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EDITORIAL

Acute mesenteric ischemia after cardio-pulmonary bypass surgery

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Abstract

Acute mesenteric ischemia (AMI) is a highly-lethal surgical emergency. Several pathophysiologic events (arterial obstruction, venous thrombosis and diffuse vasospasm) lead to a sudden decrease in mesenteric blood flow. Ischemia/reperfusion syndrome of the intestine is responsible for systemic abnormalities, leading to multi-organ failure and death. Early diagnosis is difficult because the clinical presentation is subtle, and the biological and radiological diagnostic tools lack sensitivity and specificity. Therapeutic options vary from conservative resuscitation, medical treatment, endovascular techniques and surgical resection and revascularization. A high index of suspicion is required for diagnosis, and prompt treatment is the only hope of reducing the mortality rate. Studies are in progress to provide more accurate diagnostic tools for early diagnosis. AMI can complicate the post-operative course of patients following cardio-pulmonary bypass (CPB). Several factors contribute to the systemic hypo-perfusion state, which is the most frequent pathophysiologic event. In this particular setting, the clinical presentation of AMI can be misleading, while the laboratory and radiological diagnostic tests often produce inconclusive results. The management strategies are controversial, but early treatment is critical for saving lives. Based on the experience of our team, we consider prompt exploratory laparotomy, irrespective of the results of the diagnostic tests, is the only way to provide objective assessment and adequate treatment, leading to dramatic reduction in the mortality rate.

Key words: Acute mesenteric ischemia; Non-occlusive; Cardio-pulmonary bypass; Laparotomy; Prognosis; Mortality

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INTRODUCTION

Acute mesenteric ischemia (AMI) is a life-threatening surgical emergency in which the outcome is closely dependent on the elapsed time to diagnosis and treatment. The diagnosis is typically difficult and delayed due to non-specific results of biological and radiological tests. Since prompt treatment is the key to a better outcome, AMI remains a challenging condition because of controversial algorithms and numerous therapeutic options.

When AMI occurs after a cardio-pulmonary bypass (CPB) procedure, the condition has a more subtle clinical presentation, is more difficult to diagnose and treat, leading to a higher mortality rate.

This report is an updated review of AMI with respect to the pathophysiologic events, diagnostic tests, therapeutic options, mortality rate and promising new areas of research. A separate section is dedicated to AMI in CPB patients and focuses on the main differences in the diagnosis, management and outcome.

AMI IN STANDARD CONTEXT

Definitions

AMI is caused by a sudden decrease in the blood flow to the bowel and abdominal viscera. Important features of AMI include: bacterial translocation, systemic

inflammatory response syndrome and reperfusion injury, which exacerbate the ischemic damage of the intestinal microcirculation and negatively impact the outcome. Although rare, the incidence of AMI is increasing, in parallel with the aging population[1].

Pathophysiology

AMI is the result of four distinct pathophysiologic mechanisms: arterial embolus, arterial thrombosis, splanchnic vasoconstriction, known as non-occlusive mesenteric ischemia (NOMI) and venous thrombosis.

Arterial embolus is the most common cause, responsible for almost half of all cases[2,3]. The source of the embolus is usually the heart, and the affected vessel is the superior mesenteric artery in 50% of cases. In general, the obstruction occurs at the mid to distal bifurcation points of the blood vessel[1,4].

Arterial thrombus is the underlying cause in approximately 30% of patients[2,3], with rupture of an atherosclerotic plaque in the mesenteric arteries. The site of the occlusion tends to occur at the origin of the blood vessels. The patients can tolerate major visceral artery obstruction because of the slow progressive nature of atherosclerosis, with the development of collaterals. Nearly 75% of patients have pre-existing chronic mesenteric ischemia[5], and acute bowel ischemia or infarction only ensues if the last remaining visceral artery or an important collateral artery occludes. The extent of bowel ischemia or infarction is typically greater than that seen with embolism.

In NOMI, diffuse vasospasm of the mesenteric and other visceral arteries occurs as a result of a sustained hypoperfusion state[6,7]. No vascular occlusion is usually demonstrated because pulsatile blood flow is present in larger arteries[8]. There are several predisposing factors, which are often interrelated, such as heart failure, arterial hypotension, elevated sympathetic activity, hypovolemia, sepsis, use of vasopressors and pre-existing atherosclerotic lesions[7]. Catecholamines and medications such as digitalis[9], by interfering with the auto-regulation of mesenteric circulation, can also cause vasospasm[10].

Mesenteric venous thrombosis accounts for approximately 10% of all AMI cases[2] and involves the superior mesenteric vein in over 90% of patients. Mesenteric venous thrombosis is usually secondary to an underlying coagulopathy, while in 10% the cause is idiopathic[11-15]. Patients should be screened for genetic thrombophilias. Compromised venous return leads to interstitial swelling in the bowel wall, with subsequent arterial flow disturbances and eventual necrosis. The etiologic factors responsible for venous thrombosis include portal hypertension, intra-abdominal sepsis, cirrhosis, pancreatitis, malignancy and trauma.

Other rare causes of mesenteric ischemia are aortic dissection, lupus, vasculitis, median ligament syndrome, ergot administration and post laparoscopic cholecystectomy[16]. In young patients, arterial occlusion due to inherited coagulopathy is exceedingly rare and only isolated cases have been reported[17].

The clinical features of AMI originate from factors such as the site of involvement, systemic inflammatory response triggered by damage to the microcirculation, and reperfusion injury.

At the cellular level, ischemia causes mitochondrial dysfunction, loss of ion transfer regulation, and intracellular acidosis. Alterations in membrane permeability, and the release of free radicals and degradative enzymes leads to cell death and tissue necrosis[18]. In the ischemic tissue, numerous cells including neutrophils, endothelium, monocytes and platelets are activated. Proinflammatory substances are produced such as tumor necrosis factor, interleukines, platelet-activating factor and leukotrienes. Subsequently, the injury is due to leukocyte adhesion, platelet aggregation[19] and nitric oxide production impairment[20]. The activated neutrophils release superoxide substances such as superoxide O_2, peroxide H_2O_2 and hydroxyl radicals OH[21], along with neutrophil enzymes, which result in further damage to the surrounding tissues.

Ischemic/reperfusion double-hit injury consists of an initial hypoxic episode followed by the subsequent reperfusion injury due to reestablishment of forward flow[10]. Superoxide molecules, neutrophil enzymes and pro-inflammatory substances are carried in the bloodstream, causing distant organ damage. Moreover, reperfusion causes swelling of the corresponding organs since capillary permeability is considerably increased during ischemia[22]. Finally, damage to the intestinal micro-vessels and the disruption of the intestinal mucosal barrier results in leakage of water and bacteria, with resulting endotoxemia[23] and bacteremia[24,25]. Ultimately, multi-organ failure ensues and involves the liver[18], heart[26], kidneys[27] and lungs[28]. Acute pulmonary edema resulting from mesenteric ischemia/reperfusion is caused by an increase in pulmonary microvascular permeability to fluids and proteins, as well as smooth muscle dysfunction[29,30].

Clinical presentation

Abdominal pain is the primary symptom. The pain is characteristically out of proportion to the clinical findings. It is described as colicky and is most severe in the paraumbilical region. Other symptoms are present inconstantly and include nausea (93%), vomiting (80%) and diarrhea (48%)[31]. Physical examination is unremarkable unless peritonitis has developed. During the late stages, abdominal distension and guarding, as well as systemic complications may be encountered.

Laboratory tests

Soon after onset but prior to the development of mesenteric infarction, the sensitivity of laboratory tests in detecting mesenteric ischemia is poor[32]. Even at the time when ischemia is confirmed at laparotomy, elevation of serum lactate, amylase, creatine kinase and C-reactive protein (CRP), as well as leucocytes may be absent[31]. At present, no laboratory test is available for accurately establishing or eliminating the diagnosis[33,34]. One study

reported that hemoconcentration and hyperamylasemia were independent predictive factors of massive ischemic infarction[35].

Imaging studies

It is important to remember that when intestinal ischemia is clinically suspected, diagnostic imaging studies should be performed if peritoneal signs are absent.

Plain abdominal radiographs are of little help in the diagnosis of mesenteric ischemia. The presence of dilated loops is non-specific[36,37], and thickened bowel loops, "ground-glass" appearance suggesting ascites, or "thumbprinting" caused by submucosal edema or hemorrhage are seen in less than 40% patients. Twenty-five percent patients with bowel infarction have negative plain radiographs of the abdomen[38].

Barium enema has no place in the diagnosis of AMI since it may increase intra-luminal pressure and reduce perfusion to the bowel wall, causing translocation of bacteria and potentially, perforation. In addition, the presence of barium may compromise subsequent diagnostic tests, such as computed tomography (CT) and angiography[32].

Magnetic resonance imaging has shown promising results in detecting mesenteric ischemia but remains a slow-processing technique that seems to be inadequate in an emergent situation such as AMI[39,40].

Mesenteric duplex sonography is a highly user-dependent modality that can only confirm diminished blood flow in the trunks of the mesenteric blood vessels. Mesenteric duplex scanning identifies stenosis of the superior mesenteric and celiac arteries by the mean of elevated peak systolic velocities. A velocity > 275 cm/s is indicative of >70% stenosis with a sensitivity of 92% and a negative predictive value of 99%[41]. Doppler sonography is useful in diagnosing chronic mesenteric arterial occlusive disease but has limited role in AMI[42-45]. Other applications for duplex sonography are detection of reversible celiac flux alteration such as in median ligament syndrome, and follow-up of mesenteric bypass grafts and stents[41]. The new technique of contrast-enhanced ultrasonography is a promisingly non-invasive tool for the diagnosis of bowel ischemia[46].

Angiography is the gold standard diagnostic test in acute mesenteric artery occlusion[47], providing both anatomical visualization of the vessels and therapeutic options[48]. The sensitivity and specificity are 74% to 100% and 100%, respectively[49]. When used in the absence of peritonitis signs, angiography has been shown to improve the survival rate[50,51]. Mesenteric angiography can usually identify the underlying pathophysiologic event, by differentiating between embolic and thrombotic occlusion[52]. NOMI characteristically shows narrowing and multiple irregularities of the major SMA tributaries recognized as the "string of sausages" sign. Mesenteric venous thrombosis is characterized by a generalized slowing of arterial flow (up to 20 s) in conjunction with a lack of opacification of the corresponding mesenteric or portal venous outflow tracts. However, angiography is an invasive, time consuming and potentially nephrotoxic procedure. Its routine use is controversial in emergency situations[53] and therefore, it is employed only in selected patients.

Since CT is a fast, widely available non-invasive modality, it is considered as the initial imaging test[54]. It is useful in detecting intestinal signs suggestive of ischemia, as well as vascular abnormalities such as occlusion and stenosis. It is also useful in assessing other causes of acute abdominal pain. Still, the CT findings of mesenteric ischemia and infarction are not pathognomonic, and a direct correlation between CT findings and the final diagnosis is not accurate[55]. Overall, the sensitivity and specificity of contrast-enhanced CT for mesenteric ischemia are 64% and 92%, respectively[31,56,57]. Because of these drawbacks, the American Gastrointestinal Association[49] concluded that CT is of limited use in the diagnosis of AMI and that unremarkable CT findings in the context of a high suspicion of mesenteric ischemia should prompt an angiography without delay. An exception to this rule is when superior mesenteric vein thrombosis is suspected; a situation where CT scan remains the test of choice with sensitivity rate in the range of 90%[14,50].

