosis of liver cancer, pancreatic cancer and mammary cancer, and prognosis of patients positive for RECK expression is better than that of those with negative expression[2-5]. The effects of RECK during the course of tumor generation and metastasis may be more widespread than has been discovered to date. It is now considered that RECK may affect tumor invasion and metastasis through inhibiting tumor angiogenesis, and is thus a cancer-inhibiting gene[12-14]. The results of this experiment indicate that the RECK gene is expressed in normal esophageal tissues, esophageal para-carcinoma atypical hyperplastic

Table 3 Correlation of CD105 expression with the clinical and biological behavior of ESCC (reciprocal) (mean ± SD)

	CD105			
	n	MVD	*t*/*F*	*P*
Histology grading				
Ⅰ	15	37.87 ± 3.60		
Ⅱ	25	37.44 ± 3.99	F = 0.885	0.418
Ⅲ	22	39.00 ± 4.47		
Invasion depth				
Superficial muscularis	7	32.57 ± 3.46	-4.326	0.000
Deep muscularis	55	38.80 ± 3.60		
Lymph metastasis				
N	42	36.33 ± 3.76	4.760	0.000
Y	20	41.00 ± 3.26		

Table 4 Correlation of RECK, VEGF and CD105 expressions in ESCC (mean ± SD)

RECK	*n*	VEGF		γ_s	*P*	MVD	*t*	*P*
		+	-					
+	37	19	18	-0.427	0.001	36.00 ± 3.80	-2.969	0.004
-	25	23	2			39.10 ± 3.86		

tissues and cancer tissues, but its expression level in cancer tissue is significantly lower. This indicates that tumors with low RECK expression have greater invasive capacity.

VEGF is a polypeptide cell factor discovered in recent years (also called vascular permeability factor), which has a double function: (1) it directly stimulates vascular endothelial cell reproduction through its receptor, and induces production of the proteolytic enzyme interstitial collagenase and tissue factor to promote angiogenesis; and (2) it increases vascular permeability, promotes exosmosis of fibrinogen to cause tumor interstitial edema and extracellular matrix changes, and consequently provides a basis for tumor invasion and metastasis[15,16]. VEGF is an angiogenic factor that has been studied at the most with the most specific effect at present, and which is related to generation and metastasis of various human tumors[17-23]. The results of this study show that VEGF expression is inversely related to ESCC lymphatic metastasis, i.e. the positive protein rate of VEGF for the lymph metastasis group significantly higher than that of no lymph metastasis group, which indicates that the positive protein expression of VEGF maybe correlated with metastasis of ESCC lymph. The results of this study also show that VEGF expression is not obviously related to the degree of tumor differentiation, which indicates that VEGF expression is not related to ESCC histology.

CD105 is a gene located in human chromosome 9q34, which is a homotype dipolymer membrane glycoprotein with a molecular mass of 180 kDa, participating in signal transmission inverting growth factor β (TGF-β) receptor and angiogenesis. Previous studies indicate that strong expression of CD105 in tumor-related, newly generated vascular endothelium is a more accurate index for judging endothelial reproductive state, and it is closely related to tumor generation and metastasis[24-27]. Contrasting with endothelial cell markers such as CD31, CD34 and Ⅷ factor-related antigens, the difference is that CD105 as a marker of angiogenesis is only strongly expressed in vascular endothelial cells of tumor tissues at the reproduction stage (i.e. newly generated vascular endothelium), but it is not expressed in the blood vessels of normal tissues[28-30]. A quantitative test has been carried out on the MVD of newly generated vessels in ESCC with anti-CD105 single clone antibody, the results of which indicated that MVD is positively correlated with depth of invasion and lymphatic metastasis in ESCC. That is, the MVD of tumors with deep invasion is significantly higher than that with superficial invasion, and the MVD of tumors with lymphatic metastasis is significantly higher than that of those without metastasis; however, MVD is not related to histological grading. Thus, the fixed vessel quantity in tumors is considered as a significant and separate prognosis index. The level of angiogenesis in cancer can be evaluated through MVD measurement[31], and MVD measurement in ESCC may help to judge its potential for invasion and metastasis. The MVD in tumor tissues was tested with CD105, the results of which indicate that RECK expression is inversely related to tumor angiogenesis; further, MVDCD105 for tumors with high RECK expression is obviously lower than that for those with low RECK expression, which indicates that RECK can inhibit angiogenesis. There is a co-adjustable mechanism between MVDCD105 and RECK.

Angiogenesis inhibition by RECK has also been verified in some clinical studies that have discovered that MVD in tumor tissues is inversely related to RECK expression[10,32]. However, such an inverse correlation only occurs when the expression of VEGF is much higher, i.e. for tumors with higher expression of VEGF, the influence of RECK also increases, which indicates that RECK can inhibit VEGF-induced angiogenesis. The results indicate that RECK expression is closely related to tumor prognosis. Also, the inhibitory effects of RECK on tumor angiogenesis have a certain pertinence. The results of this study indicate that positive expression of RECK is inversely correlated with VEGF expression and MVD. In cases with negative RECK expression, VEGF expression and MVD are significantly higher than in cases with positive RECK expression ($P < 0.05$). This indicates that decreasing or losing RECK expression may increase VEGF expression, which consequently promotes tumor angiogenesis, and provides the conditions for the generation and metastasis of ESCC. This study also discovered that VEGF expression is consistent with MVD, i.e. if VEGF expression is high, MVD will rise accordingly ($P < 0.05$), and the VEGF's positive stained cells at the front of tumor infiltration, which are consistent with CD105's expression positions, which further verifies that VEGF is an angiogenesis factor with specific effects, and can specifically promote tumor angiogenesis. This study shows that decreasing or losing RECK expression and increasing VEGF expression are two of the significant events during the generation and development of ESCC, and RECK, as a cancer-inhibiting gene, may inhibit angiogenesis in ESCC, through affecting the signal transmission path of VEGF expression. The combined test on RECK, VEGF and MVD can be used as an

objective index to determine the invasion and metastasis capabilities of ESCC, which is of great significance for judging prognosis.

COMMENTS

Background

The RECK gene was discovered by Takahashi *et al* in NIH3T3 cell lines transfected by the v-Ki-Ras gene, which plays an important role in regulating MMPs and participating in tumor invasion, metastasis and angiogenesis.

Research frontiers

No studies have been published in China or abroad on the correlation of the RECK gene with invasion and metastasis of esophageal cancer, and the relationship between RECK and expression of VEGF and CD105.

Related publications

Expression of RECK gene is closely related to tumor invasion and metastasis and angiogenesis. Previous studies indicate that RECK gene expression is inversely correlated with tumor invasiveness in liver cancer, pancreatic cancer, mammary cancer and pulmonary carcinoma, and for patients with higher RECK gene expression, the prognosis is apparently better than that of patients with low RECK expression. Therefore, RECK is considered to be an cancer-inhibitory gene, which can affect tumor metastasis through inhibiting the activity of MMPs and angiogenesis.

Innovations and breakthroughs

No reports on this subject have been published in China or abroad. The immunohistochemistry SP method was used to perform a combined test on expression of RECK, VEGF and CD105 genes in 62 cases of ESCC, 31 specimens of para-carcinoma atypical hyperplastic tissues, and 62 specimens of normal esophageal mucous membrane, to ascertain the role of RECK, VEGF and CD105 in the generation and development of esophageal cancer, so as to establish a molecular index for early diagnosis and prognosis judgment.

Applications

The further investigation of RECK helps us understand more about the biological behavior of esophageal carcinoma and it gives us a new guide for earlier diagnosis and therapy of esophageal carcinoma. RECK may be a molecular target for the early diagnosis and prognostic judgment of esophageal carcinoma.

Terminology

RECK is a new cancer-inhibiting gene first discovered in NIH3T3 cell lines transfected by v-Ki-Ras.

Peer review

This manuscript reports expression of three genes in ESCC, in which they appear to have prognostic value as they are correlated with histological grade, lymphatic metastasis and invasion.

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RAPID COMMUNICATION

DNA methyltransferase 3B promoter polymorphism and its susceptibility to primary hepatocellular carcinoma in the Chinese Han nationality population: A case-control study

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Abstract

AIM: To investigate the correlation between C/T single nucleotide polymorphism (SNP) in the promoter of the DNA methyltransferase 3B (*DNMT3B*) gene and risk for development and progression of primary hepatocellular carcinoma (HCC).

METHODS: One hundred case subjects were selected consecutively from Tongji Hospital (Wuhan, China). from March to November 2006. They did not receive radiotherapy or chemotherapy for newly diagnosed and histopathologically confirmed HCC. One hundred and forty control subjects having no history of cancerous or genetic diseases were healthy volunteers to Wuhan Blood Center in the same period. Frequency was matched for sex, age, alcohol consumption and cigarette smoking status of the case subjects. C/T polymorphism of the *DNMT3B* promoter was analyzed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing analysis. The association between genotypes of *DNMT3B* and clinicopathological parameters among cases was also studied.

RESULTS: The CC genotype was not detected in both HCC patients and controls. In control subjects, the frequency of TT and CT genotypes was 99.3% and 0.7% respectively, and that of T and C alleles was 99.6% and 0.4% respectively. The frequency of CT genotype was higher in HCC (3.0%). The frequency of T and C alleles was 98.5% and 1.5% respectively. However, the genotype and allelotype distribution in HCC patients was not significantly different from that in controls.

CONCLUSION: C/T polymorphism is not associated with the increased risk of HCC. *DNMT3B* genetic polymorphism is variable in different races, ethnic groups or geographic areas. Further study is needed to clarify the role of *DNMT3B* SNP in the development of HCC among other populations.

Key words: DNA methyltransferase; Single nucleotide polymorphism; Susceptibility; Primary hepatocellular carcinoma

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INTRODUCTION

Primary hepatocellular carcinoma (HCC) occurs frequently in Southeast Asia, especially in China. It ranks second and approximately accounts for 42.5% of all malignancies worldwide[1].

Although hepatitis B virus (HBV) is the major cause of HCC, only a fraction of patients with chronic HBV infection develop HCC during their lifetime, suggesting that genetic and epigenetic factors are important in determining individuals' susceptibility to HCC.

DNA methylation plays an important role in chromatin structure stability, genome integrity, modulation of tissue-specific gene expression, embryonic development, genomic imprinting, X-chromosome inactivation and is essential for the development of mammals[2,3]. DNA methylation is mediated by DNA methyltransferases (DNMTs), of which three active forms have been identified: DNMT1, DNMT3A and DNMT3B. DNMT1 is thought to be primarily responsible for maintaining pre-existing methylation patterns after DNA replication because of its preference to hemimethylated DNA substrates and targeting replication foci. DNMT3A and DNMT3B have an equal preference to hemimethylated and unmethylated DNA substrates, and therefore they are believed to be principally required for *de novo* methylation[4-6]. Recent studies have shown that three different mechanisms play a role in the effect of methylation: global hypomethylation, hypermethylation of individual gene segments and deregulated expression of DNA methyltransferases. Changes in the methylation pattern correlate with the

development of cancer. *De novo* hypermethylation in promoter CpG islands has been identified as a possible mechanism for tumor suppressor gene and DNA repair gene inactivation of in human cancer cells[7-12]. DNMT3B, regarded as a *de novo* DNA methyltransferase, is thought to play an important role in the generation of aberrant methylation in carcinogenesis[13,14].

DNMT genes are up-regulated in various human cancers, including HCC[15-20]. Significant over-expression of DNMT3B is observed in tumor tissues while over-expression of DNMT1 and DNMT3A is more modest[15,18,20].

A C-to-T transition polymorphism (C46359T, GenBank accession no. AL035071) in the promoter region of the *DNMT3B* gene, -149 base pairs from the transcription start site, is reported to greatly increase promoter activity. Many reports have shown that the C/T polymorphism is associated with an increased risk for lung cancer and decreased postsurgical survival in patients with small cell carcinoma of the head and neck. Carriers of T allele, particularly heterozygotes, have a significantly increased risk for such cancers[21-24].

Several polymorphic genes are reported to be correlated with modification of susceptibility to HCC. To our knowledge, the association between *DNMT3B* polymorphism and development of HCC has not been reported. Since the *DNMT3B* promoter polymorphism that is responsible for regulating genomic methylation is possibly associated with an increased risk for cancers, we evaluated the relationship between *DNMT3B* C46359T polymorphism and risk of HCC in a hospitalization-based case-control study in a Chinese Han nationality population.

MATERIALS AND METHODS

Subjects

One hundred case subjects were selected consecutively from Tongji Hospital (Wuhan, China) from March to November 2006. They did not receive radiotherapy or chemotherapy for newly diagnosed and histopathologically confirmed HCC. One hundred and forty control subjects having no history of cancerous or genetic diseases were healthy volunteers to Wuhan Blood Center in the same period. Frequency was matched for sex, age, alcohol consumption and cigarette smoking status of the case subjects. All the cancer patients and control subjects were unrelated Han nationality individuals from Wuhan or from its surrounding regions. Blood was taken from all recruits who consented to the epidemiology survey. Each subject was scheduled for an interview after informed consent was obtained, and a structured questionnaire was used to collect information on demographic data and risk factors, such as hepatitis B infection history and family history of any cancers.

DNMT3B genotyping

Genomic DNA was extracted from peripheral blood lymphocytes by proteinase K digestion and phenol-chloroform extraction. DNMT3B C/T polymorphism was determined by PCR-RFLP. The primers of 5'-TGCTGT GACAGGCAGAGCAG-3' (nt 46151-46170) and 5'-GG TAGCCGGGAACTCCACGG-3' (nt 46530-46511) were synthesized as previously described[21]. This 380-bp target DNA fragment contains the upstream region and the first exon of the *DNMT3B* gene. Amplification reaction was carried out in a 25 μL PCR mixture containing 50-200 ng of genomic DNA, 12.5 pmol of each primer (Shanghai Sangon Company), 0.1 mmol/L each deoxynucleotide triphosphate, 1 × PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, and 0.1% Triton X-100), 2.0 mmol/L $MgCl_2$, and 1.25 U Taq polymerase (Beijing Sbsbio Company). The PCR profile consisted of an initial denaturation at 95℃ for 5 min, 35 cycles of denaturation at 95℃ for 30 s, annealing at 65℃ for 30 s and extension at 72℃ for 30 s and a final extension at 72℃ for 10 min. The 380-bp product was then digested with *Bln* I (TaKaRa Biotechnology Company) for 12-16 h in 37℃ bath water. The digested product was separated by vertical electrophoresis at 200V of constant voltage for 1.5 h through 8% polyacrylamide gel (29:1) and silver staining, and then determined under ultraviolet irradiation. The variant T allele has a *Bln* I restriction site that results in two bands (207 bp and 173 bp), and the wild-type C allele lacks the *Bln* I restriction site, thus producing a single 380-bp band (Figure 1). More than 10% of the samples were randomly selected for repeated assays, and the results were 100% concordant. Restriction fragment length polymorphism (RFLP) analysis was confirmed by PCR-based sequencing with an Applied Biosystem automated sequencer (Figure 2).

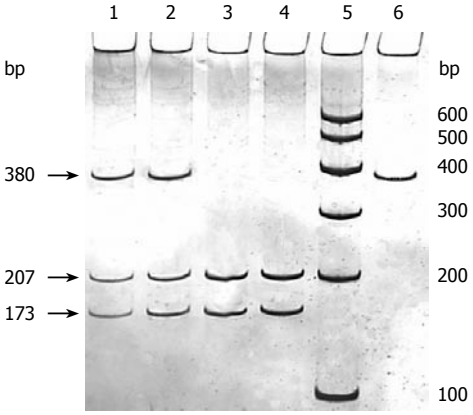

Figure 1 PCR-RFLP-polyacrylamide gel electrophoresis for genotyping of DNMT3B promoter C46359T. Lanes 1 and 2: CT heterozygote; lanes 3 and 4: TT genotype; lane 5: 100 bp molecular marker; lane 6: PCR product.

Statistical analysis

Data were presented as mean ± SD. A database was developed by Epi Info 6.0 and the analysis of data was accomplished using SPSS 10.0 software. The difference in frequency distributions of genotypes and allelotypes between the two groups was detected by chi-square test. *P* values of less than 0.05 were considered statistically significant.

RESULTS

The characteristics of 100 HCC case patients and 140 control subjects are summarized in Table 1. In the case group, the age ranged 18-70 years, the mean age was (55.86 ± 10.12) years, and the gender ratio was 4:1 while the mean

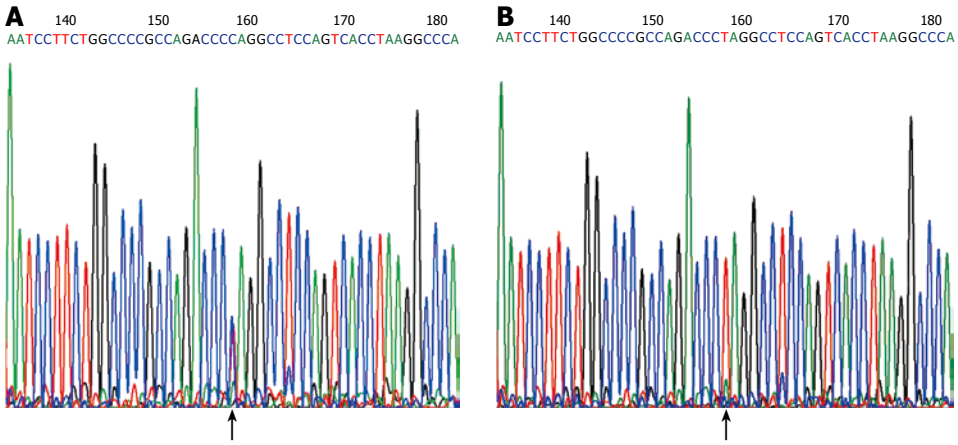

Figure 2 CT genotype (**A**) and TT genotype (**B**) Sequencing-confirmed.

Table 1 Frequency distributions of selected variables in HCC patients and control subjects, *n* (%)

Variable	Case (*n* = 100)	Control (*n* = 140)
Sex		
Male	80 (80)	114 (81.4)
Female	20 (20)	26 (18.6)
Age (yr)	55.86 ± 10.12	57.12 ± 10.01
Smoking status		
Current smoker	60 (60.0)	92 (65.7)
Former smoker	21 (21.0)	26 (18.6)
Non smoker	19 (19.0)	22 (15.7)
Pack/year	11.26 ± 6.25	14.89 ± 7.56
Alcohol consumption		
Current drinker	55 (55.0)	70 (50.0)
Former drinker	19 (19.0)	29 (20.7)
Non drinker	26 (26.0)	41 (29.3)
Alcohol/year	10.01 ± 5.21	13.58 ± 5.09

Table 2 *DNMT3B* genotype and allele frequencies in case patients and control subjects and their association with HCC, *n* (%)

Genotypes	Case patients (*n* = 100)	Control subjects (*n* = 140)
CT	3 (3)	1 (0.7)
TT	97 (97)	139 (99.3)
C allele	3 (1.5)	1 (0.4)
T allele	197 (98.5)	279 (99.6)

$\chi^2 = 1.86$, $P = 0.17$ (genotype); $\chi^2 = 1.84$, $P = 0.17$ (allele).

time of alcohol consumption and cigarette smoking was (10.01 ± 5.21) years and (11.26 ± 6.25) years, respectively. In the control group, the age ranged was 15-68 years, the mean age was (57.12 ± 10.01) years, and the gender ratio was 4.4:1 while the mean time of alcohol consumption and cigarette smoking was (13.58 ± 5.09) years and (14.89 ± 7.56) years, respectively. The two groups had a similar frequency of distribution in age, sex, alcohol consumption and cigarette smoking status. In addition, 95% of the case patients had a chronic infection with HBV but others had no infection with any hepatitis virus.