Recently, the multi-detector row CT has emerged as a widely established non-invasive technique that not only delineates the blood vessels, but also shows an anatomical three dimensional relationship with the surrounding tissues, and allows evaluation of tissue perfusion[58]. The sensitivity and specificity rates are 92%-96% and 94%-100%, respectively[31,59,60], with positive and negative predictive values of 90% and 98%, respectively[61]. Moreover, when a cardiac source of mesenteric emboli is suspected, scanning of the heart provides a method for concomitant detection of the source of the embolus[62].

Therapeutic approaches

Therapeutic decisions are taken based on four main considerations: the presence or absence of peritonitis, the presence or absence of irreversible ischemia or infarcted segments of the intestine, the general condition of the patient, and the pathophysiologic phenomenon responsible for the event.

Once a diagnosis of AMI is made, treatment should be initiated without delay. This should include active resuscitation and treatment of the underlying condition, along with efforts directed toward reducing the associated vasospasm. Broad-spectrum antibiotics and intravenous heparin at therapeutic doses should be initiated as early as possible. If the diagnosis was established through angiography, intra-arterial infusion of papaverine, a phosphodiesterase inhibitor, is recommended for NOMI and for occlusive arterial AMI, since arterial vasospasm persists even after successful treatment of the precipitating event[1,3]. When angiography is not performed, intravenous glucagon may help reduce the vasospasm[52].

In the setting of a hemodynamically stable patient, with no signs of peritonitis, conservative medical

management may be attempted. For embolus- and thrombus-induced events, thrombolytic agents such as streptokinase, urokinase or recombinant tissue plasminogen activator are effective treatments[63-66]. Thrombolytic therapy seems to be most successful in distal clots, when used within 12 h after the onset of symptoms[66]. Ultimately, primary endovascular techniques and surgical resection may prevent mesenteric infarction when performed promptly in hemodynamically stable patients with arterial mesenteric ischemia[67]. For NOMI, especially when diagnosed by angiography, selective intra-arterial infusion of papaverine at the usual dose of 30 to 60 mg/h, is an adequate treatment[1,52,53]. It reduces the mortality rate from 70% to 50%-55%[68]. Early treatment with continuous intravenous high dose prostaglandin E(1)[69] or a prostacyclin analogue[70] have shown promising results in the treatment of NOMI. As for venous mesenteric ischemia, the standard treatment is anticoagulation, while venous thrombectomy has not improved the outcome and is controversial[71,72]. Heparin should be initiated as soon as the diagnosis is established, and is associated with reduction in the recurrence rate and mortality[13,14]. Another appropriate therapeutic modality is thrombolysis[73-75].

At any time during evaluation, should signs of peritonitis develop, the patient should undergo exploratory laparotomy without delay. First, the intestine is assessed for viability. Visual evaluation of the bowel relies on arterial pulsations and intestinal peristalsis and colour, although these findings are not specific[52]. Another technique is the use of sodium fluorescein, which is injected intravenously; it is detected with a Wood's lamp in the presence of hypoxic damage. Both retrospective analysis and randomized trials have shown that this technique is more reliable than clinical evaluation of mesenteric viability[76-78]. When compared with histological results, intraoperative laser Doppler flowmetry has been shown to be 100% accurate in assessing bowel viability[79]. Doppler ultrasound can be used intraoperatively but does not provide a quick assessment of the entire length of the intestine and thus does not carry any advantage over clinical judgment[80].

In patients with arterial occlusive AMI, when sufficient bowel is potentially viable, revascularization prior to resection of the infarcted bowel may improve the survival[2]. Although surgical revascularization is the standard procedure[81], embolectomy, thrombectomy, endarterectomy[38], as well as endovascular techniques such as antegrade percutaneous stenting[82], and open retrograde stenting[83-85] provide attractive alternatives with good short-term outcome. A high stent restenosis rate is the drawback of these techniques, requiring close follow-up of the patients[85,86]. Contraindications to revascularization include obvious infarction of the bowel supplied by the affected artery, patient's instability precluding further resection, and mesenteric vein thrombosis[53,87].

Surgical bowel resection must include all of the clearly non viable and infarcted portions of the bowel. Primary anastomosis can be performed if perfusion is adequate. A second-look laparotomy is scheduled within 12 to 24 h, if large portions or multiple segments of intestine of questionable viability were left behind[88], provided that complete resection should not result in a short bowel syndrome. Although widely approved[1,3], some authors question its routine use, and limit second-look laparotomy to individual cases[89].

Alternatively, second-look laparoscopy has emerged as a minimally-invasive, technically simple procedure that can provide diagnostic and therapeutic advantages[90] despite the fact that the evaluation is limited to the serosa and that mucosal lesions can be missed[91]. As a result, the value of second-look laparotomy in preventing morbidity is uncertain[90,92-94].

Outcome

Despite advances in the identification of mortality risk factors and greater therapeutic options, the overall mortality associated with AMI is as high today as it was several decades ago[31,95], ranging from 60% to 90%[49,96-99]. When specific etiologies are considered separately, arterial thrombosis has the highest mortality rate of 70% to 100%[3,98,100] in part because of the extensive ischemia-infarction of the bowel, and the need for more complex surgical revascularization. The mortality associated with NOMI is also within this range[98], whereas arterial embolism and venous thrombosis have much better prognosis, with mortality rates of 0% to 50%[15,98,101] and 20%[5], respectively. The peri-operative factors predicting mortality after mesenteric ischemia have been extensively studied[7,99]. Of the various factors examined, age > 70 (where diagnosis is more frequently overlooked), and prolonged duration of symptoms were independent predictors of mortality[97,102-104]. It cannot be overemphasized that a high index of suspicion, prompt diagnosis and aggressive early treatment are the only surgeon-dependent factors that have a positive influence on the outcome.

Perspectives for the future

A number of biochemical and genetic studies are in progress, designed to elucidate the pathophysiologic changes in an ischemic/reperfused intestine. Several molecules have been identified which attenuate intestinal injury and reduce the production of proinflammatory cytokines[105-110]. Intra-luminal infusion of hyperoxygenated solution during ischemia may improve the functional and structural status of the enterocyte mitochondria associated with ischemia/reperfusion syndrome[111]. Most therapies have to be initiated prior to the onset of ischemia, making their clinical application difficult to foresee.

Since the prognosis is closely related to delay in the treatment, and since the diagnostic tools currently available are not very accurate, great effort is being focused on identifying more accurate methods of early diagnosis. Serum assays such as D-dimers[112-114], alcohol dehydrogenase[115], glutathione S-transferase[116] and cobalt-albumin binding[117], and measurement of pH and potassium in peritoneal irrigation fluid[118], as well

as liver tissue oxygenation index[119] are good examples of current research. Seidel *et al* showed that mesenteric electrical activity may detect mesenteric ischemia with a high degree of sensitivity and specificity[120]. However, promising studies in animals remain to be validated under clinical conditions.

Experimental studies in which laparoscopy was combined with ultraviolet light and IV injection of fluoresceine showed that this technique may be useful in detecting mesenteric ischemia and viability of the intestine at an early stage[121,122]. Moreover, trans-serosal pulse oximetry may help determine bowel viability and resection extension prior to laparotomy[123].

AMI AFTER CPB

Incidence and frequency

Abdominal complications after CPB for cardiac surgery are seen in < 1% of patients[124-132] but carry a high mortality of 14.1%[131]. AMI represents 10%-67% of these complications[129,133,134], and is the most lethal, with a case-fatality rate of 70% to 100%[129,131,132,135,136].

Pathophysiology

AMI occurring after CPB, is due to NOMI in the vast majority of cases[137-139]. The various contributory factors are: low cardiac output (frequent in this category of patients), use of vasopressors and underlying atherosclerotic disease. It is well established that CPB is responsible for mesenteric endothelial dysfunction and microcirculation disturbances even under stable hemodynamic conditions. An increase in the contractile response to alpha1-adrenergic agonist and an early release of pro-inflammatory substances has been observed after CPB[140-142]. Nevertheless, the effect of pulseless extracorporeal circulation on bowel hypoperfusion is still under debate[143,144], and off-pump coronary artery bypass does not prevent subsequent mesenteric ischemia[134,145,146]. The physiologic changes in intestinal perfusion during cardiac surgery remain to be elucidated. It has been observed that there is significant mesenteric hypoperfusion followed by hyperemic response[147] after off-pump cardiac surgery along with an increase in the resistive and pulsatility indexes[148]. Moreover, studies focusing on the identification of predisposing factors for mesenteric ischemia after CPB[135,124-126], have produced different, and even opposite results. Rare cases of embolic acute arterial infarction can be prevented when a calcified aorta is detected on pre-operative CT scan[149].

Clinical presentation

In the context of CPB, patients are usually sedated and mechanically ventilated for a few days. Consequently, symptoms are not reported and the physical examination is equivocal due to masked, late-appearing, or missing clinical signs[125,139,150]. This accounts for the delay in diagnosis, and the disease may progress to a late, even irreversible stage by the time clinical signs become obvious (i.e. cyanosis). Finally, extracorporeal circulation induces a systemic inflammatory response with vasodilatation, such that hemodynamic instability can no longer be interpreted as a clue to an underlying mesenteric ischemia. A high index of suspicion in detecting subtle clinical evidence of AMI is the key to reducing the delay in diagnosis.

Laboratory tests

Pulseless perfusion during extracorporeal circulation causes systemic hypoperfusion, as illustrated by major derangements in the biochemical tests. These abnormalities are difficult to distinguish from those related to an underlying AMI. Moreover, laboratory test abnormalities are observed inconstantly in AMI. Even if unexplained metabolic acidosis with elevation of lactate level is considered as an early sign[151], several studies have shown that serum lactate may remain normal in the presence of extensive mesenteric infarction[139]. As indicated by Edwards *et al*[130], neither routine clinical investigations nor biological tests (such as leucocytosis, and elevation in serum creatinine, creatine kinase, hepatic or pancreatic enzymes) are discriminatory for mesenteric ischemia when the diagnosis is clinically suspected.

Imaging

When AMI is suspected after CPB, imaging studies should be such that they provide a rapid and accurate diagnosis, while being safe and avoid further morbidity in an already fragile patient. Traditional radiologic studies are not accurate for the diagnosis of AMI (as stated above) and are not recommended. Abdominal ultrasound is a non-invasive technique but remains highly operator-dependent. Its accuracy decreases significantly in emergent situations, especially in the presence of ileus and dilated bowel loops, which have a negative impact on image quality. Multi-detector abdominal CT scan theoretically provides good diagnostic results in AMI, but its specificity and sensitivity after CPB are reduced dramatically; accurate diagnosis correlates with laparotomy findings in < 50% of patients[139]. This is in part due to the limitation in the use of intravenous contrast because of borderline renal function and frequent presence of diabetes in these patients. This consideration applies also to angiography that remains the gold standard diagnostic tool in peripheral splanchnic disease[152], despite its invasive nature and time-consumption.