The *DNMT3B* T allele frequency and genotype distributions in the case patients and control subjects are summarized in Table 2. Only one genotype of CT was found in 140 control subjects (0.7%) and three in 100 case subjects (3.0%), respectively. The variant C allele frequency was 1.5% for the case patients and 0.4% for the control subjects. The CC genotype was not detected in both HCC patients and controls. Although the frequency of CT genotype and C allele was higher in HCC patients than in control subjects, but there was no significant difference ($P > 0.05$).

DISCUSSION

Alterations in DNA methylation can cause changes in gene transcription patterns and also promote mutational events leading to malignant tumors. Recent studies have shown that several mechanisms, including DNA hypomethylation on pericentromeric satellite regions, DNA hypermethylation on CpG islands of genes such as *p16*, *E-cadherin*, and *HIC-1* (hypermethylated-in-cancer), over-expression of DNA methyltransferases, and reduced expression of methyl-CpG-binding proteins play a role in hepatocarcinogenesis[25-27]. It was reported that genetic disruption of both *DNMT1* and *DNMT3B* in human cancer cells results in global and gene-specific demethylation, and abrogation of silencing of tumor suppressor genes. Both the wild type and catalytically inactive *DNMT3B* mutant can suppress rDNA promoter irrespective of its methylation status[28,29], suggesting that altered activities of DNMTs contribute to the generation of aberrant methylation in cancer.

Single nucleotide polymorphisms (SNPs) are the most common form of human genetic variation. SNPs in the promoter region of genes may affect either the expression or the activity of enzymes and therefore may be mechanistically associated with cancer risk. The *DNMT3B* gene contains a C-to-T transition polymorphism (C46359T) in a novel promoter region, -149 bp from the transcription start site, which in *in vitro* assays confers a 30% increase in promoter activity[23]. It was postulated that the T variant might up-regulate *DNMT3B* expression, resulting in a predisposition towards aberrant *de novo* methylation of CpG islands in tumor suppressor and DNA repair genes. These findings encouraged us to examine the relationship between a novel polymorphism in the human *DNMT3B* promoter and risk of HCC.

To select China as a research field to analyze the relationship between *DNMT3B* genetic polymorphisms and HCC is a new attempt. In this study, the *DNMT3B* genetic polymorphism was not susceptible to HCC. The

CC genotype was not detectable in both HCC patients and controls, while the T allele was predominant. In control subjects, the frequency of TT and CT genotypes was 99.3% and 0.7% respectively, while that of T and C alleles was 99.6% and 0.4% respectively. The frequency of CT genotype was higher in HCC (3.0%), while that of T and C alleles was 98.5% and 1.5%. Although the frequency of CT genotype and C allele was higher in HCC patients than in control subjects, but there was no significant difference.

The frequency of *DNMT3B* genotypes is much different from the reported frequency in Caucasians[21]. In the control group, the frequency of TT, CT and CC was 23.2%, 41.8% and 35.0% respectively, while the T allele frequency was 44.1%. C allele was very common. The frequency of TT, CT and CC in lung cancer patients was 21.0%, 56.7% and 22.3% respectively, indicating that a different race has a different genetic composition.

The *DNMT3B* allele frequency of people in North China is slightly different from that of people in other areas of China[30]. In the present study, the CC genotype was also not detectable. In control subjects, the frequency of TT and CT genotypes was 94.9% and 5.1% respectively, while that of T and C alleles was 97.4% and 2.6% respectively, suggesting that *DNMT3B* genetic polymorphism is variable in the same race living in different geographic areas. Only the TT genotype was detectable in all control subjects as compared with other ethnic groups such as Japanese[31], also suggesting that a different ethnic group has a different genetic composition.

The PCR product was digested with *Bln* I for 12-16 h to complete the reaction of restriction enzyme. Since the sensibility of electrophoresis through polyacrylamide gel is much higher than that of electrophoresis through agarose gel, we chose the former to separate the DNA fragments.

In our study, only one of the case subjects with CT genotype had a family history of HCC, but HBsAg, HBeAb and HBcAb in the other case subjects remained positive for a period of over 10 years without any treatment. Although we designed experiments to assess the correlation of the distribution of *DNMT3B* genotypes to the transcription and expression of *DNMT3B* and HBV infection status in selected tumor and normal hepatic tissues, we could not carry out the experiments due to the insufficient number of samples with CT genotype in our study.

The very similar distribution of *DNMT3B* genotypes in HCC patients and healthy controls suggested that the C/T polymorphism of *DNMT3B* gene might not independently affect the risk for HCC. A study in North China showed that *DNMT3B* SNPs are not associated with the susceptibility to gastric cardiac adenocarcinoma[30], although this polymorphism has been demonstrated to be associated with the susceptibility to cancers of the lung, head, neck and breast.

Recently, several candidate SNPs in the *DNMT3B* gene have been deposited in public databases (http://www.ncbi.nlm.nih.gov/SNP). Although the functional effects of these polymorphisms have not been elucidated, we hypothesize that some of these variants, particularly their haplotypes, may influence DNMT3B activity on DNA methylation, thereby modulating the susceptibility to HCC. The C/T SNP (C46359T) in the promoter of the *DNMT3B* gene may not be associated with up-regulation of DNMT3B and an increased risk of HCC as observed in this study, but probably there are other polymorphisms in *DNMT3B* which are susceptible to HCC.

In the present study, the *DNMT3B* gene was not negligible in the study of hepatocarcinogenesis in different areas. Whether *DNMT3B* SNPs are associated with different tumor types needs to be further studied in China. To clarify the role of *DNMT3B* SNP in the development of HCC, investigations in other populations need to be performed as well. The potential usefulness of *DNMT3B* genotyping needs further studies on a larger scale.

Saito[32] has found that over-expression of a splice variant of DNMT3B, DNMT3B4, which may lack DNA methyltransferase activity and compete with DNMT3B3 for targeting pericentromeric satellite regions, results in DNA hypomethylation on these regions, even at precancerous stages, and plays a critical role in the development of human HCC because of chromosomal instability and aberrant expression of cancer-related genes. Further investigation on the mechanism of aberrant expression of DNMT3B in tumors, including HCC, should be performed.

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COMMENTS

Background

Genetic and epigenetic factors are important in determining individuals' susceptibility to hepatocellular carcinoma (HCC). Alterations in DNA methylation can cause changes in gene transcription patterns and also promote mutational events leading to malignant tumors. DNA methylation is mediated by DNA methyltransferases (DNMTs), of which three active forms have been identified: DNMT1, DNMT3A and DNMT3B. DNMT3B, regarded as a *de novo* DNA methyltransferase, is thought to play an important role in the generation of aberrant methylation in carcinogenesis.

Research frontiers

Studies have shown that significant over-expression of DNMT3B is observed in tumor tissues while over-expression of DNMT1 and DNMT3A is more modest. A C-to-T transition polymorphism (C46359T) in the promoter region of the *DNMT3B* gene, -149 base pairs from the transcription start site, greatly increases promoter activity and is associated with an increased risk of cancers, such as cancer of the lung, head and neck. Carriers of the T allele, particularly heterozygotes, have a significantly increased risk foe such cancers.

Innovations and breakthroughs

Since the *DNMT3B* promoter polymorphism that is responsible for regulating genomic methylation is possibly associated with an increased risk for cancer, we evaluated the relationship between *DNMT3B* C46359T polymorphism and risk of HCC using PCR-RFLP and sequencing analysis.The PCR product was digested with *Bln* I for 12-16 h to complete the reaction of restriction enzyme. Since the sensibility of electrophoresis through polyacrylamide gel is much higher than that of electrophoresis through agarose gel, we chose the former to separate the DNA fragments.

Applications

C/T polymorphism of the *DNMT3B* gene may not independently affect the risk for HCC and probably there are other polymorphisms in *DNMT3B* which are susceptible to HCC. Whether *DNMT3B* SNPs are associated with different tumor types and different races needs to be further studied. The potential usefulness of *DNMT3B* genotyping needs further studies in a large scale.

Terminology

Single nucleotide polymorphisms (SNPs) represent a natural genetic variability at a high density in the human genome. A synonymous expression is "biallelic marker" corresponding to the two alleles that may differ in a given nucleotide position of diploid cells. A single SNP represents an alternative nucleotide in a given and defined genetic location at a frequency exceeding 1% in a given population. This definition does not include other types of genetic variability like insertions and deletions, and variability in copy number of repeated sequences. SNPs are considered the major genetic source to phenotypic variability that differentiates individuals from one another within a given species. Restriction fragment length polymorphism (RFLP) is a technique by which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of fragments produced differs when DNA is digested with a restriction enzyme. The similarity of patterns generated can be used to differentiate species (and even strains) from one another.

Peer review

Dr. Wu and co-investigators looked at C/T SNP in the promoter region of *DNMT3B*. They could not demonstrate a significant difference in C/T polymorphism between HCC patients and normal Chinese population. However, *DNMT3B* gene is not negligible in study of HCC in different races, ethnic groups or geographic areas as well as in study of different tumor types.

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CASE REPORT

Mycophenolate mofetil for drug-induced vanishing bile duct syndrome

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Abstract

Amoxicillin/clavulanate is associated with liver injury, mostly of a cholestatic pattern. While outcomes are usually benign, progression to cirrhosis and death has been reported. The role of immunosuppressive therapy for patients with a protracted course is unclear. We report the case of an elderly patient who developed prolonged cholestasis secondary to amoxicillin/clavulanate. Vanishing bile duct syndrome was confirmed by sequential liver biopsies. The patient responded to prednisone treatment, but could not be weaned off corticosteroids, even when azathioprine was added. Complete withdrawal of both prednisone and azathioprine was possible by using mycophenolate mofetil, an inosine monophosphate dehydrogenase inhibitor. Sustained remission has been maintained for more than 3 years with low-dose mycophenolate mofetil.

Key words: Amoxicillin and clavulanate; Drug-induced cholestasis; Ductopenia; Mycophenolate mofetil; Vanishing bile duct syndrome

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INTRODUCTION

Acute liver injury caused by amoxicillin/clavulanate occurs in 1.7 cases per 10000 prescriptions written and is mostly of a cholestatic type[1]. Outcomes are usually benign with resolution of cholestasis in 1-4 mo following drug withdrawal[2]. However, some patients develop prolonged drug-induced cholestasis, defined as the persistence of jaundice for more than 6 mo or persistently high alkaline phosphatase and gamma-glutamyl transpeptidase for more than 1 year, despite withdrawal of the causative drug, and in the absence of pre-existing liver or biliary tract disease[3]. Patients who develop progressive destruction of the small interlobular bile ducts ("vanishing bile duct syndrome") may ultimately require liver transplantation[4,5], given the lack of effective treatment. As an immunological reaction is suspected, corticosteroids have been used empirically[6], although the precise mechanism of amoxicillin/clavulanate-induced cholestatic hepatitis is unknown. We report a case illustrating that mycophenolate mofetil can be a successful and safe alternative to corticosteroids for amoxicillin/clavulanate-induced prolonged cholestasis.

CASE REPORT

A 69-year-old man presented with fatigue, upper abdominal discomfort and a pruritic rash involving his torso. He had a history of long-standing and well-controlled polycythemia vera. His medications included aspirin and hydroxyurea, and he drank alcohol sparingly. Three weeks prior to this examination, he had undergone a course of amoxicillin/clavulanate, 875 mg twice daily, for treatment of bronchitis. Physical examination revealed a fine maculopapular rash on his torso. The liver edge was palpable under the costal margin, and was smooth and not tender. No hepatosplenomegaly was noted. Initial laboratory data showed: alkaline phosphatase 624 U/L, aspartate aminotransferase (AST) 89 U/L, alanine aminotransferase (ALT) 82 U/L, total bilirubin 1.6 mg/dL, direct bilirubin 1.0 mg/dL, gamma glutamyl transpeptidase (GGT) 360 U/L, and albumin 3.3 g/dL. Abdominal ultrasound examination showed that the liver had a heterogeneous texture, with normal bile ducts and gallbladder. Over the next 2 weeks, he became jaundiced, with a peak of alkaline phosphatase of 988 U/L, total bilirubin 7.4 mg/dL, direct bilirubin 6.7 mg/dL, AST 235 U/L, and ALT 310 U/L. Prothrombin time (PT)/international normalized ratio (INR) remained normal. Autoimmune and viral serologic studies were all negative. Iron studies and alpha-1-antitrypsin levels were within normal ranges. Endoscopic retrograde cholangiography was normal. Liver biopsy, performed 5 mo after

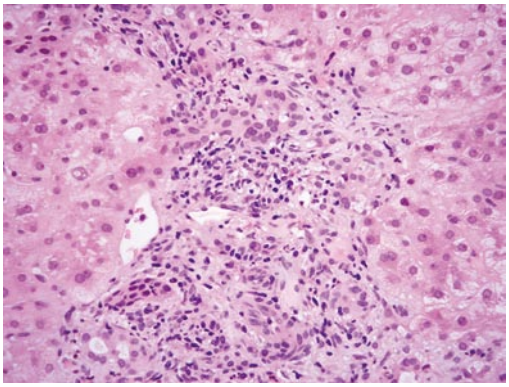

Figure 1 Initial biopsy. The triad is expanded by portal and periportal fibrosis, and there is a mixed inflammatory infiltrate. Injured bile ducts are present (bottom left and top center) and there is a marked ductular reaction.

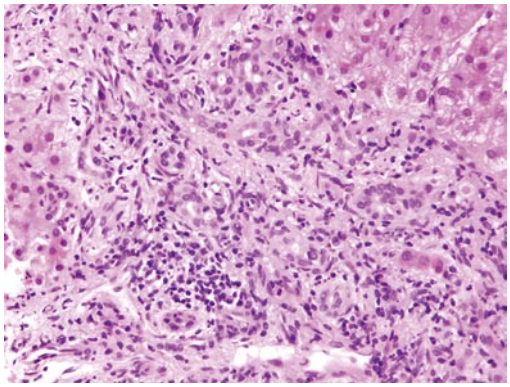

Figure 2 Second biopsy. The portal tracts appear similar to those seen in the initial biopsy, but with greater fibrous expansion ands more inflammation, including a loose aggregate of plasma cells and lymphocytes (bottom left). There is ductopenia and a prominent ductular reaction.

exposure to amoxicillin/clavulanate, showed destructive cholangiopathy of the small/medium-sized bile ducts, with ductopenia, duct proliferation, and bilirubinostasis. An infiltrate of lymphocytes and plasma cells was mostly confined to the portal tracts. Portal, periportal and focal bridging fibrosis were present, but hepatic architecture was preserved (Figure 1).

Treatment with prednisone 30 mg/d and ursodiol 1200 mg/d resulted in marked clinical and biochemical improvements. Tapering off the prednisone dose was attempted over the next few months, but resulted in increased alkaline phosphatase, AST and ALT, every time the prednisone dose was decreased to 12 mg/d. Azathioprine, titrated to 100 mg/d for several months, could not achieve prednisone tapering.

A second liver biopsy, taken after more than 1 year of treatment, showed chronic destructive cholangiopathy, persistent portal inflammation, and progression of fibrosis, with portoportal bridging and distorted hepatic architecture (Figure 2). Repeat endoscopic retrograde cholangiopancreatography (ERCP) revealed no extrahepatic biliary abnormalities.

Mycophenolate mofetil was added at a dose of 1 g twice daily and the patient was rapidly weaned off azathioprine. Over the next 6 mo, prednisone was successfully tapered, with liver tests remaining normal, except for a mild elevation in alkaline phosphatase (Figure 3). Over 1 year, the dose of mycophenolate mofetil was reduced to 250 mg twice daily, without adverse effects. Efforts to withdraw treatment completely resulted in recurrence of mild cholestatic abnormalities.

DISCUSSION

About 30 drugs, including amoxicillin/clavulanate, have been reported to cause vanishing bile duct syndrome with protracted clinical courses, the prototype being chlorpromazine[4]. Ductopenia, when interlobular bile ducts are absent from at least 50% of the small portal tracts, carries a poor prognosis[7]. The mechanism of progression from acute liver injury to ductopenia is unclear. However, it is suggested that bile ducts, as complete epithelium-lined tubes, are only rarely reconstructed once they have been completely destroyed[8]. A patient's unique immune response likely plays a role in the intensity and duration of injury, as certain HLA haplotypes have been found to be markedly overrepresented in patients who develop drug-induced cholestatic hepatitis[9,10].

Our case had the typical characteristics of amoxicillin/clavulanate-induced liver injury. These include advanced age, male sex, a cholestatic pattern of liver injury, delay between cessation of therapy and onset of jaundice, repeatedly negative tests for viral, autoimmune and metabolic diseases, and negative imaging studies[2,11-14]. Primary biliary cirrhosis and autoimmune cholangiopathy were considered unlikely, given the patient's age, gender, repeatedly negative serology, and histopathology.

A distinctive feature of our patient was his first liver biopsy that showed destructive cholangiopathy with portal and periportal fibrosis. The second biopsy, obtained 1 year later, revealed persistence of the destructive cholangiopathy but worsening fibrosis, with portoportal bridging and architectural distortion. In our patient, early fibrosis at 5 mo after exposure to amoxicillin/clavulanate may explain the prolonged cholestasis and immunosuppressant dependency. Patients reported complete recovery from prolonged amoxicillin/clavulanate-induced cholestasis did not have fibrosis on liver biopsy[2,11-14]. By contrast, in Degott's review of drug-induced cholestasis, all patients with persistent cholestasis had moderate to severe fibrosis[15].