Therapeutic options

An early diagnosis and prompt treatment based on a high index of suspicion are the only hope for reducing the mortality and improving the outcome[153,154]. The initial treatment consists of hemodynamic support, but if these measures fail, prompt intervention is mandatory. Some experts believe that surgery within the first 6 h has a positive impact on the prognosis[151]. Since most mesenteric ischemic episodes after CPB are due to NOMI, some authors argue in favour of selective angiography as the initial test, as it provides the potential for both diagnosis and therapy. Intra-arterial infusion

of papaverine[155] or tolazoline with heparin[125] are both effective treatments.

Perspectives for the future

AMI occurring in the post CPB period remains a challenging surgical emergency, characterized by extremely high mortality and a controversial management approach. As stated above, clinical assessment as well as laboratory and radiological tests are typically unreliable in establishing the diagnosis.

In view of these considerations, we have adopted a uniform treatment strategy. When a diagnosis of AMI after CPB was suspected, exploratory laparotomy was carried out, irrespective of the results of the diagnostic tests. We performed a retrospective analysis on 1634 consecutive patients undergoing CPB for coronary artery bypass alone or combined with valvular surgery, between January 1st, 2000 and July 31st, 2007. A total of thirteen patients were suspected to have mesenteric ischemia, based on clinical and/or laboratory and/or radiological findings. All patients underwent exploratory laparotomy and were divided into two groups (Group 1 and Group 2) depending upon whether or not ischemic bowel was present. There was no difference in the clinical findings, laboratory tests and radiological results between the two groups. The mean delay in laparotomy was 13.7 h and 51.4 h in Group 1 and Group 2, respectively; the difference was statistically significant. Mortality rates in Group 1 and Group 2 were 42.8% and 50%, respectively. Based on these findings, we concluded that in the context of post-CPB AMI, diagnostic tests do not provide any information of practical value, but instead consume valuable time. By performing early exploratory laparotomy, we were able to reduce the mortality rate considerably. Since all of our patients had NOMI, no revascularization was required and resection of the irreversibly ischemic and infarcted segments of the bowel helped in preventing the vicious circle leading to multi-organ failure and death.

CONCLUSION

Although AMI has been known for several decades, it remains a highly lethal emergency, characterized by numerous controversies. The pathophysiologic process has not been completely resolved, the clinical presentation is often subtle and misleading, and despite the introduction of new technologies the diagnostic tools are often inaccurate. A high index of suspicion and prompt treatment are the only means to reduce mortality.

AMI in cardiac patients undergoing CPB is an extremely challenging surgical emergency. The role of clinical evaluation becomes even more relevant since the laboratory and radiological tests are no longer effective. In this context, prompt laparotomy is the only method of providing objective assessment and targeted treatment. Using this approach we achieved considerable improvement in the mortality rate. Although promising, this practice needs to be confirmed in a larger series of patients.

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REVIEW

Satiety testing: Ready for the clinic?

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Abstract

Drink tests are advocated as an inexpensive, nonivasive technique to assess gastric function in patients with a variety of upper digestive symptoms. Many patients with dyspeptic complaints will achieve satiation or develop symptoms at ingested volumes below those typically required to achieve these endpoints in controls. Substantial variation in test performance exists and a greater degree of standardization is required. Additionally, it remains unclear exactly what drink tests measure as correlations with measures of gastric sensation, accomodation and emptying are modest at best. Finally, results of drink tests do not guide therpay. At present, these tests are best reserved for research studies and are not advocated for use in clinical practice.

Key words: Drink test; Dyspepsia; Gastroparesis

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OVERVIEW AND RATIONALE

Drink tests were originally developed as a noninvasive means to assess upper digestive sensation and, perhaps, gastric accommodation. These tests are most commonly performed in patients with symptoms of functional dyspepsia or gastroparesis and many patients with these conditions will achieve satiation or develop dyspeptic symptoms at ingested volumes below those typically required to achieve these endpoints in controls. Drink tests are well tolerated, inexpensive and easy to perform. They are variously performed using either water or nutrient-containing solutions administered at different rates. This variability in test performance has limited our understanding of the exact physiologic parameters measured by the test. Drink tests are often used in clinical studies evaluating patients with functional dyspepsia or gastroparesis. Although patients often report satiation or develop symptoms at substantially smaller ingested volumes than controls, it remains unclear exactly what physiologic processes are assessed by the drink test. Additionally, results of drink tests do not guide therapy. As such, these tests are probably best reserved for research studies and are not advocated for use in clinical practice.

Drink tests and symptoms

Drink tests were originally developed as a symptom provocative technique for patients with dyspeptic complaints. Patients with dyspepsia will generally drink less and report more symptoms than do healthy subjects. Symptom reporting is influenced to a large degree by the endpoint of the drink test. For example, the 5-min water load test asks subjects to drink room temperature water *ad libitum* over a 5 min period until they become full[1]. Patients rate symptoms of fullness, nausea and bloating at the end of the drink test and then again 20 min and 30 min after the conclusion of the test. Not surprisingly, scores for the endpoint of fullness do not differ as greatly between patients with functional dyspepsia and controls while patients with functional dyspepsia do report significantly greater scores for symptoms of bloating and nausea (Figure 1)[2]. Compared with controls, symptoms induced by the drink test are more likely to persist in patients with functional dyspepsia compared with controls.

Boeckxstaens *et al*[3] have evaluated symptom responses to both water and Nutridrink consumed at a rate of 100 mL/min in healthy subjects and patients with functional dyspepsia. After each 100 mL, symptoms of satiety, epigastric bloating, nausea, and pain were scored on a scale from 0 (no sensation) to 5 (discomfort). The test was ended when a score of 5 was reached for at least one of the symptoms and the maximal ingested volume calculated. Subjects also rated these symptoms

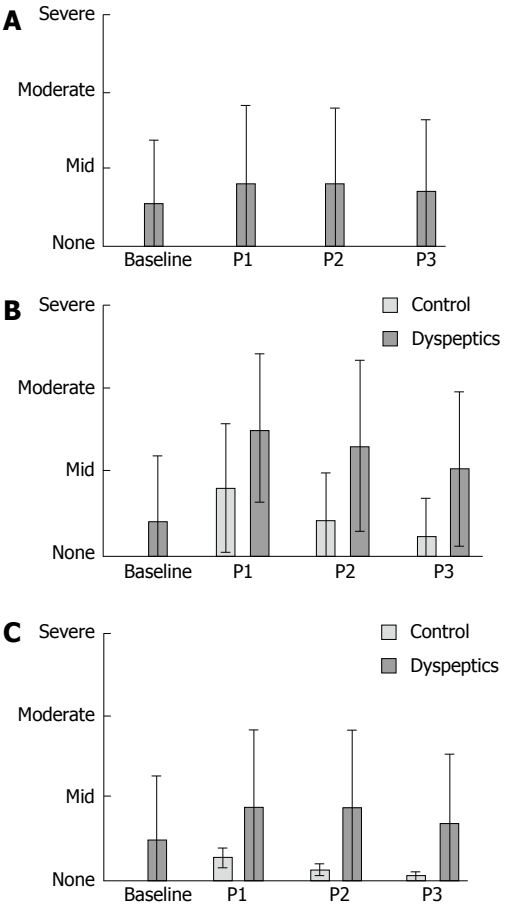

Figure 1 Symptoms before and after a 5-min water load test in controls and patients with functional dyspepsia. Patients with dyspepsia were significantly more symptomatic in terms of nausea (**A**), fullness (**B**), and bloating (**C**) both at baseline and after the water load test. Symptom scores at all time points were significantly different between the two groups. P1: 10 min after WL5; P2: 20 min after WL5; P3: 30 min after WL5. Data are expressed as mean ± SD. Adapted from Jones *et al*[2].

1 and 2 h after the end of the drink test. Again, patients with functional dyspepsia reported greater and more persistent symptoms during the drink test than did controls. Nutridrink was more symptom-provoking than water. Importantly, subjects in this study also underwent gastric barostat testing and were classified as having either normal physiology, visceral hypersensitivity or impaired accommodation. Symptom scores during drink tests were not influenced by the results of the barostat study.

While patients with functional dyspepsia will often achieve satiation at lower drink test volumes than controls and will report greater symptoms during the test, specific dyspeptic symptoms are not associated with an abnormal drink test. Jones and Maganti[4] evaluated the relationship between 15 common dyspeptic symptoms and volume to fullness as measured by a 5-min water load test. The only symptom significantly correlated with volume to fullness was nausea which showed a weak inverse correlation with nausea severity (r = -0.3, P = 0.05). Similarly, Boeckxstaens *et al*[5] reported that while patients with functional dyspepsia were more likely to report more symptoms during drink testing, no specific symptom was more likely to be associated with an abnormal drink test. Finally, Kindt *et al*[6] recently reported that maximal drink test volume was inversely associated with dyspeptic symptom scores prior to the study. For specific dyspeptic symptoms, only early satiety was significantly associated with the endpoint of the first satiety drinking test (r = 0.25, P = 0.02).

Psychiatric comorbidity is common in functional dyspepsia. In healthy subjects, experimentally induced anxiety is associated with decreased gastric compliance and meal-induced accommodation as well as increased symptom scores during a standard nutrient drink challenge[7]. However, in patients with functional dyspepsia, correlations between drink test volumes and general psychiatric distress (measured using either the SCL-90R or the Psychological General Well Being Scale) have been modest at best[2,4]. We do often encounter patients who report fullness at volumes that clearly defy physiologic parameters (< 50 mL), suggesting that central factors clearly influence test results. Finally, patients with functional dyspepsia, compared with controls, patients with gastroparesis or patients with gastroesophageal reflux often demonstrate poor self-efficacy and are less capable of estimating the volume required to produce fullness (Figure 2)[2].

Drink tests and accommodation

Assessment of gastric sensation and accommodation is most rigorously measured using a barostat. This is a cumbersome, expensive device that is decidedly patient unfriendly. Logically, it would seem that incrementally distending the stomach by drinking could achieve a result similar to incrementally distending the stomach using a balloon on the end of a catheter. Indeed, Tack *et al*[8] have reported a good correlation between barostat-measured accommodation and total calories consumed during a nutrient drink test administered at 15 mL/min. For both patients with functional dyspepsia and controls, the correlation was 0.76 (P < 0.001) and the nutrient drink test was calculated to have a sensitivity of 92% and a specificity of 86% in predicting impaired gastric accommodation.

Not all authors have agreed with these findings. Boeckxstaens *et al*[5] found no correlation between drinking capacity and fundal accommodation to a meal. These authors used both water and nutrient drink tests to evaluate subjects. The sensitivity of the water load test and nutrient drink test to detect impaired accommodation was 73% and 81%, respectively. The discrepant results between these two studies may reflect the methods used. Tack *et al*[8] had subjects ingest Nutridrink at a rate of 15 mL/min while Boeckxstaens *et al*[5] had subjects consume Nutridrink or water at 100 mL/min.

Accommodation can also be assessed using single-photon emission computed tomography (SPECT). Using a nutrient drink test administered at a rate of 120 mL/4 min, Gonenne *et al*[9] found that after controlling for covariates in a convenience sample of controls and patients with functional dyspepsia, the maximal tolerated nutrient drink test volume explained

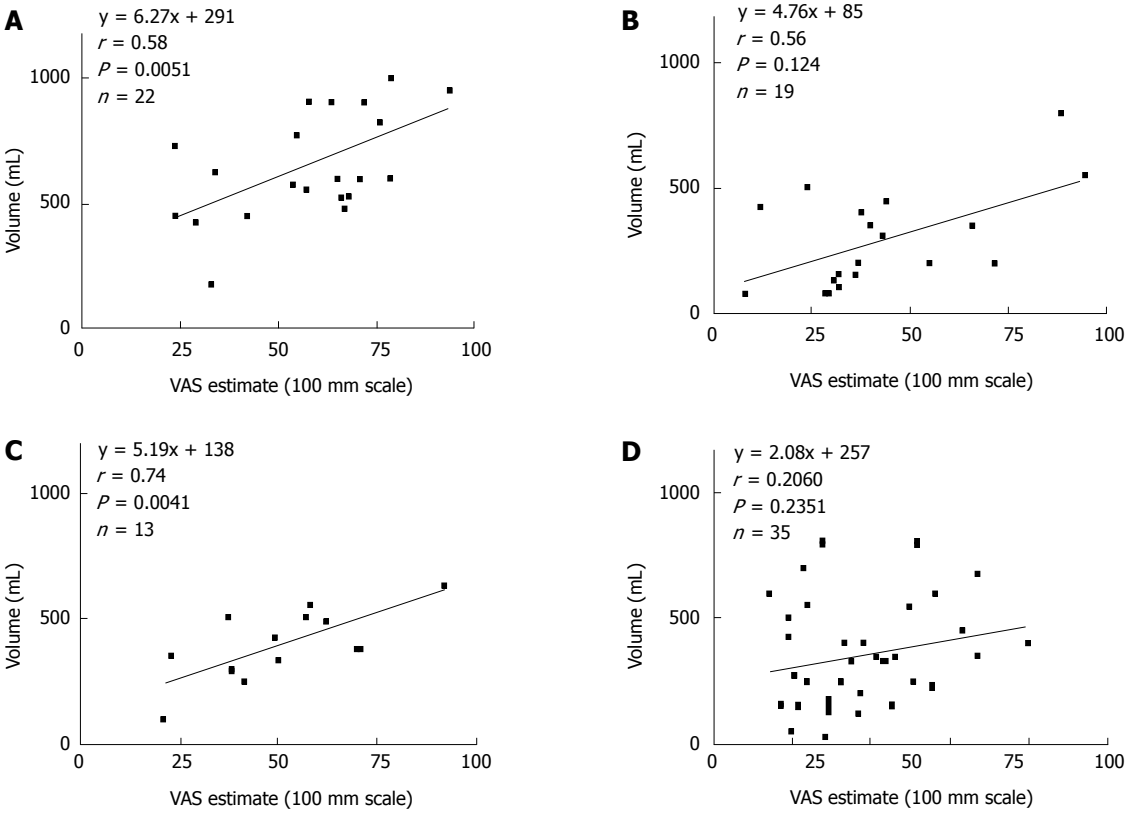

Figure 2 Drink test self-efficacy. Controls (**A**), and patients with either gastroparesis (**B**) or gastroesophageal reflux disease (**C**) are able to accurately estimate drinking capacity, while patients with functional dyspeptics (**D**) cannot. VAS: Visual analog scale. Adapted from Jones *et al*[2].

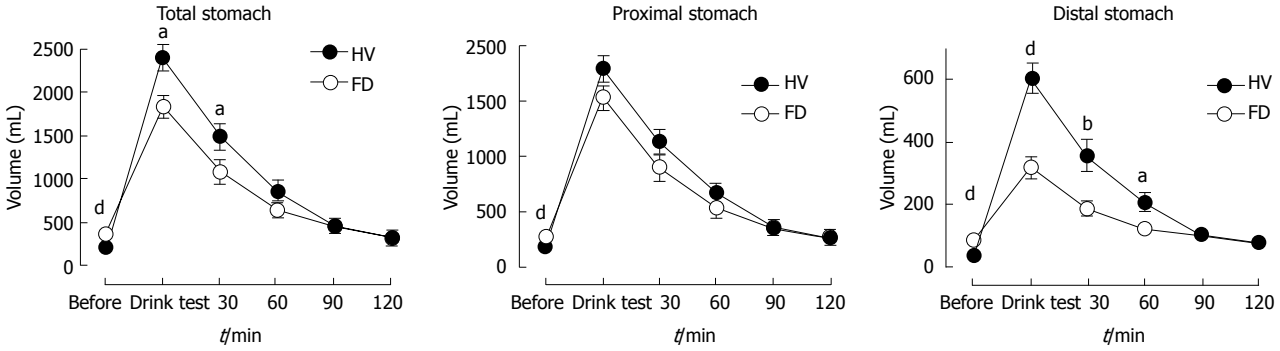

Figure 3 Volumes measured by gastric volume scintigraphy for total, proximal and distal stomach volume over time. Patients with functional dyspepsia had higher fasting volumes but reduced maximal tolerated volumes during drink test and reduced lower distal stomach volumes ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{d}P < 0.001$; Mann-Whitney *U*-test). Adapted from van der Elzen *et al*[10]. HV: Healthy volumes; FD: Functional dyspepsia.

only 13% and 3% of the variations in fasting and postprandial volumes measured by SPECT.

Recently, van den Elzen *et al*[10] have shown that drinking capacity may be more related to distal rather than proximal stomach function. Compared to controls, patients with functional dyspepsia ingested significantly less water ($P < 0.001$) and had reduced filling of the distal stomach ($P < 0.001$) after the drink test (Figure 3).

Drink tests and gastric emptying

Only a few studies have examined the relationship between gastric emptying and maximal tolerated volume and that relationship appears modest at best. Cuomo *et al*[11] reported that in females with functional dyspepsia, the correlation between maximal tolerated drink test volume and the fractional rate of gastric emptying was 0.48 ($P = 0.0003$, Figure 4). Tack *et al*[8] also reported a weak but significant correlation between maximal tolerated volume for the nutrient drink test and the half time of gastric emptying for a solid meal when pooled controls and patients with functional dyspepsia were studied ($r = -0.40$, $P = 0.001$). The correlation was not statistically significant when only evaluating patients with functional dyspepsia. Jones *et al*[2] did not find a correlation between volume to fullness using a 5-min

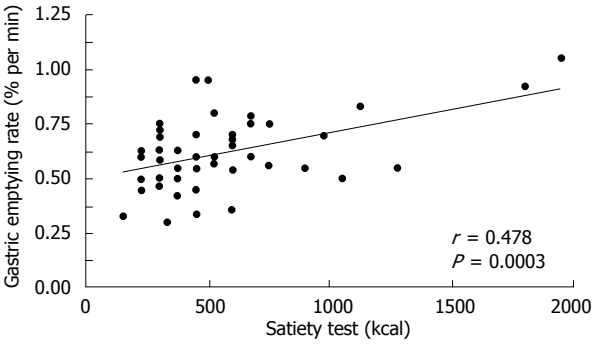

Figure 4 Correlation between nutrient drink test (kcal) and gastric emptying rate (% per min of gastric content) in the dyspeptic patients. Adapted from Cuomo *et al*[11].

water load test and T_{lag} (r = 0.1532, P = 0.4549) or $T_{1/2}$ (r = 0.1489, P = 0.4679) using a stable isotope gastric emptying breath test.

COMPARISONS BETWEEN DRINK TESTS

Drink tests are performed using either water or nutrient-containing beverages which are consumed at various rates. No method has proven superior although nutrient drink tests appear to be performed more often in clinical research. Presently, there is a need for a consensus on drink test methodology so that observations made by various investigators will be uniformly interpretable. Limited data exist regarding the performance characteristics of drink tests. Males ingest greater volumes than females but there appears to be less of an influence with respect to age and BMI[2,5,6,8].

Results of drink tests are reproducible at least in the short term. For healthy subjects, the correlations between 5-min water load tests at baseline and repeated 2 wk and 2 mo later were 0.78 (P < 0.0001) and 0.33 (P = 0.16)[2]. Cuomo *et al*[11] repeated a nutrient drink test between 2 d and 5 d in 10 controls and 5 patients with functional dyspepsia. The resulting inter-day variation of kcal ingested was 4.7% ± 1.5%.

Water loading at different rates produces comparable results in healthy subjects. The correlation between volumes to fullness for the 5-min water load and 100 mL/min water load was 0.79 (P < 0.0001)[2]. For the same subjects, the correlation between the 5-min water load and a 5-min nutrient drink test was 0.20 (P = 0.48). Boeckxstaens *et al*[5] found a significant correlation between the maximal volume ingested in the water test and the nutrient drink test. The correlation was greatest among controls (r = 0.67, P = 0.0001) and weakest among patients with functional dyspepsia (r = 0.57, P = 0.0001).

Using a 15 mL/min Nutridrink test, Kindt *et al*[6] demonstrated excellent test-retest reliability for a group that included 34 controls and 78 patients with FD (r = 0.88, P < 0.0001). During repeat testing, controls tended to consume higher volumes while patients with functional dyspepsia showed less variability.

PUBLISHED VALUES AND RANGES FOR DRINK TESTS

For the 5-min water load test, the mean volume required to produce fullness in a group of 73 controls was 648 ± 204 mL[2]. Males (703 ± 217 mL) drank more than females (611 ± 188 mL), but the difference was not statistically significant (t = 1.907; P = 0.0605). No healthy subject consumed < 300 mL of water and that volume was proposed as a cut-off for an abnormal test. A subsequent study demonstrated that the 300 mL cut-off value for the 5-min water load test discriminated controls from patients with functional dyspepsia with a sensitivity of 98% and a specificity of 46%[12].

For healthy subjects, the mean volume to fullness for the 100 mL/min water load test in controls has been reported as 1128 ± 355 mL[2]. In the same population, the mean volume to fullness for the 5-min nutrient drink test was 688 ± 187 mL. The nutrient drink test was performed using Boost™ (Mead Johnson Nutritionals, Evansville, Indiana) which contains 1.1 kcal/mL and is 70% carbohydrate, 15% fat, and 16% protein. Boost differs somewhat from Nutridrink™ (N.V. Nutricia, Zoetermeer, Netherlands) which contains 1.5 kcal/mL and is 39% fat. Boost™ is comparable to Ensure™ (Abbott Laboratories, Abbott Park, Ill.) which contains 1.06 kcal/mL and is 65% carbohydrate, 20% fat, and 15% protein. These test meals have not been directly compared but Tack *et al*[8] have shown that with increasing caloric density, maximum satiety occurs at progressively higher caloric intakes while satiety scores according to ingested volumes do not differ significantly. This suggests that volume may be a greater stimulus than caloric density. No study has assessed the influence of caloric composition or palatability.

Boeckxstaens *et al*[5] have reported normal values for both Nutridrink and water load administered at 100 mL/min. Males consumed significantly more water (2084 ± 181 mL *vs* 1367 ± 97 mL, P = 0.0001) and Nutridrink (1405 ± 81 mL *vs* 946 ± 74 mL, P = 0.002) than females. Using the 10th percentile as the lower limit of the normal range, volumes < 1100 mL of water for men and < 800 mL of water for women were considered abnormal. Similarly, volumes < 800 mL of Nutridrink for men and < 600 mL for women were considered abnormal. The difference in results for the 100 mL/min water load tests between these two studies likely reflects the fact that Jones *et al*[2] had subjects stop drinking when they first experienced fullness while Boeckxstaens *et al*[5] had patients continue to drink until they developed very severe or uncomfortable sensations of symptoms of satiety, epigastric bloating, nausea or pain.