Given the small number of patients reported with drug-induced vanishing bile duct syndrome and the unpredictability of its occurrence, there have been no clinical trials of treatment regimens. Short courses of corticosteroids have been used[6], based on a suggested immune pathogenesis of drug-induced cholestatic hepatitis. Mycophenolate mofetil, a non-competitive inhibitor of purine synthesis that acts by inhibiting inosine monophosphate dehydrogenase, blocks T- and B-lymphocyte proliferation. Approved for prophylaxis of rejection in solid organ transplantation, it is used against various immune-mediated diseases. Small clinical trials have reported its efficacy as a corticosteroid-sparing agent in autoimmune hepatitis[16,17], but it has not been evaluated for use in drug-induced liver disease. Our case illustrates

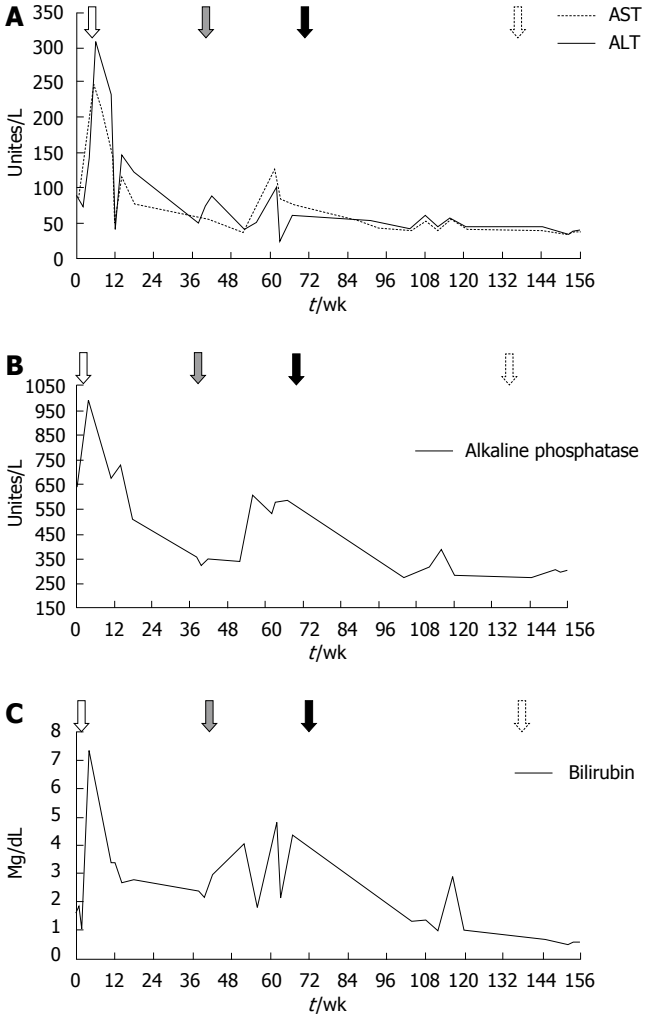

Figure 3 Three-year follow-up: liver enzymes and bilirubin during treatment with prednisone (white arrow), azathioprine/prednisone (grey arrow), and mycophenolate mofetil/prednisone (black arrow). Prednisone was stopped at wk 140 (dashed arrow). **A**: AST/ALT; **B**: alkaline phosphatase; **C**: bilirubin.

that mycophenolate mofetil can be a successful and safe alternative to corticosteroids for drug-induced prolonged cholestasis. Our patient has, at the time of this report, maintained normal liver function for over 3 years on low-dose mycophenolate mofetil, though efforts to withdraw treatment completely resulted in recurrence of mild cholestatic abnormalities.

In summary, we reported a case of severe amoxicillin/clavulanate-induced cholestatic hepatitis that resulted in progressive bile duct destruction and development of bridging fibrosis. Clinical and biochemical resolution was achieved using long-term immunosuppression, initially with prednisone and finally with low-dose mycophenolate mofetil. This suggests that cautious use of immunosuppressive therapy may be of benefit in those rare cases with persistent cholestasis. Further studies are needed to determine if early therapy can prevent irreversible bile duct injury, and to identify patients in whom such therapy is indicated.

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CASE REPORT

Sirolimus-induced drug fever and ciclosporin-induced leukencephalopathia with seizures in one liver transplant recipient

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Abstract

We describe the first case of sirolimus-induced drug fever in a female liver transplant recipient, with a history of hepatitis C-induced end-stage liver cirrhosis in 1999. In 2005, six years after transplantation, she developed calcineurin inhibitor-induced renal function impairment. Immunosuppression was switched from tacrolimus to sirolimus. Two days after the intake of sirolimus, she developed daily fever spikes, but no infectious focus was found. Antibiotic therapy had no influence on the fever. After fourteen days, sirolimus was switched back to tacrolimus and the fever disappeared. In history, the patient developed ciclosporin-induced generalized seizures eleven days after liver transplantation, followed by the development of a motoric speech disorder. Magnetic resonance imaging (MRI) findings were consistent with leucoencephalopathy, therefore immunosuppressive therapy was changed from ciclosporin to tacrolimus and the neurologic symptoms improved significantly. Our case is the first reported case of sirolimus-induced drug fever. In addition, the patient showed the rare occurrence of ciclosporin-induced leukencephalopathy with seizures.

Key words: Liver transplantation; Immunosuppression; Side effects

Schacherer D, Zeitoun M, Büttner R, Gelbmann C, Obed A, Schlitt HJ, Schölmerich J, Kirchner GI. Sirolimus-induced drug fever and ciclosporin-induced leukencephalopathia with seizures in one liver transplant recipient. *World J Gastroenterol* 2007; 13(45): 6090-6093

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INTRODUCTION

Ciclosporin and tacrolimus are very potent immunosuppressive drugs which have been used in organ transplantation for more than 10 years. Both are calcineurin-inhibitors and have the same mode of action. They are mainly metabolized by cytochrome P450 3A4 in bowel and liver[1]. The main side effects of ciclosporin and tacrolimus are renal toxicity, neurotoxicity, arterial hypertension, diabetes mellitus and hyperlipidemia[2]. Combination therapy of these two drugs is therefore not useful. Until now, many immunosuppressive regimens contain either ciclosporin or tacrolimus in combination with other immunosuppressive drugs. For several years, the mammilian target of rapamycin (mTOR) inhibitor, sirolimus (Rapamune®), has also been used as an immunosuppressive drug in organ transplantation. Similar to ciclosporin, it is also metabolized in bowel and liver by cytochrome P450 3A[1]. The main advantage of sirolimus is its virtual lack of nephrotoxicity. The main side effects are hyperlipidemia, anemia, and thrombocytopenia. Ciclosporin and sirolimus are substrates of the efflux-transporting pump P-glycoprotein, which is among others localized in gut and liver[3]. Ciclosporin and sirolimus have different modes of action and synergistic effects[4]. Therefore, in patients with renal impairment, combination therapy allows a dose reduction of both drugs which results in reduced side effects, especially on renal toxicity. Ciclosporin, tacrolimus as well as sirolimus can be given as monotherapy or in combination with other drugs. Patients with progressive impairment in renal function due to ciclosporin- or tacrolimus-induced nephrotoxicity can be switched to sirolimus monotherapy to prevent further loss of renal function[5].

CASE REPORT

We present the case of a 63-year-old female patient, who was first diagnosed with chronic hepatitis C virus infection in 1996 (genotype 1b). Infection was most likely due to several blood transfusions which were necessary after resection of a cyst of the left ovary in 1973. In 1997

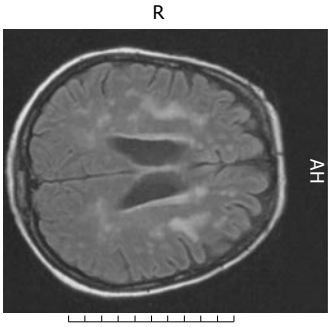

Figure 1 Magnetic resonance imaging (MRI) of the head showing almost symmetric periventricular white matter lesions consistent with leucoencephalopathy and a beginning encephalitis (FLAIR TR 8000 ms, TE 110 ms).

histopathological liver examination was performed for the first time and showed inflammation grade Ⅳ, as well as fibrosis grade Ⅲ (Ishaak-Score). Therefore, interferon monotherapy was given for one year (relapse after end of treatment). Six months later, she presented with decompensated liver cirrhosis and esophageal bleeding due to varices grade Ⅲ. After re-compensation she was listed for liver transplantation in January 1999. Neurologic status was normal prior to liver transplantation. Liver transplantation was successfully performed in August 1999. The explanted liver demonstrated complete cirrhosis (716 g). The liver graft showed no histological damage and normal perfusion as judged by duplex sonography. Immunosuppressive therapy was started with a combination therapy of ciclosporin, azathioprine and steroids. Eleven days after the start of immunosuppressive therapy, she developed a generalized seizure, which could be stopped with diazepame. Several focal and generalized epileptic fits followed and the patient developed a motoric speech disorder which finally resulted in dysarthria and complete aphasia. The patient showed no other neurologic symptoms. A magnetic resonance imaging (MRI) of the head showed periventricular white matter lesions consistent with leucoencephalopathy and a beginning encephalitis (Figure 1). The described symptoms were interpreted as side effects of ciclosporin medication. Therefore, immunosuppressive therapy was changed from ciclosporin to tacrolimus (FK 506, Prograf®) and antiepileptic therapy was initiated with 400 mg gabapentin four-times a day. The dysarthric disorder improved significantly, but residuals still existed with no occurrence of further seizures at the time when this report was written. During the treatment with steroids and tacrolimus, the patient developed an insulin-dependent diabetes mellitus.

In December 1999, transplant reinfection with hepatitis C virus was diagnosed. In July 2001, liver histology showed a beginning fibrosis of the liver graft with little infiltration of inflammatory cells, corresponding to chronic hepatitis (Yano classification: grading 4, staging 3). Therefore, antiviral therapy with interferon alpha (1.5 Mio units/wk) and ribavirin (600 mg/d) was initiated. Due to pancytopenia azathioprine medication, ribavirin was stopped one year later. Hepatitis C virus re-infection was controlled by 90 μg pegylated interferon alpha 2a once a week subcutaneously. In 2003, a control MRI showed no deterioration of leukencephalopathy.

In September 2005, the patient presented with progressive renal impairment and peripheral oedema. Serum creatinine (193 μmol/L, normal range 44-80 μmol/L) and urea levels (15 mmol/L, normal range 2.0-8.3 mmol/L) were elevated. Urinary tests confirmed chronic renal failure with a creatinine clearance of only 20 mL/min. Therefore, immunosuppressive therapy was changed from tacrolimus (Prograf®) to sirolimus (Rapamune®). Two days after starting sirolimus, the patient developed fever of 38℃, which reached up to 39℃ on the next day. No reason for the fever was found either with blood cultures or with urine examination, or with X-rays. The patient showed no other specific clinical symptoms (e.g. diarrhea, skin lesions, pharyngitis). Leucocytes (3.8/nL, normal range 4.8-10.8/nL) and thrombocytes (96/nL; normal range 130-440/nL) were reduced, most likely due to interferon therapy and splenomegaly. Erythrocyte sedimentation rate (ESR) and C-reactive protein were normal and cytomegalovirus and Epstein Barr virus screening was negative. Antibiotic therapy with piperacilline and sulbactame was given for 6 d, but did not result in any improvement of the daily spikes of fever, which occurred every evening (Figure 2). After 14 d, the fever disappeared when sirolimus was switched back to tacrolimus in combination with mycophenolate mofetil. Thus, sirolimus-induced drug fever was diagnosed.

At the time when we wrote this report, the patient was in a good clinical condition, neurologic status remained stable but dysarthria still existed, and renal function (133 μmol/L serum creatinine) improved with dose reduction of tacrolimus and intake of 3 litres fluid per day.

DISCUSSION

The clinical and radiological features demonstrated in this patient are consistent with those of leukoencephalopathy, a rare condition previously described in patients treated with ciclosporin and tacrolimus[6,7]. In our patient as in most patients previously described, leukoencephalopathy associated with immunosuppression occurred early during therapy and was reversible with good recovery. One case of late-onset leukoencephalopathy with a fatal outcome has been reported[8]. In the reported case, the symptoms due to leukoencephalopathy improved after withdrawal of ciclosporin, but unfortunately, did not completely disappear. The patient also suffered from seizures, which disappeared after withdrawal of ciclosporin and initiation of antiepileptic therapy with gabapentin.

Neurologic symptoms represent serious complications following orthotopic liver transplantation and may be caused by various perioperative factors or may develop due to drug-specific toxicity of immunosuppression. The incidence of neurotoxicity seems to be higher in patients treated with tacrolimus than in patients treated with ciclosporin in the early postoperative period, after retransplantation as well as in the late phase[9]. Watson *et al*[10] described two patients who suffered from neurological events, one with encephalopathy and the other with recurrent seizures. Both patients were on sirolimus and ciclosporin after orthotopic liver transplantation and stabilized after withdrawal of ciclosporin. Choi *et al*[11] reported that of the 367 patients who received OLT, 48 suffered from neurological complications, 17

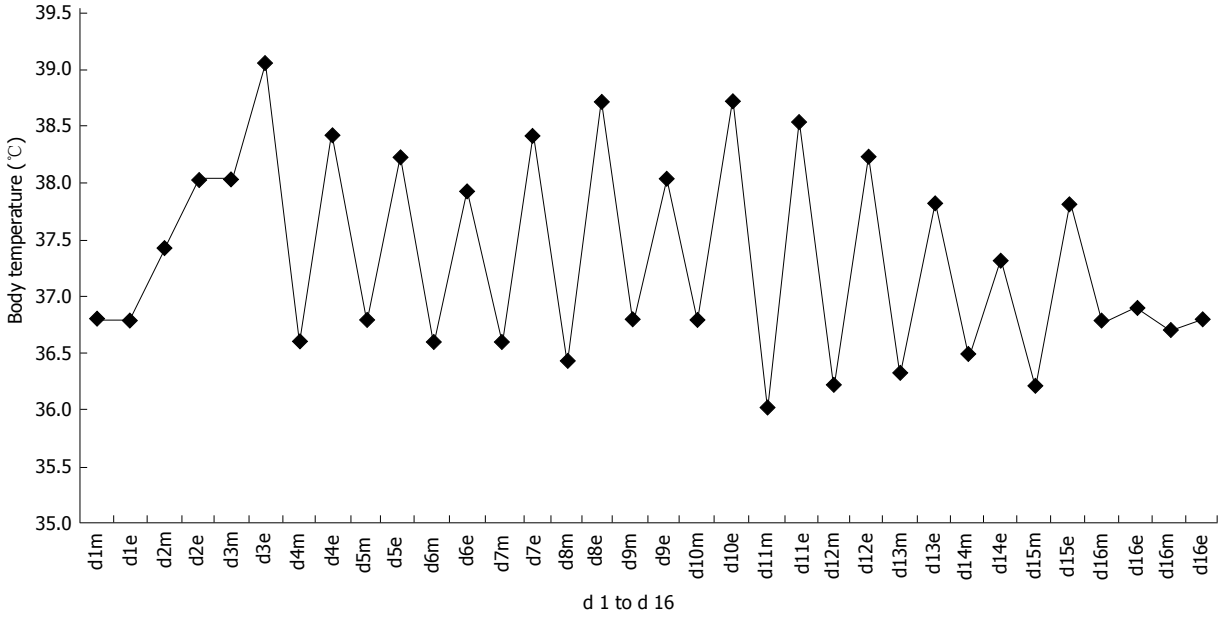

Figure 2 Temperature curve before, during (14 d) and after sirolimus therapy. Antibiotic therapy with piperacilline and sulbactame given from d 3 to 9 showing no effect. m: morning; e: evening.

developed seizures (status epilepticus occurred in two patients, generalized tonic-clonic seizures in five patients). Although neurotoxicity is not a frequent side effect of ciclosporin medication, the described cases in the reports are in accordance with our patient's symptoms (seizures and consecutive motoric speech disorder) which could be interpreted as side effects of ciclosporin medication. The motoric dysarthric disorder improved significantly after a change of the immunosuppressive regimen, but residuals still existed.

Idilman *et al*[12] described two cases of reversible posterior leukoencephalopathy manifested as headache, nausea and seizures associated with the use of immunosuppressive drugs following liver transplantation. One case of a 29-year old patient treated with ciclosporin after a liver transplant for primary sclerosing cholangitis showed late-onset progressive leucoencephalopathy due to immunosuppressive therapy and died three years later. These reports suggest that neurological side effects should be cautiously observed when alteration of immunosuppressive therapy is considered.

Although ciclosporin is an immunosuppressive agent widely used in the management of liver transplant recipients, neurological complications have been described in only few cases. The two different neurological side effects found in our patient are probably associated with ciclosporin medication.

Side effects of sirolimus include delayed wound healing, oral ulcers, hypertension, interstitial pneumonitis, infections, and most importantly, hyperlipidemia and myelosuppression[13]. Concerning the central nervous system, it has been shown that sirolimus can alter cell metabolism of primary astrocytes, thus resulting in similar neurotoxicity as experienced by tacrolimus and ciclosporin[14]. Perhaps the greatest potential benefit of sirolimus for liver transplant recipients is its lack of nephrotoxicity as compared to calcineurin inhibitors[15,16]. At present, no single immunosuppressive regimen can offer a clear advantage over another with regard to prevention of cellular rejection, graft survival, and patient survival[17]. In our patient, immunsuppression was switched from ciclosporin to sirolimus due to nephrotoxicity of ciclosporin. We clearly could show that the fever in our patient was not related to infection, but most likely to sirolimus. Two days after starting immunosuppression with sirolimus, our patient developed fever with no infectious focus found in blood cultures, urine tests or in radiologic examinations. Even the antibiotic therapy did not show any improvement of the daily spikes of fever in the evening. Due to the diagnosis of sirolimus-induced drug fever, the immunosuppressive medication was changed back to tacrolimus in combination with mycophenolate mofetil and no more fever spikes occured. To our knowledge, this is the first reported case of drug-fever obviously related to sirolimus. Two years ago, Dorschner *et al*[18] described a 2-year drug-related fever caused by everolimus, a sirolimus-derived immunosuppressant (Certican®). Their patient was 66 years old and received a cardiac transplant due to dilatative cardiomyopathy. The immunosuppressive regimen consisted of steroids, ciclosporin, and everolimus. Two weeks after the replacement of everolimus with azathioprine, all the patient's symptoms disappeared.

This is the first report of a liver transplant recipient with rare immunosuppressant-induced side effects. Until now, we could not unravel the mechanism(s) responsible for these side effects. A mutation in cytochrome P450 3A or FK-binding protein 12 (FK-BP12) seems to be unlikely, because our patient had no side effects under medication with tacrolimus, which is also metabolised by cytochrome P450 3A and bound to FK-BP12. Since ciclosporin, tacrolimus and sirolimus are substrates of the ATP-binding efflux pump P-glycoprotein, located in several organs like bowel and liver, it seems to be impossible that this protein might cause the several drug-side effects in our

patient. Therefore, we speculate that immunosuppressant drugs may have some influence on proteins in the central nervous system.

In conclusion, ciclosporin is an immunosuppressive agent widely used in the management of solid organ transplantation. Sirolimus is a powerful immunosuppressant used to prevent acute rejection episodes in patients who have undergone transplantation, particularly when nephrotoxic effects of calcineurin inhibitors become problematic.

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CASE REPORT

Carcinoembryonic antigen-producing adrenal adenoma resected using combined lateral and anterior transperitoneal laparoscopic surgery

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Abstract

A 74-year-old woman presented with symptoms consistent with hyperadrenocorticism and hypercatecholaminism. She had a cushingoid appearance and her cortisol level was elevated. Her serum dopamine and noradrenalin levels were also elevated. Computed tomography detected a left adrenal mass measuring 3.5 cm × 3.0 cm in diameter. Metaiodobenzylguanidine scintigraphy was negative. Unexpectedly, the serum Serum carcinoembryonic antigen (CEA) level was elevated. Fluorodeoxyglucose positron emission tomography showed increased uptake in the adrenal tumor only, with a maximum standardized uptake value of 2.8. Selective venography and blood sampling revealed that the concentrations of cortisol, catecholamines and CEA were significantly elevated in the vein draining the tumor. A diagnosis of CEA-producing benign adenoma was made. After preoperative management, we performed a combined lateral and anterior transperitoneal laparoscopic adrenectomy. Her vital signs remained stable during surgery. Histopathological examination revealed a benign adenoma. Her cortisol, catecholamine and CEA levels normalized immediately after surgery. We present, to the best of our knowledge, the first case of CEA-producing adrenal adenoma, along with a review of the relevant literature, and discuss our laparoscopic surgery techniques.