Using a Nutridrink test administered at a rate of 15 mL/min to healthy volunteers, Tack *et al*[8] reported that maximum satiety occurred after ingestion of 1005 ± 35 mL (mean ± SE) with a lower limit of normal of 653 mL. This observation was supported by a more recent observation from the same group[2]. In this study, controls reported maximum satiety after ingestion of 937 ± 428 mL. Increasing drink test

volumes were associated with male sex and increasing age[6]. Moreover, Chial *et al*[13] used a nutrient drink test adopted from the methodology of Tack *et al*[8]. Subjects consumed 120 mL of Ensure™ every 4 min until full, and the average volume of nutrient drink ingested (mean ± SE) was 1181 ± 50 mL. There was a weak but significant correlation ($r = 0.29$, $P = 0.02$) between volume to fullness and body mass index.

Indications for drink tests

Given that it is unclear exactly what drink tests measure, and that the test remains poorly standardized, the role of drink tests in clinical practice remains to be established[14,15]. The test has most often been employed in clinical research studies evaluating patients with functional dyspepsia. Water loading is also performed as a provocative maneuver during the performance of electrogastrography[1].

Performing a drink test

Patients should be studied in the morning after an overnight fast. While certain medications can alter digestive sensation, accommodation or gastric emptying, we do not routinely stop motility or sensory modifying medications for clinical studies.

The 5-min water load test is performed by having subjects drink room temperature tap water *ad libitum* over a 5-min period until reaching the point of fullness. Water is consumed from an unmarked flask that is taken from the subject and refilled after each drink. The volume required to refill the flask to the initial level is recorded, and the total volume consumed is calculated by summing these volumes. In this way, the flask is "bottomless" and the subject blinded as to the actual volume of water consumed. During the test, patients rate symptoms of fullness, bloating, and nausea using a 4-point Likert scale for each symptom. Scores are recorded at baseline and then every 10 min for a 30-min period after completion of the test. Individual symptoms can therefore receive a total score ranging from 0-12, and the total WL symptom score has a range of 0-36.

Nutrient drink tests can be performed in a several ways. The simplest method is that used by Chial *et al*[13], in which subjects consume 120 mL of Ensure™ every 4 min until full. Ensure is administered in a paper cup that is refilled every 4 min. At 5 min intervals, participants score fullness using a rating scale that combines verbal descriptors on a scale graded 0-5 [0: no symptoms; 1: first sensation of fullness (threshold); 2: mild; 3: moderate; 4: severe; 5: maximum or unbearable fullness]. Participants are told to stop when a score of 5 is obtained. Postprandial symptoms were measured 30 min after completing the test with participants scoring symptoms of bloating, fullness, nausea and pain using a visual analogue scale (VAS) with 100 mm lines and the words "unnoticeable" and "unbearable" as anchors. The sum of the four 100-mm VAS scales for each symptom provides an aggregate symptom score.

The nutrient drink test used by Boeckxstaens *et al*[5] had subjects who consumed Nutridrink™ at a rate of 100 mL/min. Nutridrink™ is given in beakers or paper cups filled with 100 mL aliquots. After each 100 mL, symptoms of satiety, epigastric bloating, nausea, and pain are scored on a 5-point Likert scale (0: no sensation; 1: very mild; 2: mild; 3: moderate; 4: severe; 5: very severe or discomfort). When a score of 5 is reached for any symptom, the test ends and the maximal ingested volume is calculated.

Reporting and interpreting test results

Drink test results are reported as the maximal ingested volume. Occasionally, a patient may experience emesis during the test. If emesis occurs, the volume of emesis should be recorded and subtracted from the total ingested volume. Along with the maximal ingested volume, individual and cumulative symptom scores can be reported.

While the utility of drink tests remains to be determined, we find the test most helpful when it is either normal or glaringly abnormal. In the former scenario, the patient can be reassured that gastric function is likely to be intact. The latter scenario is more subjective. Often patients will report maximal fullness after the consumption of physiologically insignificant volumes (< 50 mL). We have not found results from the water load test to be correlated with measures of psychiatric distress or somatization but maximal ingested volumes are positively correlated with quality of life[2,12]. Others have reported that maximal ingested volumes are reduced in patients with depression[16]. While drink tests are not intended as surrogates for assessing psychosocial factors or quality of life, maximal fullness at extremely low volumes may suggest that extra-gastric or central factors are playing an important role in symptom generation, perpetuation or tolerance.

Since the physiologic parameters that determine maximal ingested volumes are not well known at present, drink test results cannot be reasonably used to guide therapy. Few studies have assessed the impact of commonly used treatments for functional dyspepsia on drink test results. A brief, randomized controlled trial found that 14 d of therapy with nortriptyline, mirtazapine or placebo did not alter either maximal ingested volumes or symptom scores[17]. A similar study also found no effect on maximal ingested volume or symptoms in healthy subjects treated with either citalopram, desipramine or placebo for 11 d[18]. In contrast, in healthy subjects, the kappa-opioid agonist asimadoline has been shown to increase maximal tolerated volumes without altering gastric emptying[19].

A small trial randomized patients with functional dyspepsia to biofeedback (breathing exercises using software for vagal biofeedback) or an educational control group[20]. Drinking capacity and quality of life improved significantly more in the biofeedback group than in the control group without any significant change in baseline autonomic activity or intra-gastric volume.

Combining an incomplete understanding of relevant pathophysiologic alterations that might be measured by drink tests with limited data regarding effects of

therapy on drink test volumes leads us to conclude that drink tests are of limited utility in guiding clinical management. Until our understanding in this area has evolved, drink tests should not routinely be performed in clinical practice.

CONCLUSION

Drink tests are often used in clinical studies evaluating patients with functional dyspepsia or gastroparesis. Although patients often report satiation or develop symptoms at substantially smaller ingested volumes than controls, it remains unclear exactly what physiologic processes are assessed by the drink test. Additionally, results of drink tests do not guide therapy. As such, these tests are probably best reserved for research studies and are not advocated for use in clinical practice.

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REVIEW

Neuroendocrine tumors of the gastro-entero-pancreatic system

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Abstract

Gastro-entero-pancreatic (GEP) neuroendocrine tumors (NETs) are rare neoplasms, although their prevalence has increased substantially over the past three decades. Moreover, there has been an increased clinical recognition and characterization of these neoplasms. They show extremely variable biological behavior and clinical course. Most NETs have endocrine function and secrete peptides and neuroamines that cause distinct clinical syndromes, including carcinoid syndrome; however, many are clinically silent until late presentation with mass effects. Investigation and management should be individualized for each patient, taking into account the likely natural history of the tumor and general health of the patient. Management strategies include surgery for cure or palliation, and a variety of other cytoreductive techniques, and medical treatment including chemotherapy, and biotherapy to control symptoms due to hormone release and tumor growth, with somatostatin analogues (SSAs) and alpha-interferon. New biological agents and somatostatin-tagged radionuclides are under investigation. Advances in the therapy and development of centers of excellence which coordinate multicenter studies, are needed to improve diagnosis, treatment and therefore survival of patients with GEP NETs.

Key words: Gastro-entero-pancreatic neuroendocrine tumors; Carcinoids; Entero-endocrine tumors; Pancreatic tumors; Medical treatment; Molecular targeted therapy

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INTRODUCTION

Neuroendocrine tumors (NETs) of the gastro-entero-pancreatic (GEP) system are rare and originate from the diffused endocrine system, located in the gastro-intestinal (GI) tract (carcinoids) and in the pancreas (insular tumors), with extremely varying clinical pictures. GEP NETs represent about 2% of all the GI tumors[1], but their prevalence has increased substantially over the past three decades, only in part as a consequence of increased awareness and improved diagnostic techniques[2]. The most recent estimates suggest a global clinical incidence of 2.5-5 cases/100 000 per year[2,3], with an autoptical incidence 2-5 times higher than the clinical one, and a slight predominance in females[4,5].

The term carcinoid (from the German *Karzinoide*) was introduced in 1907 by Oberndorfer to identify some ileal tumors, originating from the enterochromaffin cells (EC) that produce serotonin, characterized by a better prognosis in comparison with adenocarcinomas. Later the term was used to describe NETs, both of the gut and extra-intestinal sites (pancreas, lung and bronchus, liver, thymus), even though the term NET should always be used specifying the tumor's origin site, in order to avoid misunderstanding. The term carcinoid should be used to indicate the serotonin-secreting tumors[6].

The diffused endocrine system of the GEP tract is the widest of the whole organism, with at least 16 different types of endocrine cells that produce more than 50 peptides or amines[2,6,7]. GEP NETs arise within the GI tract, but NETs can also occur elsewhere such as in the bronchus and lung (bronchial epithelium), hypophysis, thyroid, parathyroids, thymus, adrenal cortex and medulla, and paraganglia. GEP NETs can preserve and amplify the activity of the origin cells

characterized by secretion of a number of peptides and neurotransmitters, which can lead to the development of typical clinical syndromes by the so called "functioning" tumors, or they can be biologically inactive ("non-functioning" tumors)[1,2,8] for several reasons (defect of hormonal synthesis/secretion, rapid hormone degradation, synthesis of precursors/inactive hormones, co-secretion of antagonist hormones).

GEP NETs are usually sporadic, but they may also be multiple and may occur in some genetic syndromes such as multiple endocrine neoplasia (MEN) type 1, von Hippel-Lindau syndrome, neurofibromatosis type 1 and tuberous sclerosis[2,9,10]. Their frequency in these syndromes varies from very low (< 1%) for carcinoid to high (80%-100%) for pancreatic endocrine tumors (insulinomas 5%-20%, gastrinomas 25%-30%, non-functioning > 50%)[6].

CLASSIFICATION

As GEP NETs represent a heterogeneous group of tumors, their classification is still a critical point. In the past, GEP NETs were classified according to their embryonic origin and, according to the classification of William and Sandler[11], three distinct groups have been identified: (1) carcinoids derived from the proximal GI tract (foregut), located in the stomach, proximal duodenum, biliary tract and pancreas fed by the celiac tripod; (2) carcinoids derived from intermediate GI tract (midgut), located in the distal duodenum, small intestine, appendix and right colon, fed from the superior mesenteric artery; (3) carcinoids of the distal intestine (hindgut) localized into the descending colon, sigmoid colon and rectum, fed from the inferior mesenteric artery.

The most recent WHO classification[12] (Table 1) categorized all GEP NETs on the basis of clinical-pathological criteria as follow: (1) well-differentiated endocrine tumors, with benign or uncertain behaviour; (2) well-differentiated endocrine carcinomas, with a low-grade malignant behaviour; (3) poorly differentiated endocrine carcinomas (small cells carcinomas), with a high-grade malignant behaviour; (4) mixed endocrine-exocrine carcinomas, with characteristics of both endocrine and exocrine tumors. Each category includes functioning and non-functioning tumors.