Key words: Carcinoembryonic antigen; Laparoscopy; Adenoma; Adrenal gland; Cushing syndrome

Hori T, Taniguchi K, Kurata M, Nakamura K, Kato K, Ogura Y, Iwasaki M, Okamoto S, Yamakado K, Yagi S, Iida T, Kato T, Saito K, Wang L, Kawarada Y, Uemoto S. Carcinoembryonic antigen-producing adrenal adenoma resected using combined lateral and anterior transperitoneal laparoscopic surgery. *World J Gastroenterol* 2007; 13(45): 6094-6097

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INTRODUCTION

Serum carcinoembryonic antigen (CEA) level is widely used as a reliable tumor marker in cancer patients. CEA level is rarely elevated in benign disease, and to the best of our knowledge there have been no reports of CEA-producing benign adrenal tumors. We present the first reported case of a CEA-producing adrenal adenoma, along with a review of the relevant literature, and discuss a useful technique for the removal of adrenal tumors.

CASE REPORT

A 74-year-old Japanese woman was referred to our hospital

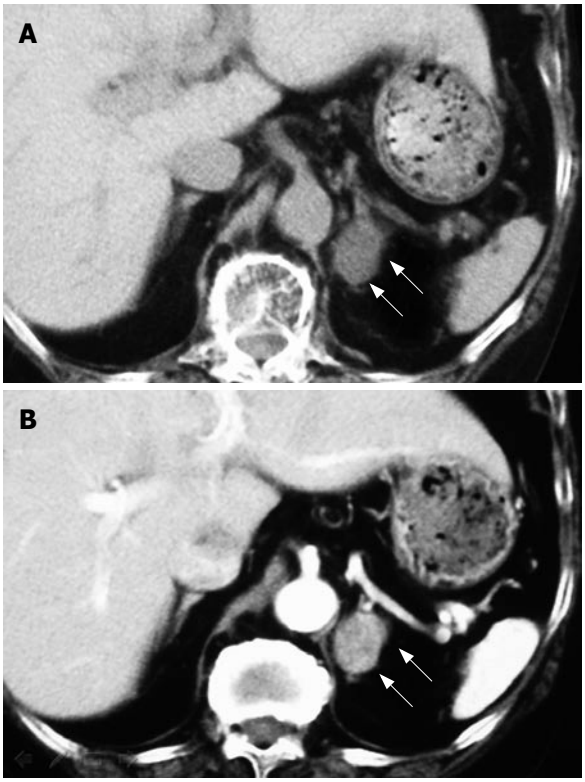

Figure 1 Abdominal CT. Plain (**A**) and enhanced (**B**) CT revealed a left adrenal mass (white arrows) measuring 3.5 cm × 3.0 cm in diameter.

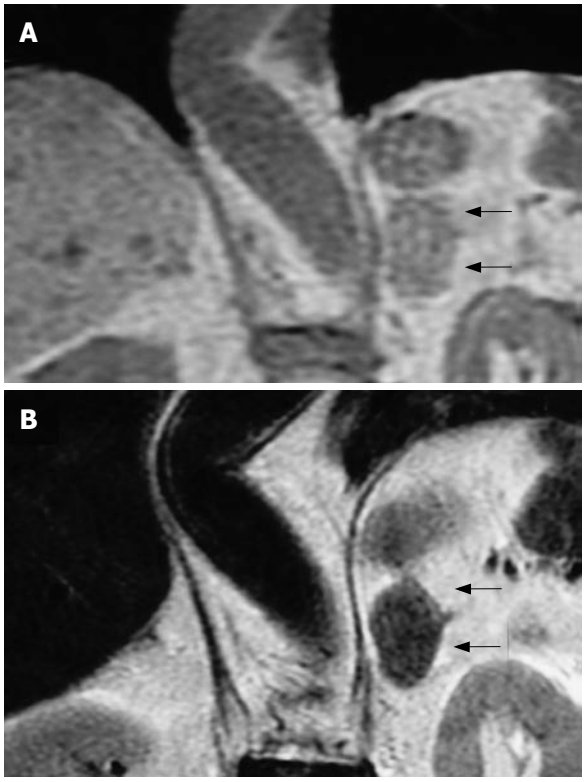

Figure 2 Abdominal MRI. MRI showed an adrenal mass (black arrow) with slightly low intensity on T1-weighted images (**A**) and low intensity on T2-weighted images (**B**).

because of fatigue and frequent episodes of palpitations, sweating, headache and weight gain over the past year. She had a history of hypertension and hyperglycemia, for which she had been treated at another hospital for 18 years. Although the number of medications and their dosages had slowly increased during the year before this consultation, her diseases and symptoms were not well controlled. Physical examination showed truncal obesity, moon face, buffalo hump and proximal muscle weakness. On admission, her blood pressure was 202/108 mmHg, and pulse rate 96 beats/min. X-ray images revealed osteoporosis. Serum biochemistry showed elevated levels of glucose (243 mg/dL; normal, 70-109 mg/dL), Hemoglobin A1c (HbA1c) (8.9%; normal, 4.3-5.8%) and total cholesterol (271 mg/dL; normal, 50-149 mg/dL), and reduced levels of total protein (5.5 g/dL; normal, 6.5-8.3 g/dL) and albumin (3.2 g/dL; normal, 3.7-5.3 g/dL). Abdominal computed tomography (CT) showed a left adrenal mass measuring 3.5 cm × 3.0 cm in diameter (Figure 1). Magnetic resonance imaging (MRI) showed an adrenal mass with slightly low intensity on T1-weighted images and low intensity on T2-weighted images (Figure 2). Her physical appearance was cushingoid, as described above. Adrenal cortical hormone analysis revealed a markedly elevated cortisol level, but aldosterone and estradiol levels were normal. The regulatory factors for these hormones were all within normal ranges (Table 1). Catecholamine analysis revealed markedly elevated dopamine and noradrenalin (NA) levels but the adrenalin level was within the normal range. The levels of catecholamine-breakdown products homovanillic acid (HVA) and vanillylmandelic acid (VMA) were both increased (Table 1). ^{123}I-metaiodobenzylguanidine scintigraphy showed no adrenal or extra-adrenal hot spots.

Unexpectedly, the CEA level was also found to be elevated (Table 1), although other tumor markers including carbohydrate antigen (CA) 19-9, CA125, CA15-3, alpha-fetoprotein and squamous cell carcinoma antigen were all normal. Upper gastrointestinal and colorectal endoscopy, neck/chest CT, and genital and breast examination did not reveal any abnormal findings. F-18 fluorodeoxyglucose (FDG)-positron emission tomography (PET)/CT revealed increased FDG uptake in the left adrenal gland (Figure 3). The maximum standardized uptake value was 2.8, and there was no FDG uptake elsewhere. We performed selective venography and blood sampling at various locations around the inferior vena cava to measure the concentrations of factors not within a normal range. The left adrenal vein (AV) was the drainage vein of the tumor and flowed into the left renal vein (RV). Results from the selective blood sampling clearly showed that the concentrations of these factors were dramatically increased in the left adrenal 'drainage' vein. This revealed that the left adrenal tumor was an obvious source of the CEA (Figure 4).

We diagnosed the tumor as a benign adrenal adenoma, which caused hyperadrenocorticism and hypercatecholaminism[1] and produced a large amount of CEA. After preoperative management[2], we removed the tumor by combined lateral and anterior transperitoneal laparoscopic adrenectomy (LA). The patient was placed in the right lateral position. A trocar was inserted at the umbilicus, and a carbon dioxide pneumoperitoneum (10 mmHg) was established. We introduced one trocar into the abdominal cavity through the lateral abdominal

Table 1 Time course of serum endocrine profiles and CEA levels before and after surgery

	Normal range	Before surgury	Postoperative day			
			1	3	5	7
Adrenal hormones						
Cortical secretion						
Hormones						
Aldosterone (pg/mL)	30.0-160.0	30.4	50.3	113.5	43.3	72.7
Cortisol (μg/dL)	5.0-15.0	54.2	11.2	9.9	10.6	7.2
Estradiol (pg/mL)	10.0-20.0	12.2	10.8	18.6	17.8	11.7
Regulatory factors for cortical hormones						
Adrenocorticotrophic hormone (pg/mL)	5.0-52.0	5.2	5.4	9.2	5.8	14.8
Angiotensin (pg/mL)	9.0-47.0	12.3	24.6	33.1	36.6	24.9
Renin (pg/mL)	2.5-21.4	3.7	13.9	16.1	8.9	17.6
Medullary secretion						
Catecholamines						
Dopamine (pg/mL)	< 14.0	15.1	6.5	8.5	6.2	11.5
NA (pg/mL)	46.0-60.0	102.7	52.7	47.1	50.9	59.1
Adrenalin (pg/mL)	< 70.0	40.0	45.8	65.2	64.6	61.2
Catecholamine breakdown products						
HVA (ng/mL)	4.0-7.8	8.8	4.5	4.2	5.2	6.5
VMA (ng/mL)	3.3-8.6	24.5	7.8	7.5	7.7	8.1
Tumor marker						
CEA (ng/mL)	< 5.0	12.6	4.5	2.5	2.1	2.2

Values outside the normal range are underlined.

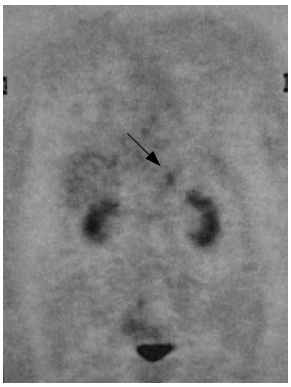

Figure 3 FDG-PET/CT. FDG uptake in the left adrenal gland (black arrow). The maximum standardized uptake value was 2.8, and no FDG uptake was seen anywhere else.

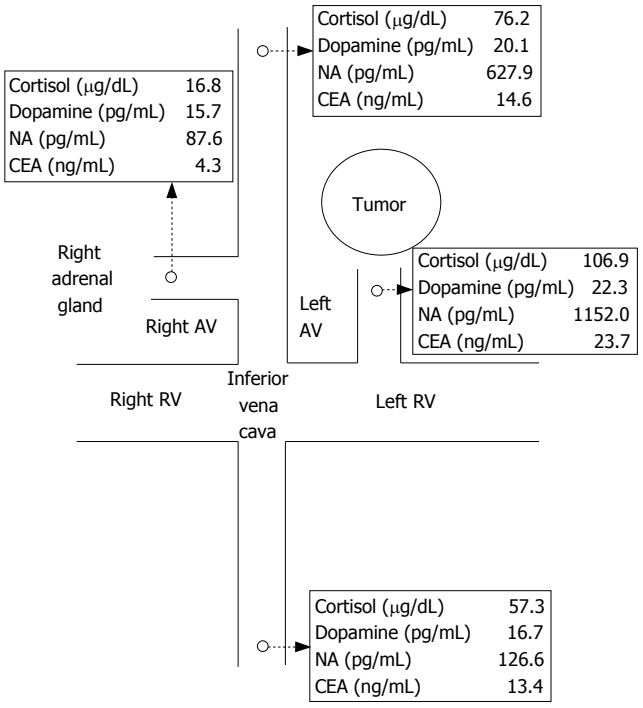

Figure 4 Selective venography and direct blood sampling. Measurements of serum cortisol, dopamine, NA and CEA were performed at four locations (proximal and distal inferior vena cava and both AVs). White circles represent sampling points.

wall, and two trocars through the subcostal wall. Using an electrothermal vessel sealing system (LigaSure, Tyco Healthcare, CO, USA), we isolated the spleen and distal pancreas by resecting the phrenic, colic and renal ligaments. The left RV and splenic vein were exposed by retracting the distal pancreas, spleen and left kidney. The left AV was separated from the surrounding tissue and ligated at its entry into the RV using a clip. The tumor was found to be located as indicated on preoperative imaging studies, without adhesion to the adjacent organ, and was removed with complete hemostasis. Vital signs remained stable during LA. Operative time was 2 h 45 m, and total blood loss was 85 mL. Histopathological examination of the resected specimen confirmed a benign adenoma. Chromaffin staining was negative. Cortisol, catecholamine and CEA levels normalized immediately after surgery (Table 1).

DISCUSSION

Based on hormonal evaluations of the adrenal cortex, we considered that this patient's hyperadrenocorticism was adrenocorticotrophic hormone-independent and renin-angiotensin-system-independent. Phenylethanolamine N-methyltransferase (PNMT), which is found in the adrenal glands, is required for the conversion of NA to adrenalin during catecholamine synthesis[3]. Catecholamine metabolism produces the breakdown products HVA (from dopamine) and VMA (from NA). Hormonal evaluations of the adrenal medulla suggested that this adrenal

adenoma either lacked PNMT or the optimal environment for the activation of PNMT.

LA was first performed in 1992[4,5], and this safe and effective treatment is now used worldwide for the management of functioning and non-functioning adrenal tumors for many reasons[6]. The minimal skin incisions provide a sufficient surgical field, anastomosis and reconstruction are not required, hemostasis can be achieved using laparoscopic devises, and the resected tumor can be removed through the small skin incision. Many previous reports on LA have already described the advantages and shortcomings of the transperitoneal and retroperitoneal approaches. There are two transperitoneal approaches, lateral and anterior[7]. Especially in left adrenal tumors and cases with more retroperitoneal fat, we have a clear impression that the combined lateral and anterior transperitoneal LA has the advantage of providing a sufficient surgical field and anatomical orientation in a timely manner. Surgery for catecholamine-releasing tumors differs from that for non-functioning tumors, because of the risk of intraoperative hypertensive and tachycardiac events[8]. In the present case, the combined approach allowed easy and early ligation of the drainage vein, which was the source of the catecholamines. We suggest that this procedure is effective for avoiding intraoperative iatrogenic complications.

CEA, the first tumor-associated antigen to be described, has many features that make it attractive for active vaccination against cancer. This useful biomarker is expressed in > 50% of all human cancers[9], including colorectal, lung, stomach, breast, pancreas, gallbladder, biliary tract, cervix, uterus, ovary, head/neck, bladder, kidney and prostate cancer. In the present study, we performed FDG-PET/CT to rule out adrenal cancer and to search for extra-adrenal tumors to explain the raised CEA level, as the conventional investigations for evaluation of an elevated CEA level showed no significant findings. We eventually hypothesized that the left adrenal tumor was producing a large amount of CEA, and performed selective venography and direct blood sampling of the drainage vein of the tumor to verify this. This method was useful for detecting the source of CEA in the present case. Previous reports have demonstrated that smoking, inflammatory diseases and benign tumors uncommonly produce CEA; but that benign tumors rarely progress to become malignant[10,11]. We are unable to explain how this patient's benign adenoma acquired the ability to secrete CEA, even after reviewing the relevant literature. This appears to be the first reported case of a CEA-producing adrenal adenoma. As a benign adrenal adenoma rarely secretes CEA, more cases need to be studied to better understand CEA production in benign adrenal tumors.

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CASE REPORT

Heterotopic pancreas in the stomach: A case report and literature review

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Abstract

Ectopic pancreas is defined as pancreatic tissue found outside the usual anatomic location of the pancreas. It is often an incidental finding and can be found at different sites in the gastrointestinal tract. It may become clinically evident when complicated by pathologic changes such as inflammation, bleeding, obstruction, and malignant transformation. In this report, a 40 years old woman with epigastric pain due to ectopic pancreatic tissue in the stomach is described. The difficulty of making an accurate diagnosis is highlighted. The patient has remained free of symptoms since she underwent wedge resection of the lesion three years ago. Frozen sections may help in deciding the extent of resection intraoperatively. Although ectopic pancreas is rare, it should be considered in the differential diagnosis of a submucosal gastric tumour.

Key words: Ectopic pancreas; Stomach; Histology; Surgery

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INTRODUCTION

Pancreatic heterotopia was first described in 1727 when it was found in an ileal diverticulum[1]. It is a rare entity, defined as the presence of extrahepatic tissue without any anatomic or vascular continuity with the pancreas. It may occur at a variety of sites in the gastrointestinal tract having a propensity to affect the stomach and small intestine. Usually, it is a silent anomaly but it may become clinically evident when complicated by inflammation, bleeding, obstruction or malignant transformation[2]. We report a case of a 40-year-old female with an ectopic pancreatic lesion in the antrum of the stomach.

CASE REPORT

A 40-year-old woman was admitted to our hospital due to a 2-mo history of recurrent episodes of epigastric pain, nausea and vomiting. Physical examination, routine blood tests including amylase, plain chest and abdominal X rays along with abdominal ultrasound were unremarkable. Esophagogastroduodenoscopy revealed a sessile polypoid mass with benign features located in the gastric antrum to the posterior wall measuring approximately 2 cm in diameter. The mucosa appeared normal throughout the stomach. Biopsy confirmed the presence of a normal gastric mucosa over the lesion. Computed tomography was not performed.

A decision was made to proceed with surgery. Endoscopic injection with methylene blue was performed to mark the lesion preoperatively.

The patient underwent exploratory laparotomy. Through a small midline incision a gastrotomy was performed. The lesion was clearly stained with methylene blue 4 h after the endoscopy. It was located approximately 10 cm from the pylorus to the greater curvature. In palpation the lesion was rubber like, fixed to the surrounding mucosa giving the feeling 'like a breast fibroadenoma'. Its dimension was approximately 5 cm × 3 cm × 4 cm. By using a stapler device a wedge resection of the lesion was performed with macroscopically clear margins. Frozen sections excluded malignancy and the possibility of ectopic pancreatic lesion. The surgical margins were clear.

The patient had no postoperative complications and was discharged 4 d later. She has remained free of symptoms with negative endoscopy since then.

Histopathologic examination of the lesion showed heterotopic pancreatic tissue in the gastric antrum with a lobular architecture characteristic of ectopic pancreas. The pancreatic lobules were located mainly in the gastric submucosa (Figure 1A) and partially in the muscularis propia (Figure 1B). They contained a mixture of pancreatic acini, ducts and islets of Langerhans. The overlying gastric mucosa was normal.

DISCUSSION

Ectopic pancreas is defined as pancreatic tissue that lacks anatomical or vascular communication with the normal

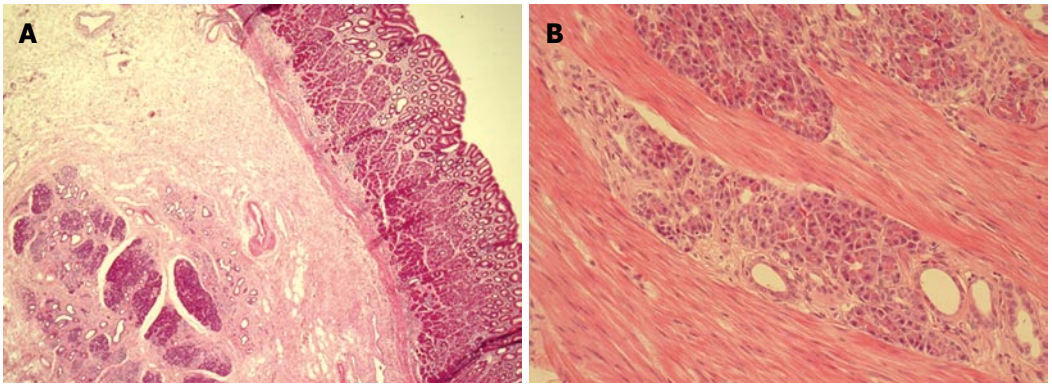

Figure 1 Heterotopic pancreatic lobules occupying the submucosa under an intact normal gastric mucosa (HE, x 100) (**A**) and between smooth muscle fibers of the gastric muscularis propria (HE, x 200) (**B**).

body of the pancreas[3]. Heterotopic pancreas has a genetic make-up, physiologic function, and local environmental exposure similar to that of the pancreas[4]. The incidence in autopsies ranges 0.5%-13.7%, being more common at the age of 30-50 years with a male predominance[3].