However this classification has prognostic limits and a suboptimal reproducibility among pathologists hence TNM classification is being developed for NETs[13,14]. Table 2 provides examples of TNM classification for pancreatic NETs and carcinoids.

CLINICAL FEATURES

Clinical manifestations of GEP NETs are very heterogeneous: indeed, they can either remain asymptomatic for years, or can occur with obstructive symptoms, such as abdominal pain, nausea, vomiting, cholestasis, or can present with metastases, found accidentally, or can occur with typical syndromes due to hormonal hypersecretion. In most cases, because of vagueness of symptoms, the diagnosis is delayed (3-10 years on average), with an increased risk of developing metastases.

Gastrointestinal NETs (carcinoids)

NETs of the small intestine according to the Surveillance, Epidemiology, and End Results (SEER) database have an incidence of 0.15-0.5 cases/100 000 per year[15]. They are usually asymptomatic or characterized by obstructive symptoms, due to the local fibrotic reaction or, rarely, to the mass itself, until liver metastases appear[6]. At this stage, the typical clinical picture is the carcinoid syndrome that occurs in 18% of patients with ileal carcinoid[2,16] and is characterized by flushing, diarrhea, abdominal pain; less frequent events are lacrymation, profuse sweating, telangiectasias, cardiac fibrosis, and cutaneous manifestations pellagra-like due to lack of niacin (Table 3). Carcinoid syndrome is caused by the release of serotonin, which is no longer metabolized in the liver, and other substances, such as tachykinins, prostaglandins, and bradykinins[2,17].

Gastric carcinoids, that account for 4.6% of all carcinoids[15], originate from gastric EC-like mucosal cells, are mostly asymptomatic and occasionally found in the course of gastroscopies[6]; rarely they can cause an atypical carcinoid syndrome (flushing of greater duration than typical, of a red colour, with scialorrea, sweating, tearing, hypotension and itching)[16-18]. These carcinoids are divided into 3 groups: those that occur in chronic hypergastrinemic conditions, such as the type 1, associated with chronic atrophic gastritis, and type 2, associated with Zollinger Ellison syndrome in MEN-1, while type 3 is not associated with hypergastrinemia and is frequently malignant, with distant metastases.

Appendiceal endocrine tumors are often small and are found incidentally during appendectomies, with a frequency of 3-9/1.000 appendectomies and are usually benign[6,19-21]. Colonic carcinoids account for 8.6% of all carcinoids. They are often large and, among the intestinal carcinoids, have the worst prognosis[6,22].

Rectal carcinoids may present as an incidental finding on sigmoidoscopy or colonoscopy (1:2.500). They are typically small, non-functioning and distant metastases are rarely present at diagnosis (probably due to the early diagnosis)[6,22].

Carcinoids have previously been reported to be associated with secondary non-carcinoid malignancies, with rates as high as 46%-55%, more frequently located in the lung, breast, prostate and colon[23,24].

Pancreatic NETs

Endocrine tumors of the pancreas can occur with typical syndromes due to hormonal hypersecretion, such as insulinoma, gastrinoma, VIP-oma, glucagonoma and somatostatinoma (Table 4), but in a percentage of 40%-50% they are non-functioning or secrete peptide with a low biological impact, such as pancreatic polypeptide (PP) and neurotensin. Moreover a metastatic disease can be present at the time of diagnosis in approximately 50% of the cases[1,2,6,8].

Table 1 WHO classification[12]

Site	Well differentiated endocrine tumor		Well-differentiated endocrine carcinoma	Poorly-differentiated endocrine carcinoma
	BB	UB		
Pancreas	< 2 cm	⩾ 2 cm	Local invasion	Small cells
	< 2 mitoses[1]	> 2 mitoses	2-10 mitoses	> 10 mitoses
	< 2% Ki-67	> 2% Ki-67	> 5% Ki-67	> 15% Ki-67
	No vascular invasion	Vascular invasion	Vascular invasion ± metastases	Vascular/perineural invasion
Stomach	Mucosa/Submucosa	Mucosa/Submucosa	Invasion of muscolaris propria ± metastases	Small cells
	⩽ 1 cm	> 1 cm		
	No vascular invasion	Vascular invasion		
Duodenum/	Mucosa/Submucosa	Mucosa/Submucosa	Invasion of muscolaris propria ± metastases	Small cells
Jejunum	⩽ 1 cm	> 1 cm		
	No vascular invasion	Vascular invasion		
Ileum/	Mucosa/Submucosa	Mucosa/Submucosa	Invasion of muscolaris propria ± metastases	Small cells
Colon/	⩽ 1 cm (ileum)	> 1 cm (ileum)		
Rectum	⩽ 2 cm (colon)	> 2 cm (colon)		
	No vascular invasion	Vascular invasion		
Appendix	⩽ 2 cm	> 2 cm	Extensive invasion of mesoappendix ± metastases	Small cells
	No vascular invasion	Vascular invasion		

[1]Mitoses expressed as number/10 high power field. BB: Benign behavior; UB: Uncertain behavior.

Table 2 TNM staging for pancreatic NETs[13], foregut and midgut gastrointestinal carcinoids[14]

	Pancreatic NETs			Foregut and midgut gastrointestinal carcinoids		
T-primary tumor						
Tx	Primary tumor cannot be assessed			Primary tumor cannot be assessed		
T0	No evidence of primary tumor			No evidence of primary tumor		
T1	Tumor limited to the pancreas and size < 2 cm			Tumor invades mucosa or submucosa and size ⩽ 1 cm		
T2	Tumor limited to the pancreas and size 2-4 cm			Tumor invades muscolaris propria and size > 1 cm		
T3	Tumor limited to the pancreas and size > 4 cm or invading duodenum or bile duct			Tumor invades subserosa		
T4	Tumor invading adjacent organs (stomach, spleen, colon, adrenal gland) or the wall of large vessels (celiac axis or superior mesenteric artery)			Tumor invades adjacent structures		
	For any T, add (m) for multiple tumors			For any T, add (m) for multiple tumors		
N-regional lymph nodes						
Nx	Regional lymph nodes cannot be assessed			Regional lymph nodes cannot be assessed		
N0	No regional lymph node metastases			No regional lymph node metastases		
N1	Regional lymph node metastases			Regional lymph node metastases		
M- distant metastases						
Mx	Distant metastases cannot be assessed			Distant metastases cannot be assessed		
M0	No distant metastases			No distant metastases		
M1	Distant metastases			Distant metastases		
Disease stage						
Ⅰ	T1	N0	M0	T1	N0	M0
Ⅱa	T2	N0	M0	T2	N0	M0
Ⅱb	T3	N0	M0	T3	N0	M0
Ⅲa	T4	N0	M0	T4	N0	M0
Ⅲb	Any T	N1	M0	Any T	N1	M0
Ⅳ	Any T	Any N	M1	Any T	Any N	M1

Criteria for carcinoids of the appendix and colon rectum differ only for the tumor size.

Insulinoma and gastrinoma are the most frequent pancreatic NETs. The incidence of insulinomas is 2-4 new cases/1 000 000 per year, whereas that of gastrinoma is 0.5-4 new cases/1 000 000 per year[8,25].

Insulinoma are usually (90%) benign tumors, most are small (> 90% are < 2 cm) and single, 6%-13% are multiple, and 4%-6% are associated with MEN-1. Clinically they are characterized by fasting hypoglycemia and neuroglycopenic symptoms. Moreover the release of catecholamines induced by hypoglycemia produces symptoms such as sweating, tremor and palpitation. Diagnostic procedures are given in Table 4.

Gastrinoma is a NET secreting gastrin. The chronic hypergastrinemia results in marked gastric acid hypersecretion that ultimately causes peptic ulcer disease, often refractory and severe, diarrhea and gastroesophageal reflux disease (Zollinger Ellison Syndrome, ZES).

At the time of diagnosis 50%-60% of gastrinomas are malignant. The tumor is preferentially located in the pancreas (24%-53%) and in the duodenum (13%-49%). Approximately 20% of gastrinomas are part of MEN-1. The diagnosis requires the demonstration of hypergastrinemia with hyperchlorhydria (Table 4).

Table 3 Carcinoid syndrome

Clinical features	Incidence (%)	Characteristics	Mediators
Flushing	90	Foregut tumors: prolonged fit, red-purple, localized to face and trunk. Midgut tumors: quick fit, pink-red.	Serotonin, histamine, P substance, prostaglandins
Diarrhea	70	Secretory	Serotonin, histamine, VIP, prostaglandins, gastrin
Abdominal pain	40	Long lasting	Obstruction, hepatomegaly, intestinal ischemia, fibrosis
Profuse sweating	15		Serotonin, histamine
Telangiectasias	25	Face	Unknown cause
Heart disease	30 (right)	Valvulopathies (tricuspid valve, pulmonary valve). Right heart failure. Dyspnea	P substance, serotonin
	10 (left)		
Pellagra	5	Dermatitis	Deficit of niacin

Table 4 Clinical features of the main endocrine pancreatic tumors

Tumor (syndrome)	Clinical features and diagnostic tests	MEN-1 (%)	Metastases (%)	SnSRS (%)
Insulinoma	Spontaneous or fasting hypoglycemia (Whipple's triad) Positive fasting test (hypoglycemia with hyperinsulinism)	8-10	10	50
Gastrinoma (Zollinger-Ellison syndrome)	Peptic ulcers, diarrhea, GERD, BAO > 15 mEq/h Positive secretin test (serum gastrinemia > 200 ng/L within 10 min from secretin venous infusion, 2 U/kg per min)	30	60	80
VIP-oma (Verner Morrison syndrome)	Severe watery diarrhea (> 1L/die), hypokalemia, hypochlorhydria	Rare	70	80
Glucagonoma	Necrolytic migratory erythema, diabetes, weight loss, anemia, hypoaminoacidemia, venous thrombosis	Rare	60	80
Somatostatinoma	Diarrhea, steatorrhea, weight loss, diabetes, cholelithiasis	Not associated	84	80
CRH/ACTH-oma	Cushing's syndrome	-	90	-
GHRH-oma	Acromegaly	-	-	-

SnSRS: Sensitivity of ^{111}In-Pentetreotide scintigraphy (Octreoscan®).

VIP-omas are NET that secretes VIP, which causes a distinct syndrome (Verner Morrison syndrome) characterized by large volume watery diarrhea, hypokalemia and dehydration. Pancreatic VIP-omas are rare (3%-8% of all pancreatic NETS)[8,25]. They are usually large (72% are > 5 cm) and malignant at the time of diagnosis (64%-92%). Extra-pancreatic VIP-omas may occur in pediatric patients and are neurogenic tumors (ganglioneuromas, ganglioneuroblastomas, neuroblastomas and pheochromocytomas).

Glucagonomas are rare (1/20 000 000 per year)[8,25,26]. They are usually large tumors at diagnosis with a size of 5-10 cm and from 50% to 82% are metastatic. The most common presenting feature is necrolytic migratory erythema, associated with glucose intolerance or diabetes, anemia, weight loss, depression, diarrhea and thromboembolism.