Of the 105 gastrectomies performed in our institution over the last five years, ectopic pancreatic tissue was found in only one case (1/105, 0.9%).

Several theories have been proposed to explain the pathogenesis and occurrence of pancreatic heterotopia. The most tenable theory implicates that during the development of normal pancreas from several evaginations, originating from the wall of the primitive duodenum, one or more evaginations may remain in the bowel wall. Migration of this embryonic remnant along with the development of the gastrointestinal tract gives rise to the ectopic pancreatic tissue[5]. Another theory suggests that during embryogenesis pancreatic metaplasia of the endodermal tissues localized in the gastric submucosa may occur[5].

Histopathologically, it is not a diagnostic problem when pancreatic acini, ducts, islets of Langerhans and intervening connective tissue are present. The most characteristic gross feature is a central ductal orifice[6].

Specifically in the stomach, the involvement of submucosal layer, muscularis and subserosal layer is 73%, 17% and 10%, respectively[7]. In the presented case the pancreatic tissue involved both the submucosa and muscularis propria. Heinrich in 1909 proposed three types of heterotopic pancreas but his classification was modified by Gaspar-Fuentes in 1973 acquiring its final form. Type Ⅰ heterotopia consists of typical pancreatic tissue with acini, ducts, and islet cells similar to those seen in normal pancreas (Figure 1). Type Ⅱ heterotopia is composed of pancreatic ducts only, referred as canalicular variety. Type Ⅲ heterotopia is characterized by acinar tissue only (exocrine pancreas). Type Ⅳ heterotopia is made up of islet cells only (endocrine pancreas)[8].

The usual location is in the stomach in 25%-38% of the cases, duodenum in 17%-36%, jejunum in 15%-21.7% and rare in the esophagus, gallbladder, common bile duct, spleen, mesentery, mediastinum and fallopian tubes. Gastric lesions are discovered in the antrum in 85%-95%, either on the posterior or anterior wall, being more common along the greater curvature[5].

The pancreatic ectopic tissue is usually silent but can also undergo complications that occur in normal pancreatic tissue such as acute or chronic pancreatitis, abscess and pseudocyst formation[9]. Malignant transformation may rarely occur. Up to 15 cases have been reported so far[10]. In order to be described as arising from heterotopic pancreas, the diagnosis of a carcinoma should fulfil three criteria: (1) the tumour must be located within or very close to the ectopic pancreatic tissue, (2) transition between pancreatic structures and carcinoma must be identified and (3) the non-neoplastic pancreatic tissue must comprise fully developed acini and ducts[11]. Adenocarcinomas arising from ectopic pancreas seem to have a somewhat better prognosis than those arising from the pancreas itself, probably due to earlier presentation[10].

Symptoms depending upon the anatomical location, such as gastric outlet obstruction in a pre-pyloric rest or obstructive jaundice in a bile duct focus, may originate from the mass effect of the tumour[12] and are also related to the size of the lesion. Lesions greater than 1.5 cm in diameter are more likely to cause symptoms[12]. Pain is one of the most common symptoms. The possible explanation is that the pain is due to endrocrine and exocrine function of the heterotopic pancreatic tissue, and relates to the secretion of hormones and enzymes, being responsible for inflammation or chemical irritation of the involved tissues[13]. Haemorrhage due to mucosal erosion, ulcer formation and perforation especially localized in the small intestine have also been reported[12].

Barium swallow study may show a typical image of a rounded filling defect with central indentation. The reported sensitivity and specificity are 87.5% and 71.4%, respectively[14]. Upper GI endoscopy can demonstrate a broad based umbilicated submucosal lesion. In the majority of cases, biopsies are superficial and non diagnostic. However, positive biopsies can establish the diagnosis[15]. Endoscopic ultrasonography has proven to be a useful adjunct in identification of pancreatic rests, localizing in the submucosa and ranging 0.5-2 cm. The combination of endoscopic ultrasonography with fine-needle aspiration allows cytologic evaluation of submucosal gastrointestinal lesions, having a sensitivity ranging 80%-100%[16,17].

Computed tomography findings are usually non specific. However, multi-slice spiral CT with oral and portovenous phase Ⅳ contrast may demonstrate the lesion which enhances similarly with the normal pancreatic tissue. CT can localize lesions with normal pancreatic tissue but cannot distinguish ectopic pancreas from other submucosal tumors[18,19].

In our case, since neither CT nor endoscopic ultra-

sonography was performed and the biopsy showed normal gastric mucosa, the diagnosis was made based on the benign endoscopic features of the lesion.

The diagnosis may be sometimes difficult intraoperatively due to the gross similarity of pancreatic heterotopia with gastrointestinal stromal tumour (GIST), gastrointestinal autonomic nerve tumour (GANT), carcinoid, lymphoma or even gastric carcinoma. If in doubt, frozen section is very helpful to establish the diagnosis intraoperatively and to avoid unnecessary extensive operations.

In conclusion, although pancreatic heterotopia is rare, it should be always considered in the differential diagnosis of extramucosal gastric lesions. Despite the development of modern diagnostic modalities, its diagnosis remains challenging. Surgical excision provides symptomatic relief and is recommended especially if diagnostic uncertainty remains. If in doubt, frozen section can help to avoid unnecessary radical operations.

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CASE REPORT

Large solitary retroperitoneal echinococcal cyst: A rare case report

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Abstract

Echinococcal disease remains a problem within some endemic areas. Echinococcal cysts usually involve the liver and lungs, but any other organ can potentially be involved. Extrahepatic localization is reported in 14%-19% of all cases of abdominal hydatid disease. We report the case of a large echinococcal cyst localized in the lower pelvis. A 28-year-old woman was admitted to a surgical ward with lower abdominal pain and discomfort lasting for a month. Ultrasonography and computed tomography scanning revealed a large retroperitoneal cystic mass (9 cm × 4 cm) in contact with the left ovary and left ureter. There were no cysts in any other location. Serological tests were positive for Echinococcus. The patient was operated on and the entire cyst was excised intact. Histopathological results confirmed the diagnosis of echinococcosis. Antihelminthics were administered postoperatively and the patient was discharged after 6 d, and is now being closely followed up. Total cystectomy when possible represents the treatment of choice for large extrahepatic echinococcal cysts.

Key words: Echinococcus cysts; Ultrasonography; Extrahepatic location; Seropositivity; Antihelminthics; Total cystectomy

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INTRODUCTION

Echinococcal disease, which produces unilocular cystic lesions, is an infection of humans caused by the larval stage of *Echinoccocus granulosus*. It is prevalent in the Middle East, the Mediterranean region, particularly in Greece and Lebanon, Australia, Argentina and Africa. Confirmed hosts are dogs that pass eggs into their feces. Intermediate hosts, e.g. sheep, cattle, humans, goats and horses, ingest the eggs and develop cysts[1]. Echinococcal cysts are mostly found in the liver (60%-70% of cases), followed by the lungs (10%-25%), spleen, ovaries, kidneys, brain, bones and heart, but rarely elsewhere in the body[1]. Hydatid disease in extrahepatic locations usually remains asymptomatic unless the cyst grows and produces symptoms due to pressure, rupture to the pleural or peritoneal cavity, secondary infection, or an allergic reaction[2]. We report the rare case of a 28-year-old woman with a large hydatid cyst in her lower pelvis, without hepatic or any other involvement.

CASE REPORT

A 28-year-old woman was admitted to our surgical ward with vague abdominal pain localized in the hypogastrium, and severe constipation, which had lasted for the previous 20 d. Her gynecologist had diagnosed a large cystic mass adjacent to the left ovary. She did not report episodes of fever, vomiting or diarrhea. Neither did she note any alterations in her menstrual cycle. Blood tests showed mild leukocytosis (12 000 white blood cells). Plain abdominal X-rays did not show any specific diagnostic findings. Abdominal ultrasonography (US) revealed a large cystic mass (9 cm × 4 cm) in contact with the left ovary and left ureter. Computed tomography (CT) confirmed the US findings (Figure 1). There were no cysts in any other location. Serological tests were positive for echinoccocal disease. The patient underwent a laparotomy, and a large cystic mass was identified retroperitoneally, firmly attached to the sacrum and in contact with the left ovary, left ureter and the sigmoid colon. Total cystectomy was performed en block with a portion of the mesosigmoid. The sigmoid vasculature remained intact, and there was no evidence of intraoperative colon ischemia. Postoperatively, antihelminthics were administered and the patient was discharged after 6 d. She had been closely followed up for 3 mo, without any abdominal pain and with significant improvement of constipation.

DISCUSSION

Echinococcosis remains a problem in endemic areas. *E.*

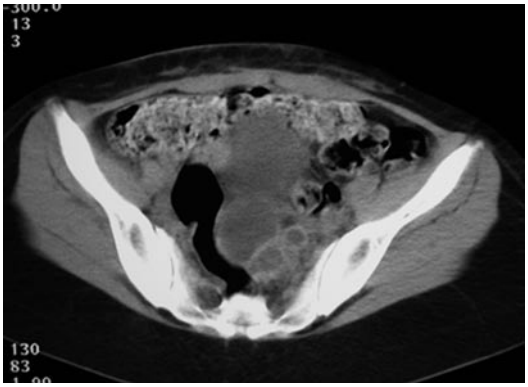

Figure 1 CT image of a large retroperitoneal echinococcal cyst in contact with the left ovary and left ureter.

granulosus is a 5-mm long worm, with a lifespan of 5-20 mo within the jejunum of dogs. When eggs are ingested by an intermediate host, the embryos escape, penetrate the intestinal mucosa, enter the portal circulation, and are then trapped in the liver[3]. A small number escape the hepatic filter, enter the systemic circulation, and are scattered to other organs. Larvae develop into fluid-filled unilocular hydatid cysts that consist of an external membrane and an inner germinal layer. Daughter cysts originate from the inner layer[3,4]. Slowly enlarging echinococcal cysts generally remain asymptomatic. Five to 20 years elapse before cysts enlarge sufficiently to cause symptoms. Abdominal pain, hepatomegaly or a palpable mass in the right upper quadrant are the most common symptoms for patients with liver echinococcosis[1]. Bile-duct compression or leakage of fluid into the biliary tree may mimic cholelithiasis. Biliary obstruction can result in jaundice. Generalized toxic reaction due to hydatid cyst rupture and secondary infection are among the most common complications[5].

Cysts in the peritoneal cavity account for 10%-16% of cases and are mainly the result of the rupture of concomitant hepatic cysts[2]. Extrahepatic locations of the echinococcus include the lungs (10%-15%), spleen (0.9%-8%), kidneys (1%-4%), pancreas (0.25%-0.75%), brain, heart, ovaries, bones and abdominal wall. Symptoms in such cases occur because of pressure or complications including rupture, allergic reaction and secondary infection[6]. Radiography, US and CT studies are important for a diagnosis of echinococcal disease. Plain abdominal X-rays may show calcifications of the cystic wall[7]. US is the method of choice for the detection of hepatic and extrahepatic echinococcal cysts. Hydatid cysts are classified by ultrasound into six categories.

Type I are defined as univesicular and < 50 mm in diameter. Type Ⅱ are univesicular with a prominent laminated layer, and tend to be seropositive. Type Ⅲ are subdivided into Ⅲa, defined as cysts with a prominent lamination that contains daughter cysts, and Ⅲb, characterized by lamination but a lower number of daughter cysts. Both Ⅲa and b are highly seropositive. Type Ⅳ appear as solid masses. Type Ⅴ are characterized by degeneration with calcifications. Type Ⅵ are defined as multiple cysts that may be univesicular and laminated, with daughter cysts involving one or more organs[8]. The sensitivity of US ranges from 93% to 98%[5].

CT confirms the diagnosis by revealing the presence of daughter cysts and plaque-like calcifications in the cystic wall. It is important as it provides information regarding the exact location of extrahepatic cysts in relation to neighboring structures. CT sensitivity ranges from 90 to 97%[5,9].

Serological tests contribute to diagnosis. Immunoglobulin G antibody detection by ELISA has a sensitivity of 95% and a specificity of 94%[8]. The sensitivity of indirect hemagglutination test has been found to be 87.5%[8,10].

Therapy for extrahepatic echinococcal disease is based on considerations regarding the size, location and manifestations of the cysts, and the overall health status of the patient. Asymptomatic small cysts once diagnosed can be treated with antihelminthic drugs, administered for 28 d in one to eight repeating cycles, separated by drug-free intervals of 2-3 wk[11].

For symptomatic or large hydatid peritoneal cysts, surgery, when feasible, is the principal method of treatment. Surgical treatment can be either radical or conservative. Total cystectomy, whenever possible, is the gold standard. For peritoneal cysts firmly attached to intraperitoneal viscera, unroofing and drainage has been proven to be a safe method[11,12]. It is important that the abdominal cavity is isolated with gauzes soaked in 20% hypertonic saline solution to avoid secondary hydatosis and allergic reaction[11].

In our case, to eradicate the disease, total cystectomy was carried out with meticulous dissection of the left ureter, gonadal vessels, hypogastric nerve plexus and the mesosigmoid vasculature. Thus, the sigmoid remained intact, free from compression, and constipation was greatly improved postoperatively.

In conclusion, a diagnosis of extrahepatic echinococcal disease is more accurate today because of the new imaging techniques available. The treatment of choice for small asymptomatic hydatid cysts is conservative by administration of antihelminthic drugs. Large or symptomatic echinococcal cysts need to be treated by total cystectomy, or unroofing and drainage followed by adjuvant antihelminthic therapy.

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CASE REPORT

Mediastinal tuberculous lymphadenitis presenting as an esophageal intramural tumor: A very rare but important cause for dysphagia

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Abstract

Dysphagia associated with esophageal mechanical obstruction is usually related to malignant esophageal diseases. Benign lesions are rarely a cause for this type of dysphagia, and usually occur either as an intramural tumor or as an extrinsic compression. Mediastinal tuberculous lymphadenitis is rare in adults, and even more rarely causes dysphagia. We report two cases of dysphagia in adult patients caused by mediastinal tuberculous lymphadenitis, presenting radiologically and endoscopically as an esophageal submucosal tumor. Based on the clinical and imaging diagnosis, the patients underwent a right thoracotomy, and excision of the mass attached to and compressing the esophagus. Pathological examination of the specimens showed a chronic granulomatous inflammation with caseous necrosis, which was consistent with tuberculous lymphadenitis.

Key words: Dysphagia; Tuberculous lymphadenitis; Esophageal tumor; Uncommon dysphagia; Esophageal benign lesion

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INTRODUCTION

In the esophagus, squamous cell adenocarcinoma as the most common type of malignant tumors and leiomyoma as the most common type of mesenchymal neoplasms, represent the frequent causes for dysphagia. However, dysphagia associated with esophageal mechanical obstruction can be caused by several conditions, mainly endoluminal/mucosal lesions, intramural tumors or extrinsic compression.

Despite technological improvements in the diagnostic work-up, the definitive diagnosis of some of these entities, particularly differentiation of submucosal parietal lesions from extrinsic lesions compressing the esophagus, may only be established after surgery[1].

Mediastinal diseases such as sarcoidosis[2], lung cancer, lymphoma, distant metastases[3,4] or mediastinal inflammatory lymph nodes[1,5-9] are the conditions that rarely compress the thoracic esophagus and cause dysphagia.

Some causes for dysphagia should always be considered in the differential diagnosis of dysphagia, because some of them if diagnosed and treated in time will completely cure the patient. We herein report two cases of dysphagia caused by mediastinal tuberculous lymphadenitis (MTL), presenting as esophageal intramural tumors.

CASE REPORT

Case 1

A 40-year-old woman who complained of a six-month history of dysphagia for solid food and paroxystic odynophagia, was admitted to our hospital for further evaluation of a submucosal mass-like lesion seen on an upper gastrointestinal (GI) endoscopy taken in another hospital. She had no other digestive symptoms, denied weight loss, fever, cough, sputum and night sweating. Her past medical history was unremarkable, namely exposure to tuberculosis and her family history was also unremarkable. Clinical examination was irrelevant, and her hematological and biochemical tests were normal.

A chest X-ray did not show any parenchymal and pleural change or mediastinal pathology. A barium swallow esophagogram (Figure 1A) disclosed a two-centimeter protruding lesion on the left lateral aspect of the mid-esophagus, distal to the aortic arch and with a smooth surface. An upper GI endoscopy (Figure 1B) showed a

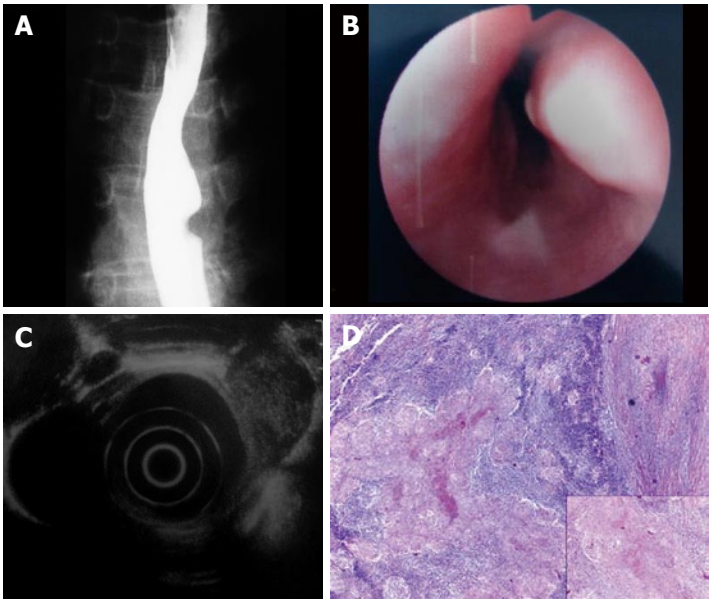

Figure 1 Barium swallow showing a two-centimeter protruding lesion on the left lateral aspect of the mid-esophagus (**A**), and endoscopic view of a two- centimeter protruding lesion covered by normal mucosa and with firm consistency (**B**), endoscopic ultrasonography revealing a two-centimeter solid hypoechoic lesion in the third layer (**C**), and lymph node parenchyma extensively occupied by necrotizing epithelioid granulomas (HE, × 40) (**D**). Insert: typical tuberculous granuloma with central caseous necrosis and multinucleated cells of Langhans type (HE, × 200).

two- centimeter deformity of the esophageal wall, covered by normal mucosa and with firm consistency, located 25 centimeters from the anterior incisor teeth. An endoscopic ultrasonography (Figure 1C) suggested that the lesion described by the other methods was an extramucosal mesenchymal tumor of the esophagus. A CT scan showed no pulmonary lesions or enlargement of mediastinal lymph nodes. The patient underwent a right thoracotomy, and excision of the mass attached to and compressing the esophagus. Surgery was performed with some difficulty due to surrounding fibrosis.

Pathological examination of the specimen showed a conglomerate of lymph nodes surrounded by chronic granulomatous inflammation with caseous necrosis. Zielh-Neelsen stain of the specimen, however, failed to disclose acid-fast bacilli (Figure 1D).

The symptoms of dysphagia and odynophagia disappeared after excision of the mass and post-operative treatment with a three-regimen anti-tuberculous chemotherapy with isoniazid, ethambutol hydrocloride and rifampicin.

A follow-up barium swallow esophagogram revealed a normal esophagus and the patient was in good health 6 years after an uneventful operation and subsequent discharge.

Case 2

A 54-year-old woman was referred to our hospital with a five-month history of dysphagia for solid food and a diagnosis of a submucosal lesion in the middle third of the esophagus. She had no other digestive symptoms, denied weight loss, fever, cough, sputum and night sweating. Her past medical history was unremarkable, namely exposure to tuberculosis and her family history was also unremarkable. Clinical examination was irrelevant, except for a palpable right supra-clavicular lymph-node. In spite of a repeatedly high erythrocyte sedimentation rate (30-73 mm in the first hour), sputum examination to disclose acid-fast bacilli and all the other routine laboratory tests revealed no abnormal findings.

Fine needle cytology of the supra-clavicular lymphadenopathy showed a necrotic lesion, without malignant cells. Zielh-Neelsen stain was negative. A barium swallow esophagogram (Figure 2A and B) disclosed a four-centimeter protruding smooth lesion on the right lateral aspect of the mid-esophagus, with apparent integrity of mucosa. An upper gastrointestinal (GI) endoscopy (Figure 2C) showed a two-centimeter deformity of the esophageal wall covered by normal mucosa and with firm consistency, located 22 centimeters from the anterior incisor teeth. An endoscopic ultrasonography (Figure 2D) revealed a two-centimeter hipoecogenic mass with well-defined limits, which was apparently dependent on the fourth ecographic layer of the esophagus, suggesting a leiomyoma. A chest X-ray and a CT scan did not show any lung parenchymal or pleural change, but the CT scan revealed enlargement of some mediastinum and pulmonary hilum lymph nodes, even though they did not form conglomerates.

Through a right thoracotomy, the mass compressing the esophagus was removed, in fact, an enlarged lymph node, and some other lymph nodes adherent to the esophageal wall were also removed.

Pathological examination of the specimen showed lymph nodes surrounded by a chronic granulomatous inflammation with caseous necrosis. Zielh-Neelsen stain of the specimen disclosed acid-fast bacilli (Figure 2E).

The patient began a postoperative three-regimen anti-tuberculous treatment with isoniazid, ethambutol hydrocloride and rifampicin.

The symptoms of dysphagia disappeared after treatment and a follow-up barium swallow esophagogram (Figure 2F) revealed a normal esophagus. The patient was in good health 4 years after discharge.

DISCUSSION

The ethiology of dysphagia associated with esophageal mechanical obstruction is usually referred to as

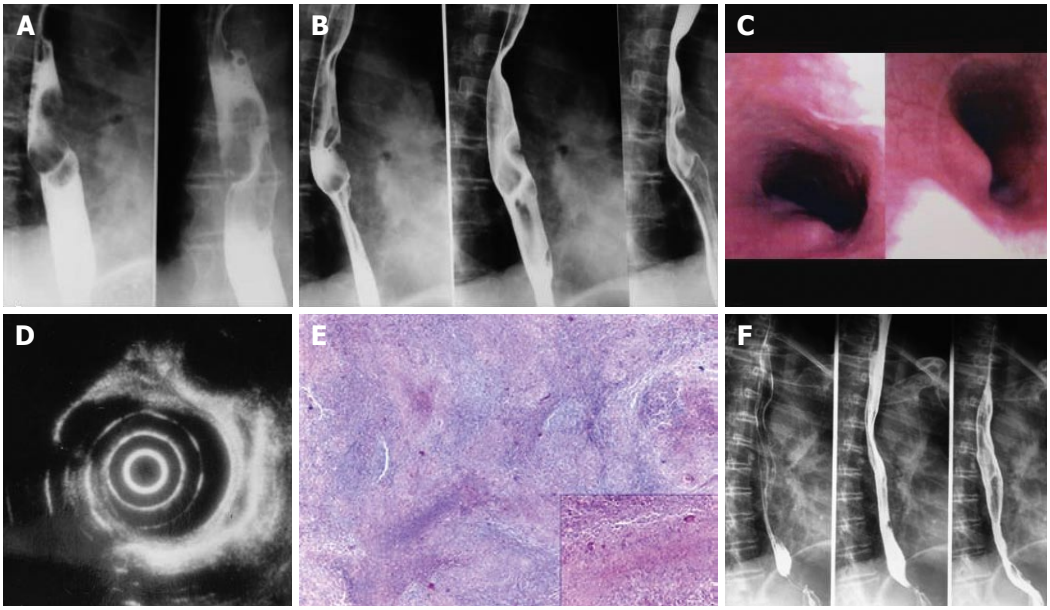

Figure 2 A barium swallow esophagogram disclosing a four-centimeter protruding smooth lesion with apparent integrity of mucosa on the mid-esophagus (**A**, **B**), esophagoscopy showing a two-centimeter deformity of the esophageal wall covered by normal mucosa and with firm consistency, twenty-two centimeters from dental arch (**C**), endoscopic ultrasonography revealing a two-centimeter well defined solid tumor in the fourth layer (**D**), lymph node parenchyma extensively occupied by necrotizing epithelioid granulomas (HE, × 40) and typical tuberculous granuloma with central caseous necrosis and multinucleated cells of Langhans type (HE, × 200) (**E**), and a two-month follow-up after surgery revealing no filling defect at the same level (**F**).

endoluminal/mucosal, intramural or extrinsic, in accordance with the location of the initial lesion in the esophageal wall. Malignant tumors of the esophagus are the most frequent cause, not only for primary dysphagia, but also for dysphagia caused by lesions originating from the esophageal mucosa[10].

Obstructive dysphagia caused by benign lesions is rare and usually results from intramural or extrinsic lesions. Even though rare, benign lesions leading to dysphagia are of great concern in the clinic. In fact, these lesions, if not treated, can lead to death due to progressive obstruction of the esophagus or pulmonary complications, but they almost always allow a curative surgical resection[10].

The endoscopic and radiological characteristics of a submucosa tumor are the endoluminal protusion of the digestive tube wall, usually covered by a normal looking mucosa. However, such features may also be caused by organ lesions or structures that extrinsically compress the digestive wall. EUS is described as the diagnostic method with a greater capacity of distinguishing between intraparietal and extrinsic lesions compressing the digestive wall, with a diagnostic accuracy superior to CT-scan and barium swallow[11,12]. Although EUS-guided fine needle biopsy is possible in these lesions, the material retrieved is sometimes insufficient for a diagnosis and, particularly for evaluation of the malignant potential of the lesions[12-15].

In our cases, even though the diagnosis of an extrinsic compression on the esophagus could not be excluded, barium swallow revealed an image compatible with a submucosa tumor protruding into the lumen. In fact, the angle between the esophageal wall and margin of the mass was almost perpendicular, which is in accordance with an intramural lesion, rather than an extrinsic compression on the esophagus[16]. Furthermore, all of the other diagnostic examinations pointed towards the diagnosis of a submucosal tumor-like intraluminal protruding mass. For these reasons, the patients were operated on with a probable diagnosis of a leiomyoma of the esophagus, suggesting that this is the most frequent intraparietal benign tumor of the esophagus.

One of our patients had a supraclavicular adenopathy and a persistently high erythrocyte sedimentation rate. The lymph node biopsy revealed necrotic material, but no Zielh-Neelson stained bacilli. Even though central necrosis of a lymph node is a frequent finding in tuberculous lymphadenitis[17], this fact does not give us a diagnosis of tuberculosis. In our cases, pulmonary Rx and CT scan of the thorax did not reveal lesions compatible with the diagnosis of pulmonary tuberculosis.

Mediastinal inflammatory lymph nodes, particularly tuberculous lymphadenitis[1,6-9] is a condition compressing the thoracic esophagus and causing dysphagia. Although mediastinal tuberculous lymphadenitis is rare, it is increasing in adults[18-20]. On the other hand, tuberculosis is an infectious disease with a rising incidence particularly in Asian and Eastern European countries and also a rising prevalence in association with HIV infection[21]. In 1993, tuberculosis was declared as a global emergency, by the World Health Organization[22].

Mediastinal tuberculous lymphadenitis should be included in the differential diagnosis of dysphagia, but it should be realized that it can present with various endoscopic and radiological findings. In fact, mediastinal tuberculous lymphadenitis can affect the esophagus by compressing the esophagus externally, causing rupture in the mediastinum and leading to an inflammatory process with secondary involvement of the esophagus, invading

the esophagus, ulcerating the mucosa and draining caseum into the esophageal lumen[9], as well as resulting in an esophageal fistula in some cases[23].

Dysphagia in mediastinal tuberculous lymphadenitis is due to the external compression on the esophagus, but the pain during swallowing - odynophagia occurring in one of our patients, suggested that the esophagus is directly involved in the inflammatory process. Interestingly, Ghmire and Walker[7] reported a case of mediastinal tuberculous lymphadenopathy who had painful dysphagia without significant involvement of the esophagus endoscopically and radiologically. They assumed that the contiguous inflammation of paraesophageal tissues resulted in disturbed esophageal motility of the patient.

In cases of tuberculous adenitis with esophageal mucosal lesion, confirmation of diagnosis should be done by histological or microbiological examination of the specimen obtained by endoscopic biopsy. In our cases without lesion of the mucosa, but with a strong suspicion that the cause for dysphagia is of tuberculous origin, EUS and endoscopic fine needle biopsy can play a role in diagnosing mediastinal masses that produce esophageal symptoms[5]. Other authors prefer biopsies guided by mediastinoscopy or thoracoscopy, when there is no mucosal lesion[24]. Nevertheless, when it is impossible to reach the affected lymph nodes through these approaches, surgery may be necessary in order to establish a diagnosis[1].

At present, minimally invasive surgery should be attempted to remove lesions diagnosed pre-operatively as probable leiomyomas. However, it was difficult, in the first case, to localize the lesion immediately below the aortic arch, on the left side of the esophagus associated with the surrounding fibrosis. In fact, it is hard to isolate the lesion in the presence of mediastinitis fibrosis resulting from rupture of an affected lymph node[25-27]. Medical treatment of most patients with tuberculosis consists of a short course in chemotherapy, using 3 or 4 essential anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, pyrazinamide and streptomycin). Anti TB regimen consists of an initial intensive phase and a continuation phase[22]. It was reported that most patients can be successfully treated with a three-drug anti-tuberculosis chemotherapy regimen[6,8,9,28]. If the diagnosis of mediastinal tuberculous lymphadenitis is made pre-operatively, such a treatment may lead to favourable outcomes[16,24]. Surgery should be reserved for patients with no pre-operative diagnosis, as in our cases, and those who develop complications, such as mediastinal abscess that is unresponsive to non operative management[1].

Since the diagnosis of a small esophageal submucosal lesion or an intraparietal lesion is usually difficult, surgery is necessary. Even though rare, the diagnosis of mediastinal tuberculous lymphadenitis should be highly considered in the presence of an uncertain esophageal lesion[29]. This is particularly important because a diagnostic non surgical approach is possible and an adequate treatment can completely cure mediastinal tuberculous lymphadenitis with or without tuberculous esophagitis[16].

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CASE REPORT

Malakoplakia of the colon associated with colonic adenocarcinoma diagnosed in colonic biopsies

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Abstract

Malakoplakia, typically involving the urinary tract, is an uncommon form of chronic inflammation caused by chronic infections and characterized by accumulation of macrophages. It has also been found in many other sites such as the gastrointestinal tract, pancreas, liver, lymph nodes, skin, respiratory tract, adrenal gland, vagina and brain. We present a case of a 64-year-old man referred to our hospital with cachexia and radiologic evidence of metastatic tumor of the liver. Colonoscopy revealed a large malignant - appearing polypoid mass of the ascending colon and multiple distinct polyps throughout the rest of the colon. Biopsies of the ascending colon mass confirmed the diagnosis of adenocarcinoma. Histological examination of two of the other polyps revealed malakoplakia which was characterized by aggregates of granular histiocytes with Michaelis - Gutmann bodies and histochemically confirmed with periodic acid-Schiff and von Kossa stains. This is a rare case diagnosed on endoscopic samples. The majority of reported cases were found in surgical specimens. In addition, the endoscopic appearance of multiple polyps is unusual in malakoplakia.

Key words: Malakoplakia; Gastrointestinal tract; Colon cancer

Karasavvidou F, Potamianos SP, Barbanis S, Stathakis E, Psychos A, Kapsoritakis AN, Koukoulis G. Malakoplakia of the colon associated with colonic adenocarcinoma diagnosed in colonic biopsies. *World J Gastroenterol* 2007; 13(45): 6109-6111

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INTRODUCTION

Malakoplakia, typically involving the urinary tract, is an uncommon form of chronic inflammation caused by chronic infections and characterized by accumulation of distinct macrophages[1,2]. It has also been found in various sites such as the gastrointestinal tract, pancreas, liver, lymph nodes, skin, respiratory tract, adrenal gland, vagina and brain[3]. We describe a case of a 64-year-old man with end-stage carcinomatosis which originated from the ascending colon and was associated with colonic malakoplakia. The endoscopic appearance of malakoplakia was very unusual, mimicking multiple polyps throughout the large bowel.

CASE REPORT

A 64-year-old man was admitted to our hospital with cachexia, ascites and multiple liver metastases, according to an abdominal CT scan. He reported a three-month history of fatigue, shortness of breath, altered bowel habits, weight loss and progressive abdominal distension. On examination he was pale and cachectic, and had a bulging abdomen with flank and shifting dullness. Blood pressure was 100/65 mmHg and pulse rate 94 beats/min. Hemoglobin was 8.1 g/dL and hematocrit 25%. Blood chemistry revealed cholestasis and 2.6 g/dL albumin and normal CEA levels. Abdominal CT scan revealed widespread hepatic metastases and a large quantity of ascetic fluid. Abdominal paracentesis and ascetic fluid cytology were compatible with peritoneal carcinomatosis. Colonoscopy revealed a large malignant polypoid lesion, almost obstructing the lumen of the ascending colon, as well as multiple (13) distinct polyps throughout the rest of the colon (5 rectal, 5 sigmoid, 3 transverse). The polyps were sessile, soft, ulcerated and hemorrhagic, measuring 5-15 mm in diameter (Figure 1). Two of them were removed in order to exclude a familial polyposis syndrome. The patient received only supportive treatment and subsequently died of liver failure-generalized carcinomatosis two weeks after hospitalization. Biopsies of the ascending colon mass confirmed the diagnosis of adenocarcinoma.

Histological examination of two of the other polyps revealed malakoplakia characterized by aggregates of granular histiocytes (Figure 2A). Several of these histiocytes contained intracytoplasmic Michaelis - Gutmann bodies (Figure 2B), which were confirmed

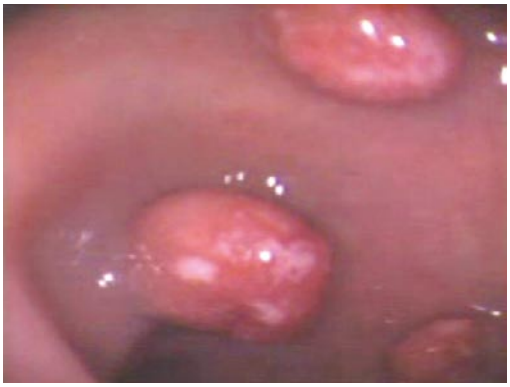

Figure 1 Endoscopic appearance of distinct polyps of the colon.

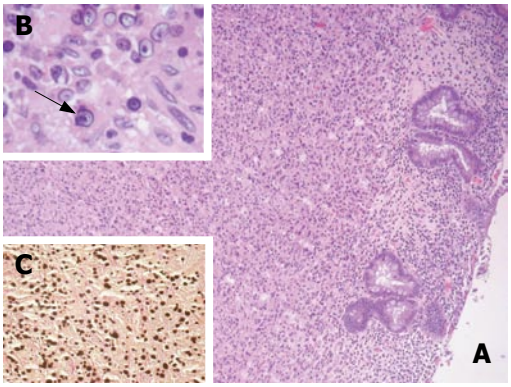

Figure 2 Aggregates of granular histiocytes in the lamina propria of large bowel mucosa (HE, × 200) (**A**), Michaelis - Gutmann bodies (HE, × 400) (**B**) and (Von Kossa, × 200) (**C**).

histochemically with periodic acid-Schiff and von Kossa stains (Figure 2C).

DISCUSSION

Malakoplakia, derived from the Greek adjective malakos (soft) and plaka (plaque), was first described in 1902 by Michaelis and Gutmann[4]. It occurs predominantly in the genitourinary tract (about 75% of the reported cases)[2]. The second most common site is the gastrointestinal tract (11% of the cases), and the majority of these cases involve the rectum and colon[5,6]. The remaining cases affect the brain, lungs, lymph nodes, adrenals, tonsils, conjunctiva, skin, bone, abdominal wall, liver, pancreas and retroperitoneum[3]. An increasing number of cases have been correlated with immunosuppression[7,8].

Definitive diagnosis of the lesion can be made by histopathologic examination. Malakoplakia is characterized by aggregates of histiocytes with abundant eosinophilic cytoplasm known as von Hansemann cells, intermingled with lymphocytes, plasma cells and neutrophiles. Finding the well-known Michaelis-Gutmann bodies is diagnostic for malakoplakia. These bodies are phagolysomes that have become encrusted with calcium and iron salts. They vary in size from 2 μm to 10 μm, have targetoid appearance due to concentric laminations and are stained with periodic acid-Schiff and von Kossa calcium stains. At the ultrastructural level, disintegrated bacteria have been occasionally observed in the Michaelis-Gutmann bodies. The origin of Michaelis - Gutmann bodies is most likely an abnormal response resulting in incompletely digested bacterial fragments and subsequent mineralization[1,9].

Indeed, malakoplakia is related to chronic bacterial infections, such as E*scherichia coli, Proteus mirabilis, Staphylococcus aureus, Mycobacterium Tuberculosis* and *Shigella boydii*. Fungi such as *Paracoccidiodes brasiliensis* and viruses have also been implicated[1,9,10]. In patients with AIDS, *Rhodococcus equi* has also been reported[11]. The pathogenesis of malakoplakia remains unknown. Three possible pathogenetic mechanisms have been suggested: an unusual causative organism, an abnormal or altered immune response and an abnormal macrophage response due to defective lysosomal function[12].