Somatostatinomas are rare tumors of either the pancreas or the upper small intestine, usually duodenum, near the ampulla of Vater. Somatosatinomas can be part of neurofibromatosis 1. Pancreatic tumors are usually large and metastatic (70%-92%) at diagnosis. The clinical symptoms include: diabetes, cholelithiasis, diarrhea with steatorrhea, hypochloridria, abdominal pain, weight loss and anemia.

Other rare tumors include CRH/ACTH-omas, GRF-omas, calcitoninomas and neurotensinomas[26]. Non functioning tumors constitute 30%-50% of all pancreatic NETs and differentiation from pancreatic adenocarcinomas is extremely important because prognosis is clearly different. The tumors are usually large, can be multifocal when are part of MEN-1 and malignancy rate varies from 62% to 92%[25].

DIAGNOSIS

Hormonal dosages

Several circulating or urinary tumor markers can be used for the diagnosis and follow-up of GEP NETs.

Among the generic markers, chromogranin A (CgA), a glycoprotein contained in secretion granules of neuroendocrine cells, has become the most important circulating tumor marker for the diagnosis and follow-up of NETs[27,28]. Elevated circulating levels of CgA are found in about 60%-80% of GEP NETs, both functioning and non-functioning[29], even if other non-neoplastic conditions, such as renal insufficiency, atrophic chronic gastritis, therapy with proton pump inhibitors[30,31] can determine false-positive results, reducing its specificity. Other generic markers include neuron-specific enolase (NSE), PP and human chorionic gonadotropin, with lower diagnostic accuracy than CgA[6,32].

5-hydroxyindoleacetic acid (5-HIAA) is the specific marker for carcinoids producing serotonin[2,6,18,32]; it is a metabolite of serotonin that can be determined in 24 h urines. The sensibility of the urinary 5-HIAA is about 65%-75%, while its specificity between 90%-100%[6].

Certain foods and drugs will affect the urinary excretion of 5-HIAA if they are taken in the 3-5 d before collection

of the urine sample. Bananas, avocados, aubergines, pineapples, plums, walnuts, cough syrup, paracetamol, fluorouracil, methysergide, levodopa, aspirin, 5-aminosalicylic acid (5-ASA), naproxen and caffeine may cause false-positive results. Adrenocorticotrophic hormone (ACTH), glucocorticoids, heparin, isoniazid, methyldopa and phenothiazines may give false-negative results[6].

For functioning NETs, the dosage of the specific hormone that causes the characteristic syndrome represents the specific tumor marker[1,6,8]. In particular in patients with suspected insulinoma, glycemia, insulin, peptide C and pro-insulin must be tested. Further biochemical tests include the prolonged fast (48-72 h), which is the gold standard for establishing the diagnosis of insulinoma. Indeed, 98% of patients with insulinoma will develop symptomatic hypoglycemia within 72 h.

In Zollinger Ellison syndrome, serum gastrin and basal gastric acid output should be evaluated[33,34]. If the gastrin is $\geqslant$ 1000 ng/L and gastric pH $<$ 2.5, the diagnosis is established. The secretin test is the provocative test of choice in patients with gastrin levels $<$ 1000 ng/L (Table 4). Plasma vasointestinal polypeptide (VIP) determination is used to diagnose VIP-oma in the suspicion of Verner-Morrison syndrome, plasma glucagon for glucagonoma, and serum somatostatin for somatostatinoma[1,6,8].

Imaging

Different integrated techniques can be used for diagnosis[1,2,6,35]. Imaging has an important role in localizing the primary tumor, identifying sites of metastatic disease and assessing response to treatment. The gastric and intestinal tumors are usually well studied with endoscopic techniques and endoscopic ultrasound. The tumors of the small intestine may require, besides enforcement of traditional radiological techniques (small bowel barium studies), the use of the most current techniques for studying small bowel (double balloon enteroscopy, video endoscopic capsule). Both for carcinoid and pancreatic tumors, computer tomograghy (CT) and magnetic resonance imaging (MRI) are important in defining the extent of metastatic disease and assessing response to treatment. Both techniques appear to have similar sensitivities for detection of these tumors, ranging from 30% to 94%[35]. Endoscopic ultrasound has an important role in the preoperative assessment of the pancreas where a small functioning tumor or the possibility of multiple tumors is suspected. This technique is very successful in expert hands, with sensitivities as high as 79%-100% being reported[35].

Functional imaging modalities, such as somatostatin receptor scintigraphy (SRS, Octreoscan®), have great impact on patient management by providing tools for better staging of the disease, visualization of occult tumor, and evaluation of eligibility for somatostatin analogue (SSA) treatment. In fact NETs generally express somatostatin receptors and by administering a radiolabelled SSA, the tumor is highlighted by the scintigraphic investigation. The SRS is a highly specific examination with sensitivity, for tumors of more than 1 cm, approximately of 80%-90% (with the exception of insulinoma that expresses somatostatin receptors in only 50% of cases)[1,2,6,36,37]. SRS also detects distant metastases with a sensitivity that can reach 96%[2,6]. It should be also noted that a positive SRS may lead to a possible systemic SSAs treatment or radionuclide therapy. On the other hand, even more sensitive techniques are being developed, based on methods combining PET-CT using [^{18}F] levodopa, 5HTP [^{11}C] or [^{68}Ga] linked to a SSA (^{68}Ga-DOTA-octreotide-PET)[36].

On the contrary, PET with conventional fluoro-deoxy-glucose has not proven advantageous for NET imaging, because of GEP NETs' low metabolic activity, with the exception of tumors with high proliferative activity and low differentiation[36].

Finally angiographic techniques, with the possible establishment of hormonal gradients, are currently used only in special cases and adequately equipped centers.

Pathology

Histopathological examination is the main criterion of the WHO classification[12] (Table 1), which takes into account: tumor size, number of mitosis, presence of cellular atypias, proliferative index, angioinvasion. Immunohistochemistry is also one of the most important techniques for the study of NETs. Several antibodies are available both against general endocrine markers such as NSE, sinaptofisin and CgA, and against specific hormones.

It is also important to discriminate well-differentiated forms from poorly-differentiated carcinomas using malignancy markers. With this aim, the immunohistochemical expression of Ki67 seems as important as the determination of the mitotic index, expressed as the number of mitoses/10 high power fields[6,38].

TREATMENT

Surgical treatment

If possible, radical surgery is the cornerstone of the treatment of primitive GEP NETs. If there is loco-regional or liver metastases a debulking surgery can be performed in patients in whom 90% of the tumor is removable. It is suggested to perform a palliative surgery in the following clinical situations: (1) on the primary tumor with non-operable liver metastases (particularly in functioning tumors) because symptoms correlate with neoplastic mass; (2) if the primary tumor is localized in the small bowel, as it can lead to bowel obstruction; (3) in the case whereby surgery allow a subsequent multimodal treatment.

A combination of several therapies can be performed for liver metastases, such as surgical resection, (chemo) embolization, radiofrequency ablation and, in selected cases, orthotopic liver transplantation may be considered[16,39,40]. Although there are few studies that compare different treatment options on liver metastases, it would seem that different treatments improve survival

Table 5 Results of studies of molecularly targeted agents in patients with neuroendocrine tumours[54,55]

Agent	Response rate (%)	PFS rate (%)/Duration
VEGF monoclonal antibody		
Bevacizumab[56]	18	95 at 18 wk
mTOR inhibitor		
RAD001 (everolimus)	13	71 at 24 wk
Temsirolimus[57]	5.6	50 at 6 mo
VEGF TKI		
Sunitinib	10	Median, 42 wk
Vatalanib	In progress	(time to progression)
Sorafenib	In progress	
Pazopanib	In progress	
PDGFR/Kit/Abl inhibitor		
Imatinib[58]	4	Median, 5.9 mo
EGFR inhibitor		
Gefitinib	4	61 (carcinoids) and 31 (pancreatic tumor) at 6 mo
Other		
Bortezomib[59]	0	Median, 3 mo (Time to treatment failure)

PFS: Progression free survival.

rate at 5 years globally from 30% for the untreated tumor to 50%-70%[39,40].

Medical therapy

Medical treatment of NETs is different depending on whether the tumor is a well-differentiated or a poorly differentiated one. Functioning tumors are usually well differentiated and the first target of therapy is the control of symptoms. As these tumors are generally slow in growth, with a relatively long life expectancy, it is essential to ensure patients a good quality of life.

Treatment of gastrinomas is based on the use of proton pump inhibitors at an appropriate dosage (omeprazole and lansoprazole 40-60 up to 120 mg/d)[41,42]. Insulinomas are treated with diazoxide associated with hydrochlorothiazide; if this therapy is ineffective calcium channel blockers, beta blockers and glucocorticoids can be used[43]. For other well-differentiated cancers therapy is based on the use of SSAs, interferon and, more recently, targeted therapy[44,45].

Somatostatin is a hormone that inhibits the secretion of various hormones and peptides; somatostatin receptors are present in most well-differentiated GEP NETs (70%-95% of tumors), with the exception of insulinoma. SSAs allow control of hormonal-related symptoms and should be used both in a preoperative setting and in inoperable tumors[44]. They are sometimes used as antiproliferative agents, even if clinical studies have given disappointing results with regard to tumor regression and tumor shrinkage is demonstrated in less than 10% of the patients at standard dosage, although about 50% of patients can show stabilization of tumor size[46]. A possible positive effect on tumor volume regression with high-dose SSAs has yet to be demonstrated. Two different SSAs, octreotide and lanreotide, are used clinically. These analogues bind principally to the receptor subtypes 2 and 5. Recently pasireotide, a somatostatin analog with high affinity for all types of somatostatin receptors, has been introduced and has been shown to be effective in patients who do not respond to the currently available SSAs octreotide and lanreotide[47]. However, its use is still restricted to clinical studies. Altogether, SSAs are safe, easy to use, and well tolerated by patients experiencing only mild and infrequent side effects, among which are diarrhea, abdominal pain, steatorrhea, and cholelithiasis[48].

In addition, alpha interferon, such as monotherapy or in combination with SSAs, can be used to inhibit hormone hypersecretion and to stabilize the disease, with variable response rates. There has been biochemical response in 40%-60% of patients, symptomatic improvement in 40%-70% of patients, and significant tumor shrinkage in a median of 10%-15% of patients[48,49]. Interferon is used for the same indications as are SSAs in NETs of the gut, except for carcinoid crisis. Side-effects are generally mild, flu-like syndrome, fatigue, weight loss, polyneuropathy, myositis, thrombocytopenia, anemia, leukopenia, hepatotoxicity and neutralizing antibodies.

Poorly differentiated tumors are generally treated with different chemotherapy schedules. The role of chemotherapy in the treatment of GEP NETs is still uncertain, as variable response rates in different studies have been reported. While well-differentiated tumors are not responsive to chemotherapy (based on streptozotocin, doxorubicin, dacarbazine and 5-flurouracile variously associated with each other)[6,50] with only about 10% of carcinoids having a positive response, the best response rates (40%-70%) have been reported in some studies for anaplastic cancer, using different schemes based on cisplatin and etoposide, although there is no unequivocal evidence of survival improvement[51-53]. Furthermore, randomized controlled trials on chemotherapy *versus* biological treatment (SSAs with/without interferon) are still lacking.