Colonic malakoplakia was first described by Terner and Lattes in 1965[13] and has been reported to occur in conjunction with tumors and non-tumoral conditions[14-16]. Since 1965 about 95 cases of colonic malakoplakia have been published. Notably, 24 of them had a coexistent colonic adenocarcinoma, similarly to our case[6]. All of the reported cases were found in surgical specimens, most of them in conjunction with the tumor. Half of the cases occured as a pericolic mass and only 3 cases as a single nodule or a microscopic focus[15].

However, our patient is a rare case in which the diagnosis of malakoplakia was made preoperatively on biopsy samples as previously described[17,18]. The additional biopsies were prompted by the presence of multiple small polyps in addition to the main cancerous one. The coexistence of multiple colonic polyps related to malakoplakia has not been described previously, the previously reported endoscopic appearances have been described as unifocal or nodular lesions and large masses, and the presence of a pericolonic mass associated with a fistula has been noted[6].

Notably, von Hasselman histiocytes can mimic adenocarcinoma cells in frozen sections. A helpful clue for the correct diagnosis is the presence of Michaelis-Gutmann bodies which are not seen in mucin vacuoles.

Our case may serve as a reminder of the clinical significance of malakoplakia coexisting with colonic adenocarcinoma, which increases the risk of over-staging the tumor and over-treating the patient.

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CASE REPORT

Iatrogenic colorectal perforation induced by anorectal manometry: Report of two cases after restorative proctectomy for distal rectal cancer

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Abstract

There are no reports regarding perforation of the colorectum induced by anorectal manometry. We report two cases of colorectal perforation that occurred during manometry in the patients undergoing restorative proctectomy for distal rectal cancer. In the first patient, computed tomography showed an extraperitoneal perforation in the pelvic cavity and a rupture of the rectal wall. A localized perforation into the retroperitoneum was managed conservatively. In the second patient, a 3 cm linear colon rupture was detected above the anastomotic site. A primary closure of the perforated colon and proximal ileostomy were conducted, but the patient died 2 wk later. We hypothesize that the perforation induced by anorectal manometry may be associated with the relative weakening of the proximal bowel wall due to anastomosis, decreased compliance, and abnormal rectal sensation. We suggest that measurement of the maximum tolerable volume should not be routinely performed after restorative proctectomy for distal rectal cancer.

Key words: Iatrogenic perforation; Anorectal manometry; Rectal cancer; Low anterior resection

Park JS, Kang SB, Kim DW, Kim NY, Lee KH, Kim YH. Iatrogenic colorectal perforation induced by anorectal manometry: Report of two cases after restorative proctectomy for distal rectal cancer. *World J Gastroenterol* 2007; 13(45): 6112-6114

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INTRODUCTION

Anorectal manometry is an objective test for evaluating a patient's resistance to spontaneous defecation provided by the sphincter mechanism, as well as the sensory capabilities of the rectum in terms of the sensation of imminent defecation[1]. Currently, anorectal manometry is widely performed for the tracking of anorectal physiological changes occurring after low anterior resection for rectal cancer, as the test allows for a numerical value evaluation of pre- and post-operative anorectal function, including rectoanal inhibitory reflex, rectal compliance, and anal resting pressure[2-4]. This procedure is generally thought to be safe, and the incidence of critical complications associated with anorectal manometry has not been reported. We recently encountered two unusual cases of iatrogenic perforation occurring following anorectal manometry in rectal cancer resection patients.

CASE REPORT

Case 1

A 72-year-old male patient received ultra-low anterior resection with coloanal anastomosis for the treatment of rectal cancer 22 mo ago. The patient's primary tumor was located 4 cm from the anal verge. He complained of frequent defecation in excess of 10 bowel movements a day, as well as urgency and tenesmus. We performed anorectal manometry in order to measure changes in the patient's anorectal function. Anorectal manometry (Model UPS-2020 Stationary GI Motility System, MMS, Netherlands) was conducted using the water-perfusion technique, with an 8-channel micro tip catheter connected to a perfusion pump. We evaluated the rectal sensation *via* inflation of a latex balloon with an air flow of 1 mL per second. The threshold volumes for the first minimum sensation, defecatory desire, urge, and maximum tolerance were determined. In this study, the maximum resting pressure (48.25 mmHg) determined was significantly lower than that observed in the normal controls (normal value:

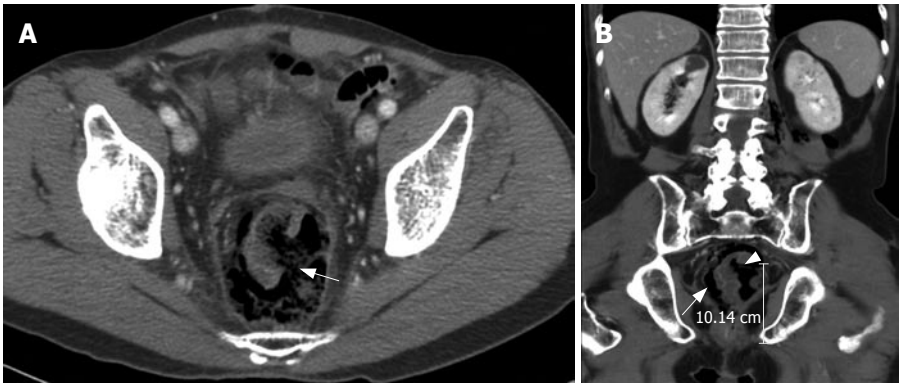

Figure 1 Abdomino-pelvic CT at the level of acetabula showing air bubbles mixed with small solid particles surrounding both lateral aspects of the rectum (arrow) (**A**) and coronal view of extraluminal air in the pelvic cavity (arrow) and rupture of the left lateral wall of the rectum (arrow head) (**B**).

53-90 mmHg), and the maximum squeezing pressure (117 mmHg) was not reduced in comparison with the normal controls (normal value: 100-200 mmHg). During the test, the patient complained of slight discomfort in the lower abdomen during measurement of the maximum tolerable volume. When the balloon catheter was removed, however, the surface of the balloon observed was slightly blood stained. As the patient had normal vital signs and appeared to be relatively healthy, he was discharged after examination. Seven hours later, the patient revisited the emergency room because of persistent lower abdominal pain, anal pain, and a sensation of "chilling". Upon physical examination, the patient experienced mild lower abdominal tenderness with palpation but no symptoms of generalized peritonitis. His temperature was 39.2℃ initially, and decreased within three hours to 38.5℃. His heart rate was 110 per minute and no hypotension was found. The most noteworthy feature of his laboratory studies was an elevated white blood cell count of 17 000/mm^3. Upright chest and abdomen films were normal. However, abdominal CT showed a moderate amount of extraperitoneal air in the pelvic cavity and a rupture of the rectal wall (Figure 1A and B). Perforation into the retroperitoneum was localized, and no signs of intraperitoneal perforation were observed. The patient was hospitalized and received no treatment by mouth, total parenteral nutrition, and intravenous broad-spectrum antibiotics. Daily physical examinations were conducted. We verified improvement in radiologic signs on a CT examination conducted seven days later. The patient was discharged on the 14th d of hospitalization.

Case 2

A 78-year-old female patient underwent an ultra-low anterior resection and coloanal anastomosis following preoperative radiotherapy (50.4 Gy during 5 wk) coupled with infusion of 5-FU for low rectal cancer 23 mo ago. The patient had a history of angioplasty due to unstable angina 4 years ago. She presented at the hospital for frequent defecation and urgency to defecate, which persisted after surgery. We performed anorectal manometry to measure the function of her anorectum in the same manner as in Case 1. No abnormalities were detected with the exception of loss of rectoanal inhibitory reflex and a reduction in resting pressure. However, when the rectal balloon was gradually inflated with 130 mL air for measurement of the maximum tolerable volume, a steep fall in intra-balloon pressure (from 130 mmHg to 65 mmHg) was detected, and the examiner could actually feel her resistance against the decrease in air injection. During the test, she complained of an abrupt discomfort in the abdomen and abdominal distension. An urgent CT scan of the abdomen and pelvis was conducted, which evidenced a large quantity of free intraperitoneal gas and fluid within the abdomen consistent with the perforation of a gas-containing viscous body (Figure 2). Emergency laparotomy was immediately conducted, and a 3 cm linear colon rupture was detected above the coloanal anastomosis suture area. Accordingly, primary closure of the perforation site and a diverting ileostomy were performed. The patient's underlying heart condition deteriorated rapidly after surgery, and she died two weeks later, despite aggressive resuscitation.

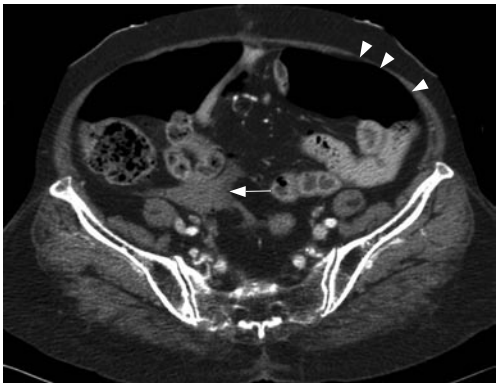

Figure 2 Abdomino-pelvic CT at pelvis level showing a large amount of extraluminal air (arrow heads) mixed with a complicated collection of fluids, probably primary extraluminal feces (arrow).

DISCUSSION

We experienced two iatrogenic colorectal perforations (0.13%) in 1501 anorectal manometry tests in the past three years. Both patients had a history of rectal cancer resection. Anorectal manometry has been widely adopted as a means for evaluating physiological changes in the anus and rectum of patients undergoing low anterior resection. To our knowledge, no iatrogenic perforation has been reported as a complication arising from anorectal manometry conducted following low anterior resection[3,5-7].

We consider that this colorectal perforation is

associated with certain characteristics of the neorectum following low anterior resection and anastomosis, including relative weakening of the proximal bowel wall due to anastomosis, decreased compliance, and abnormal rectal sensation. The pressure of balloon inflation can exert undue stress on the weakened proximal bowel wall to fibrotic anastomosis, causing rupture on the neorectum. The vulnerable part, which evidences low compliance, can be readily ruptured by the application of physical force via artificial balloon inflation. As the rectal balloon is inflated, patients are instructed to inform the examiner of the rectal sensation according to changes in the air injection level. However, patients with dull rectal sensation are not able to appropriately express it. In the case of the aged, who undergo rectal surgery or to whom radiotherapy is administered, there is some risk that the balloon may be inflated over the actual maximum threshold volume.

In treatment of iatrogenic colonic perforation, nonoperative management of colonic perforation is advocated for patients who are clinically stable with no evidence of peritonitis[8-10]. For selected patients with incidental intramural or small retroperitoneal perforations but no evidence of barium spillage, favorable results have also been reported as the result of conservative treatment consisting of bowel rest combined with total parenteral nutrition, intravenous fluid treatment, and broad-spectrum antibiotics[11,12]. On the basis of our experience with the two cases, this indication for conservative management after iatrogenic perforation may also be applied to perforation occurring during anorectal manometry. However, we believe that there may be a higher risk for perforation during anorectal manometry than for other types of perforation because (1) anorectal manometry is conducted without reasonable bowel preparation and (2) diagnostic delays are likely to occur as physicians tend not to recognize the possibility of perforation. Therefore, a more cautious approach should be taken when selecting patients who can receive conservative treatment for perforation occurring during anorectal manometry.

In order to avoid iatrogenic perforations during anorectal manometry, it is important to assess the high risk factors associated with perforation prior to anorectal manometry. History taking should focus on age, previous rectal surgery, bowel inflammation, and bowel obstruction. Meticulous digital rectal examination preceding anorectal manometry, for the detection of unsuspected anorectal abnormal lesions, is necessary for patients with a history of rectal surgery. This facilitates catheter insertion and provides information on anorectal conditions. We believe that the process of measuring the maximum tolerable volume may be omitted in patients following low anterior resection and anastomosis for distal rectal cancer. The maximum tolerable volume may be highly distorted in patients undergoing rectal resection in comparison with patients with normal rectum, as sensations of rectal distension differ in accordance with the patterns and rates of balloon inflation, which are dependent on examiners and laboratories[13]. We suggest that measurement of the maximum tolerable volume should not be routinely performed in patients undergoing restorative proctectomy for distal rectal cancer.

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CASE REPORT

Epidermal growth factor receptor antibody plus recombinant human endostatin in treatment of hepatic metastases after remnant gastric cancer resection

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Abstract

We report a 55-year-old male who developed advanced hepatic metastasis and peritoneal carcinomatosis after resection of remnant gastric cancer resection 3 mo ago. The patient only received epidermal growth factor (EGF) receptor antibody (Cetuximab) plus recombinant human endostatin (Endostar). Anti-tumor activity was assessed by ^{18}F-fluorodeoxyglucose (^{18}F-FDG) positron emission tomography/computer tomography (PET/CT) at baseline and then every 4 wk. The case illustrates that ^{18}FDG-PET/CT could make an early prediction of the response to Cetuximab plus Endostar in such clinical situations. ^{18}FDG-PET/CT is a useful molecular imaging modality to evaluate the biological response advanced hepatic metastasis and peritoneal carcinomatosis to Cetuximab plus Endostar in patients after remnant gastric cancer resection.

Key words: Hepatic metastasis; Remnant gastric cancer; Cetuximab; Recombinant human endostatin; ^{18}F-fluorodeoxyglucose; Positron emission tomography/computer tomography

Sun L, Ye HY, Zhang YH, Guan YS, Wu H. Epidermal growth factor receptor antibody plus recombinant human endostatin in treatment of hepatic metastases after remnant gastric cancer resection. *World J Gastroenterol* 2007; 13(45): 6115-6118

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INTRODUCTION

The activity of epidermal growth factor (EGF) and its receptor (EGFR) has been identified as the key driver in the process of cell growth and replication. There is now a body of evidence that EGFR-mediated drive is increased in a wide variety of solid tumors, including non-small cell lung cancer, prostate cancer, breast cancer, gastric cancer, colon cancer, ovarian cancer and tumors of the head and neck[1]. EGFR antibody (Cetuximab, Erbitux, Merck and Imclone Systems) has been approved by the Food and Drug Administration (FDA)[2] for use in treatment of colorectal cancer. Angiogenesis is the formation of new capillaries from existing blood vessels. The recognition of tumor angiogenesis as a therapeutically useful target is based on experimental evidence that tumor growth and progression are dependent on new blood vessel formation. Endostatin has been shown to inhibit potently angiogenesis *in vitro* and *in vivo* and Endostar (Medgenn Bioengineering Co. Ltd. Yantai, Shandong, P. R.China.) has been approved by the State Food and Drug Administration (SFDA)[3]. In this case, we used ^{18}FDG-PET/CT to monitor the early response of advanced hepatic metastasis and peritoneal carcinomatosis to Cetuximab plus Endostar in patients after remnant gastric cancer resection.

CASE REPORT

A 55-year-old male, with a history of subtotal gastrectomy for gastric ulcer 20 years ago, received remnant gastric cancer resection 3 mo ago. Immunohistochemistry revealed adenocarcinoma of the stomach with expression of EGFR. Episodes of fever above 40℃ appeared without evidence of infection and mild jaundice developed with weight loss of 10 kg within 1 mo. A palpable liver and tenderness of the left upper abdominal quadrant were found during physical examination. Laboratory findings during the first admission showed 55 g/L HGB, 41.4 μmol/L TBIL, 12.9 μmol/L DBIL, and 28.5 μmol/L IBIL. Advanced hepatic metastasis and peritoneal carcinomatosis were confirmed by ^{18}F-fluorodeoxyglucose positron emission tomography/computer tomography (^{18}F-FDG PET/CT) and biopsy guided by ^{18}F-FDG PET/CT. These findings were supportive for tumor-associated cachexia because his overall performance status was significantly reduced (Karnofsky index: 40%). Conventional chemotherapy was not considered the first choice of

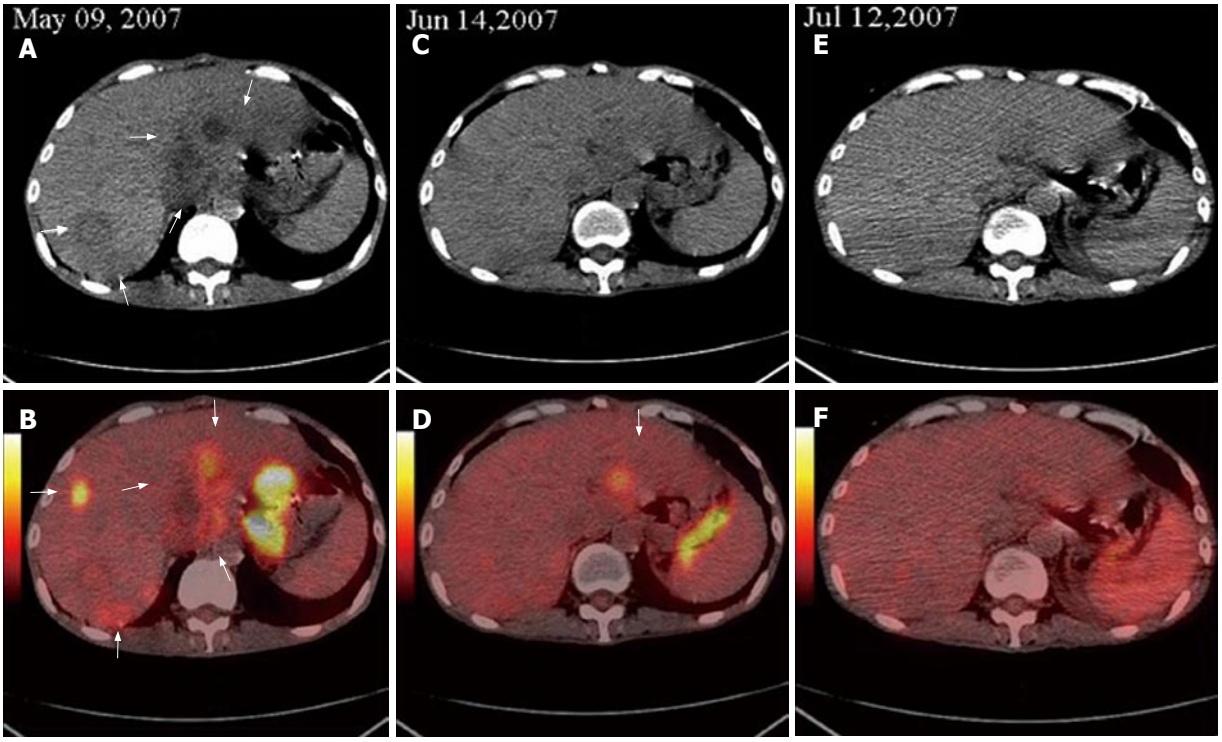

Figure 1 Base line FDG PET/CT detecting a highly metabolic metastasis in liver (white arrows, **A** and **B**), second FDG PET/CT demonstrating the decreased number and metabolism level of liver metastases and one highly metabolic metastasis in the left lobe of liver (white arrows, **C** and **D**), third FDG PET/CT displaying no lesion at the same position (**E** and **F**).

treatment for the patient. After giving informed consent, the patient received Cetuximab weekly at 400 mg/m^2 iv loading dose, followed by at 250 mg/m^2 iv maintenance dose for 8 wk, together with but separately used Endostar daily at 15 mg iv loading dose and maintenance dose for over 8 wk. Anti-tumor activity was assessed by ^{18}F-FDG PET/CT at baseline and then every 4 wk. During the 8-wk follow-up period of time, ^{18}F-FDG PET/CT was performed 3 times to monitor the treatment response. His temperature returned to normal slowly after 4 wk of treatment. Cetuximab exhibited only mild skin toxicity, and hepatic metastasis and peritoneal carcinomatosis lesions had a partial response to Cetuximab treatment (> 30% reduction, according to RECIST) after 4 wk (Figure 1 and Figure 2). Main laboratory tests showed 70 g/L HGB, 21.4 μmol/L TBIL, 13.9 μmol/L DBIL, 16.5 μmol/L IBIL. Karnofsky index was 70%. Due to responses to the treatment without serious complications, after the second treatment cycle, hepatic metastases were almost completely gone on the third ^{18}F-FDG PET/CT (Figures 1 and 2). However, a few metastatic lymphoid nodes in the abdomen could be detected (Figure 3). In parallel, main laboratory tests showed 90 g/L HGB, 20.4 μmol/L TBIL, 12.9 μmol/L DBIL, 15.5 μmol/L IBIL. Karnofsky index was 80%. Due to the confirmed responses to the treatment, the third cycle of treatment was going on when this paper was completed.

DISCUSSION

Multiple cellular pathways involved in the growth and metastatic potential of tumors may create heterogeneity, redundancy, and the potential for tumors to bypass signaling pathway blockade, resulting in primary or acquired resistance. Combined therapies are more effective in inhibiting different signaling pathways and can overcome tumor resistance[4]. Vascular endothelial growth factor (VEGF) and EGFR inhibitors have become the key components of therapies for several tumor types. There is a close relationship between these two factors. VEGF signaling is up-regulated by EGFR expression while VEGF up-regulation independent of EGFR signaling seems to contribute to resistance to EGFR inhibition. Therefore, inhibition of both pathways improves anti-tumor efficacy and overcomes resistance to EGFR inhibition[5].

EGFR belongs to a family of receptors known as the ErbB family (ErbB tyrosine kinase receptors), which comprises four proteins encoded by the c-erbB proto-oncogene. EGFR can activate a cascade of multiple signaling pathways that facilitate tumor growth process[6]. The EGFR signaling pathway regulates cell differentiation, proliferation, migration, angiogenesis, and apoptosis, all of which are down regulated in cancer cells. In a study, EGFR immunoreactivity was detected in one (3.8%) of the 26 early gastric carcinomas and in 33 (34.4%) of the 96 advanced gastric carcinomas, respectively, the incidence of expression between the two groups was significantly different[7]. In gastric cancer, the management of peritoneal dissemination in the peritoneal cavity is extremely important. However, peritoneal dissemination in the final stage of gastric cancer remains untreatable. VEGF is correlated with peritoneal metastasis from gastric cancer, and has been reported as a useful indicator of peritoneal recurrence[8].

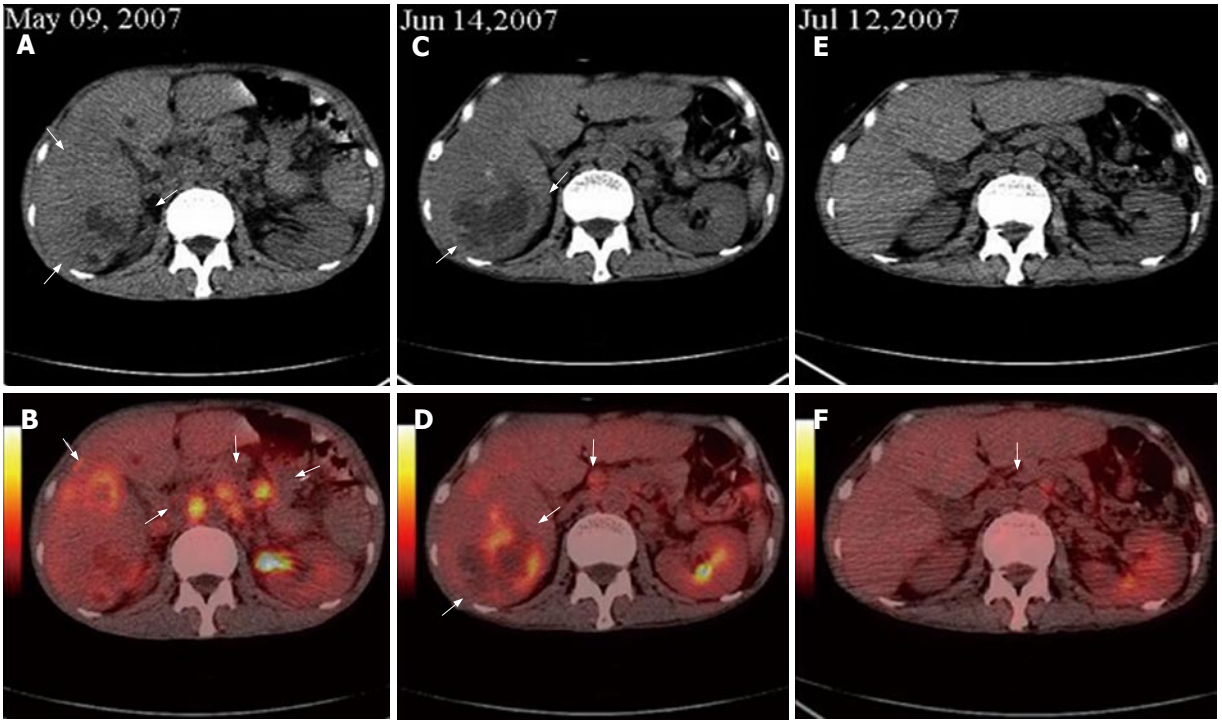

Figure 2 Base line FDG PET/CT detecting huge metastases in the right lobe of liver and highly metastatic lymphoid nodes in abdomen (white arrows, **A** and **B**), second FDG PET/CT demonstrating huge metastases in the right lobe of liver and the decreased number, size and metabolism level of metastatic lymphoid nodes (white arrows, **C** and **D**), third FDG PET/CT displaying the disappeared huge metastases in the right lobe of liver and the normal size of metastatic lymphoid nodes (**E** and **F**).

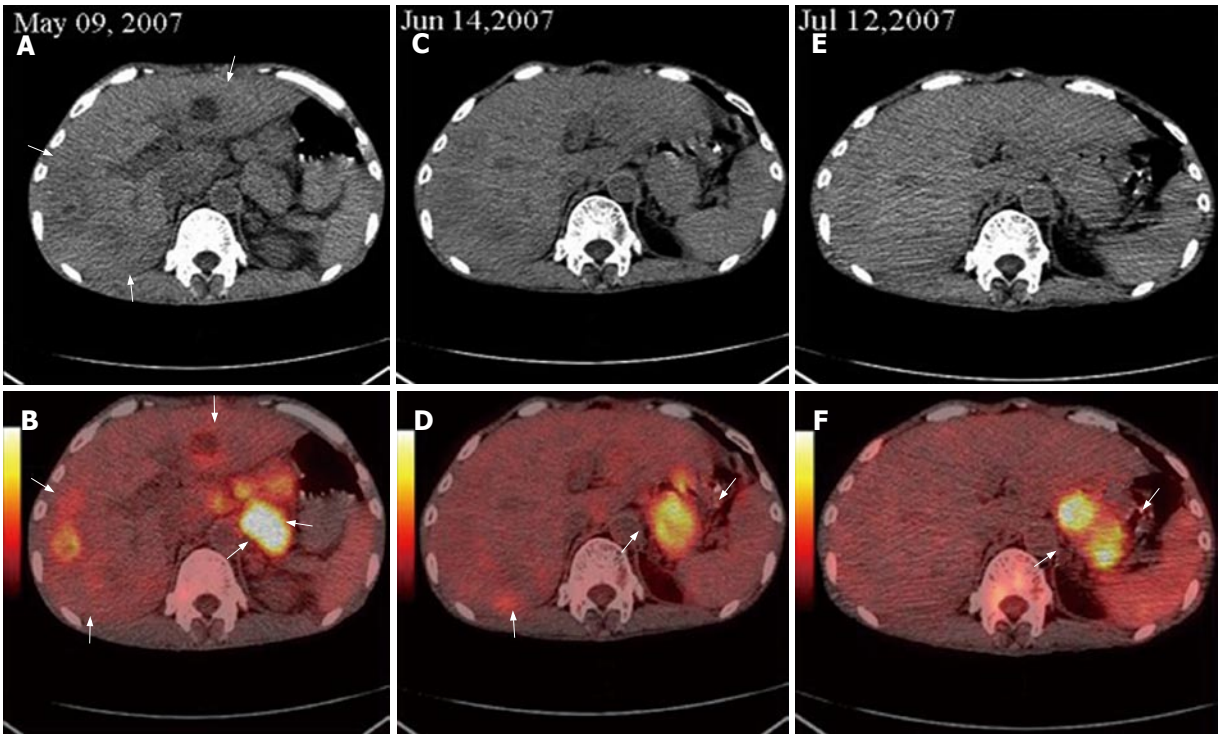

Figure 3 Base line FDG PET/CT detecting multi high metastases in both lobes of liver and highly metastatic lymphoid nodes in abdomen (white arrows, **A** and **B**), second FDG PET/CT demonstrating multi high metastases in the liver and the decreased number, size and metabolism level of metastatic lymphoid nodes (white arrows, **C** and **D**), third FDG PET/CT displaying disappeared liver metastasis and metastatic lymphoid nodes (**E** and **F**).

Cetuximab is a chimeric IgG1 monoclonal antibody and binds to EGFR with a high specificity and a higher affinity than either EGF or TGF-α, thus blocking ligand-induced phosphorylation of EGFR. Second line therapies with Cetuximab for colorectal cancer after failure of first-line regimes have shown a response rate of 17%-23% and an overall survival (median) of 8.6 mo[9]. Endostar is a new recombinant human endostatin developed by Medgenn

Bioengineering Co. Ltd (Yantai, Shandong, China) in 2005. A preclinical study indicated that endostar could inhibit tumor endothelial cell proliferation, angiogenesis and tumor growth, and results from prospective trials addressing the response of colorectal cancer to Cetuximab containing regimes have confirmed its efficacy in clinical practice[10].

Modern cancer care is critically dependent on imaging technologies, which are used to detect early tumors and guide their therapy or surgery[11]. Molecular imaging technologies provide information about the functional or metabolic characteristics of malignancies, tumor stage and therapeutical response, and tumor recurrence, whereas conventional imaging technologies predominantly assess the tumor's anatomical or morphologic features including its size, density, and shape, *etc*. Since conventional imaging technologies reveal morphology of lesions with nonspecific features, differentiation between malignant and benign lesions could be improved by molecular imaging. As our knowledge about the molecular basis of cancer increases, imaging methods that provide clinicians with telling details about the molecular environments of patients' tissues are needed. By using standard anatomic imaging technologies combined with molecular imaging technologies such as PET/CT, we can detect disease processes at the anatomic, physiologic, metabolic, and molecular levels, thereby, allowing earlier detection of diseases, monitoring of therapies, and better prognostication of disease progression[12].

The precise tailoring of treatment for patients with cancer is a challenge. PET/CT and SPECT/CT help achieve better results[13]. PET with the glucose analog ^{18}F-FDG is increasingly used to monitor the effectiveness of therapy for patients with malignant lymphomas and solid tumors. Quantitative assessment of therapy-induced changes in tumor ^{18}F-FDG uptake can predict tumor response and patient outcome early in the course of therapy. Treatment may be adjusted according to the chemosensitivity and radiosensitivity of tumor tissue in an individual patient. Thus, ^{18}F-FDG PET has a potential to reduce the side effects and costs of ineffective therapy[14]. The use of integrated PET/CT instead of PET in treatment monitoring poses some methodologic challenges against the quantitative analysis of PET scans. However, it may provide the opportunity to integrate morphologic and functional information. This integration may define new parameters for assessment of tumor response and facilitate the use of PET in studies as well as in clinical practice[14]. In our case, a partial response (> 30% reduction, according to RECIST) of hepatic metastasis and peritoneal carcinomatosis lesions was achieved after 4 wk of treatment with Cetuximab plus Endostar.

In conclusion, our case demonstrates that molecular imaging could monitor molecular treatment and combination of EGFR-specific antibodies with VEGF-specific antibodies may be a promising treatment modality. Further prospective trials are mandatory to confirm its effects and discriminate Cetuximab-induced response from Endostar-associated response. The potential of this novel approach to anticancer therapy should be elucidated in large clinical trials.

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Eosinophilic cholecystitis as a rare manifestation of visceral larva migrans

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Abstract

Eosinophilic cholecystitis is an infrequent form of cholecystitis. The etiology of eosinophilic cholecystitis is still obscure, and it is sometimes accompanied with several complications, but a simultaneous onset with pericarditis is very rare. We would like to make an alternative interpretation of our recent report "Kaji K, Yoshiji H, Yoshikawa M, Yamazaki M, Ikenaka Y, Noguchi R, Sawai M, Ishikawa M, Mashitani T, Kitade M, Kawaratani H, Uemura M, Yamao J, Fujimoto M, Mitoro A, Toyohara M, Yoshida M, Fukui H. Eosinophilic cholecystitis along with pericarditis caused by Ascaris lumbricoides: A case report. World J Gastroenterol 2007; 13: 3760-3762."

Key words: Cholecystitis; Visceral larva migrans; Parasite; *Ascaris suum; Toxocara canis*

Yoshiji H, Yoshikawa M, Kaji K, Fukui H. Eosinophilic cholecystitis as a rare manifestation of visceral larva migrans. *World J Gastroenterol* 2007; 13(45): 6119

http://www.wjgnet.com/1007-9327/13/6119.asp

TO THE EDITOR

Eosinophilic cholecystitis is an infrequent form of cholecystitis. The etiology of eosinophilic cholecystitis is still obscure, and it is sometimes accompanied by several complications, but a simultaneous onset with pericarditis is very rare[1-3].

We would like to make an alternative interpretation of our recent report "Kaji K, Yoshiji H, Yoshikawa M, Yamazaki M, Ikenaka Y, Noguchi R, Sawai M, Ishikawa M, Mashitani T, Kitade M, Kawaratani H, Uemura M, Yamao J, Fujimoto M, Mitoro A, Toyohara M, Yoshida M, Fukui H. Eosinophilic cholecystitis along with pericarditis caused by Ascaris lumbricoides: A case report. *World J Gastroenterol* 2007; 13: 3760-3762." We reported that this rare clinical manifestation was caused by *Ascaris lumbricoides*. However, from the serological diagnosis, it was likely that these clinical symptoms were visceral larva migrans (VLM) caused by *Ascaris suum* or *Toxocara canis* rather than by *Ascaris lumbricoides*. While a definitive diagnosis of parasitic diseases is established after the detection of worms or eggs from the patient, the direct detection of the worm is quite rare. As we described in the report, we could not make a direct detection of the worm either. Generally, food-borne parasitic infection can be diagnosed based on the raw food intake history, laboratory data such as hypereosinophila, and the presence of antibody against the parasite in the serum. Our patient revealed a higher titer of antibody against *Ascaris suum* and *Toxocara canis* by enzyme-linked immunosorbant assay by 150 and 100 fold as compared with the control serum. Nevertheless, we can not rule out the possibility that it is caused by *Ascaris lumbricoides*. *Ascaris lumbricoides* also could penetrate the gastrointestinal tract.

In conclusion, since the title was too high in our study, we had to consider other alternative possibilities of VLM caused by *Ascaris suum* or *Toxocara canis* as described above, and we believe that this letter would help make our previous report more easily understood and legible for the readers.

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NEWS

Science news release and its benefits to your research

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Abstract

News release to the latest science findings is beneficial to both researchers and their served institutions as well as the public. It will help to set a bridge of communication between researchers, the public and media, and publishers, making the latest research findings well known to the public. *World Journal of Gastroenterology* has currently freely opened the News Release Service System (*WJG*-NRSS) for original articles with potential significance and novelty for news release to mass media to broaden the findings to the public.

Chang YD. Science news release and its benefits to your research. *World J Gastroenterol* 2007; 13(45): 6120-6121

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News release to your research findings before publication in mass media is beneficial to both researchers and their served institutions. It will help to bridge the communication among researchers, the public and media, and publishers, particularly making the latest findings of the researchers well known to the public. An investigation from The New England Journal of Medicine shows that the citation rate of an article increase seven times when the findings of an article have been reported by New York Times.

World Journal of Gastroenterology (*WJG*) has currently freely opened the News Release Service System (*WJG*-NRSS) for the authorized reporters for news release in mass media such as EurekAlert!. As one of the registered members with identity of an international peer reviewed journal, *WJG* has been authorized to release science news on EurekAlert! (Figure 1).

As an online, global news service operated by AAAS, the science society, EurekAlert! is one of the central places through which *WJG* can bring the authors' latest findings to the media and public. *WJG* science news features the resources focused on all original articles with both significance and novelty. EurekAlert! has 923 registered entities including *WJG*, and has timely delivery of the released news to the public and its 5400 journalists worldwide.

Figure 1 *WJG* as a registered member to EurekAlert! with an identity of an international peer reviewed journal.

WJG has encouraged those articles with both significance and novelty to release news before its publication on EurekAlert! so that more readers could share the researcher's latest findings and ongoing research. The sample science news released by *WJG* on EurekAlert! are shown in Table 1. The number of hits shows that the four science news with amusive titles have been very attractive to readers.

An excellent news is very essential to achieve the expectations. In addition to basic guidelines for general news writings, we recommend some specific guidelines for our reporters in *WJG* science news writings. News titles may be different from the original article but should be attractive and informative. The main body of news is less than 1000 words with a summary of both less than 75 words. Interesting pictures are also strongly recommended. The deadline for news submission is about one week before publication. Since most readers to science news are not experts, journalists should avoid scientific jargon and use everyday language in news writings regardless of the topic or content. Finally but importantly, be sure the science news is lawful and ethical.

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Table 1 Science news from *World Journal of Gastroenterology* released to EurekAlert!

Science news	Released media	Date posted	Hits since posted
Targeting nerve growth factor may cure liver cancer[1]	http://www.eurekalert.org/pub_releases/2007-09/wjog-tng090507.php	September 7, 2007	713
Researchers discover correlation between GERD and obesity in females[2]	http://www.eurekalert.org/pub_releases/2007-09/wjog-agr090407.php	September 5, 2007	756
Who will recover spontaneously from hepatitis C virus infection[3]	http://www.eurekalert.org/pub_releases/2007-08/wjog-wwr082807.php	August 29, 2007	504
Clearance of hepatitis C viral infection[4]	http://www.eurekalert.org/pub_releases/2007-08/wjog-coh082807.php	August 29, 2007	355

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cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

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1-3 March 2007
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www.come-innsbruck.at/events/ibd2007/default.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
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easl2007@easl.ch
www.easl.ch/liver-meeting

Meeting SAGES 2007 Annual Meeting -part of Surgical Spring Week
18-22 April 2007
Paris Hotel and Casino, Las Vegas, Nevada
www.sages.org/07program/index.php

Meeting Falk Symposium 159: IBD 2007-Achievements in Research and Clinical Practice
4-5 May 2007
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Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
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espghan2007@colloquium.fr

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
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Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
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fca@netvisao.pt

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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Author(s) and editor(s)

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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/eid.htm

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