GEP NETs can over express some molecules, such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and its receptor (VEGFR) or insulin-like growth factor receptor (IGFR), that can be targeted by some new drugs under assessment in early clinical trial (see Table 5)[54-59]. Other molecular therapies currently under investigation include the Raf-kinase inhibitor sorafenib and the inhibitor of the mTOR pathway, everolimus (RAD001)[54,55].

Peptide receptor radionuclide therapy (PRRT)

Another therapeutic approach is PRRT, which uses somatostatin analogs to convey radioactivity within the tumor itself (using generally ^{90}Ittrium, ^{177}Lutetium or ^{111}Indium), through somatostatin receptors[60,61]. PRRT can be considered in patients with inoperable GEP NETs and positive nuclear medicine imaging. According to some studies a stabilization of the disease can be reached in 50%-70% of cases[62-64] and control of symptoms in 70%[60]. Data in the literature, which however are not based on randomized, comparative studies, seem to favor [^{177}Lu-DOTA, Tyr] octreotate as

the most suitable peptide and radionuclide for PRRT[65]. Currently, tolerated dose is defined by the dose tolerated by the critical organs, kidney and bone marrow; it is likely that the dose can be modified in the future by more sophisticated, individually tailored dosimetry models, and by the introduction of new protective agents, different treatment schedules and radionuclides. This treatment has to be carried out in centers properly equipped and is to be reserved for selected cases.

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RESEARCH FRONTIER

"Rescue" regimens after *Helicobacter pylori* treatment failure

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Abstract

Helicobacter pylori (*H pylori*) infection is the main cause of gastritis, gastroduodenal ulcer disease, and gastric cancer. After more than 20 years of experience in *H pylori* treatment, in my opinion, the ideal regimen to treat this infection is still to be found. Currently, apart from having to know first-line eradication regimens well, we must also be prepared to face treatment failures. Therefore, in designing a treatment strategy we should not focus on the results of primary therapy alone, but also on the final (overall) eradication rate. The choice of a "rescue" treatment depends on which treatment is used initially. If a clarithromycin-based regimen was used initially, a subsequent metronidazole-based treatment (quadruple therapy) may be used afterwards, and then a levofloxacin-based combination would be a third "rescue" option. Alternatively, it has recently been suggested that levofloxacin-based rescue therapy constitutes an encouraging second-line strategy, representing an alternative to quadruple therapy in patients with previous PPI-clarithromycin-amoxicillin failure, with the advantage of efficacy, simplicity and safety. In this case, a quadruple regimen may be reserved as a third-line rescue option. Finally, rifabutin-based rescue therapy constitutes an encouraging empirical fourth-line strategy after multiple previous eradication failures with key antibiotics such as amoxicillin, clarithromycin, metronidazole, tetracycline, and levofloxacin. Even after two consecutive failures, several studies have demonstrated that *H pylori* eradication can finally be achieved in almost all patients if several rescue therapies are consecutively given. Therefore, the attitude in *H pylori* eradication therapy failure, even after two or more unsuccessful attempts, should be to fight and not to surrender.

Key words: *Helicobacter pylori*; Rescue; Salvage; Rifabutin; Levofloxacin

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INTRODUCTION

Helicobacter pylori (*H pylori*) infection is the main cause of gastritis, gastroduodenal ulcer disease, and gastric cancer. After more than 20 years of experience in *H pylori* treatment, in my opinion, the ideal regimen to treat this infection is still to be found. Consensus conferences have recommended therapeutic regimens that achieve *H pylori* cure rates higher than 80% on an intention-to-treat basis[1-3]. However, several large clinical trials and meta-analyses have shown that the most commonly used first-line therapies-including proton pump inhibitors (PPIs) plus two antibiotics-may fail in up to 20% of patients[4,5], and in the clinical routine setting, the treatment failure rate might be even higher. Moreover, during the last few years, the efficacy of PPI-based regimens seems to be decreasing, and several studies have reported intention-to-treat eradication rates lower than 75%[6-14] and even lower than 50%[15,16]. Antibiotic resistance to clarithromycin has been identified as one of the major factors affecting our ability to cure *H pylori* infection, and the rate of resistance to this antibiotic seems to be increasing in many geographical areas[17,18].

Reports dealing with retreatment of *H pylori* after failure are difficult to analyze for several reasons[19]. Firstly, patients who fail with their first-line treatment

probably include a higher percentage of individuals who are unreliable tablet takers, others have resistant organisms and there is the "constitutional" group, where failure will be inevitable. On the other hand, some patients submitted for rescue therapy have already had more than one previous treatment for *H pylori*, and this circumstance is not always clarified in the protocols. Furthermore, the original primary treatments vary among the different studies, not only with respect to the antibiotic type, but also with respect to the dose and duration of the regimen. Finally, only a few studies have directly compared, in the same protocol, two or more second therapies[20,21].

Several rescue therapies have been recommended, but they still fail to eradicate *H pylori* in more than 20% of cases[20], and these patients constitute a therapeutic dilemma[21]. Patients who are not cured with two consecutive treatments, including clarithromycin and metronidazole, will have at least single, and usually double, resistance[18]. Furthermore, bismuth salts are not available worldwide anymore and, therefore, management of first-line eradication failures is becoming challenging. Currently, a standard third-line therapy is lacking, and European guidelines recommend a culture for these patients to select a third-line treatment according to microbial sensitivity to antibiotics[2,3]. However, cultures are often carried out only in research centers, and the use of this procedure as "routine practice" in patients who failed several treatments seems not to be feasible[20-22]. Therefore, the evaluation of drugs without cross-resistance to nitroimidazole or macrolides as components of retreatment combination therapies would be worthwhile.

All these issues are important at the present time, but they will be even more relevant in the near future, as therapy for *H pylori* infection is becoming more and more frequently prescribed. Therefore, the evaluation of second or third rescue regimens for these problematic cases seems to be worthwhile. In designing a treatment strategy, we should not focus on the results of primary therapy alone; an adequate strategy for treating this infection should use several therapies which, if consecutively prescribed, come as close to the 100% cure rate as possible[20,21,23,24].

The aim of the present manuscript will be to review the experience dealing with "non-responders" to *H pylori* eradication therapy. As, at present, the current most prescribed first-line regimens include a combination of PPI plus two antibiotics, the present review will focus only on rescue regimens when these triple combinations fail. Bibliographical searches were performed in the PubMed (Internet) database including studies available until March 2008, looking for the following words (all fields): pylori AND (retreatment OR re-treatment OR rescue OR failure OR salvage OR second-line OR third-line OR fourth-line). References of reviews on *H pylori* eradication treatment, and from the articles selected for the study, were also examined in search of articles meeting inclusion criteria (that is, dealing with *H pylori* rescue therapies).

IS IT NECESSARY TO PERFORM CULTURE AFTER FAILURE OF THE FIRST ERADICATION TREATMENT?

Pretreatment antibiotic resistance is the most important factor in nonresponse to initial treatment[25-29]. Thus, the choice of a second-line treatment depends on which treatment was used initially, as it would appear that retreatment with the same regimen cannot be recommended[30]. If a clarithromycin-based regimen was used, a metronidazole-based treatment (or at least a clarithromycin-free regimen) should be used afterwards, and *vice versa*[31]. This recommendation is based on the observation that acquired bacterial resistance to metronidazole or clarithromycin results primarily from the previous treatment failure[26,32,33], and therefore rescue therapies should avoid these antibiotics and use different combinations.

An antimicrobial susceptibility test for *H pylori* before second-line treatment is sometimes performed, although whether the test is truly necessary remains unknown. Some authors have evaluated the efficacy of susceptibility-guided *vs* empiric retreatment for *H pylori* after a treatment failure. In the study by Yahav *et al*[34], patients in whom at least one treatment regimen for *H pylori* eradication had failed underwent gastric biopsy and culture, and were retreated according to the *in vitro* susceptibility results. Findings were compared with those of control patients (where culture was unavailable). Susceptibility-guided retreatment was associated with better eradication rates (86%) than empiric treatment (63%). However, several methodological drawbacks exist in this study. Firstly, more than 50% of the patients received first-line eradication treatment with both clarithromycin and metronidazole (instead of including clarithromycin and amoxicillin), which is not the generally recommended combination; consequently, no logical empirical treatment remained afterwards (levofloxacin-based regimens were not available at that time). In this respect, when only the eradication rates in control (culture unavailable) patients treated with a first regimen of PPI-amoxicillin-clarithromycin followed by a second *empiric* quadruple regimen were considered (the generally recommended first and second-line strategies), the success figures were not significantly different from those reported in patients receiving susceptibility-guided retreatment. Secondly, because this study was nonrandomized, there might have been heterogeneity among the two groups with respect to the treatment regimens prescribed by the treating physicians. Finally, this study was limited by the lack of susceptibility data for the controls, which restricted the ability to analyze the reasons why empiric therapy did not work as well as the susceptibility-guided protocol.

In a French multicenter study[35], patients in whom one previous *H pylori* eradication therapy (mainly with PPI-amoxicillin-clarithromycin) had failed were randomized to receive one of three empirical triple therapy regimens or a strategy based on antibiotic susceptibility. The empirical regimens

were PPI-amoxicillin-clarithromycin (for 7 d or 14 d) or PPI-amoxicillin-metronidazole (for 14 d). In the susceptibility-based strategy, patients with clarithromycin-susceptible strains received PPI-amoxicillin-clarithromycin, whilst the others received PPI-amoxicillin-metronidazole. The eradication rates for empirical therapies were low, while the cure rate was higher (74%) for the susceptibility-based treatment. If the *H pylori* strain was clarithromycin-susceptible (which occurred in approximately 1/3 of the cases), a high success rate was obtained with the PPI-clarithromycin-amoxicillin rescue regimen. The study, however, was done in France, where bismuth is banned, so that the use of quadruple therapy with a PPI, bismuth, tetracycline, and metronidazole as recommended by the updated Maastricht Consensus Report[3], was not tested. In fact, as will be reviewed later, several studies have obtained relatively good results with this quadruple regimen empirically prescribed, with a mean eradication rate of 77%, which is similar to the 74% achieved for the susceptibility-based treatment in the present study. Thus, in this study, instead of not readministering any of the antibiotics against which *H pylori* had probably become resistant, the authors insist on prescribing again clarithromycin (or metronidazole) for the second-line treatment. Furthermore, statistically significant differences were not demonstrated when comparing the efficacy of the empirical PPI-amoxicillin-metronidazole and the susceptibility-based strategy, suggesting that the metronidazole-based combination may be an effective empirical alternative after failure of a clarithromycin-based combination.

In the updated Maastricht Consensus Report[3], it was recommended that culture and antimicrobial sensitivity testing should be routinely performed only after two treatment failures with different antibiotics. According to this statement, some studies have suggested that an antimicrobial susceptibility test for *H pylori* before administering second-line treatment is not necessary. In this respect, in the study by Avidan *et al*[36], after failure of first-line eradication treatment, half of the patients were randomly assigned to treatment with a different PPI-based triple regimen regardless of the culture obtained, and the other half was assigned to treatment with PPI and two antibacterial agents chosen according to a susceptibility test; the authors found that the culture results did not influence the treatment protocol employed. Similarly, in the study by Miwa *et al*[37], patients with *H pylori* infection for whom first-line treatment with a PPI-amoxicillin-clarithromycin regimen had failed were randomly assigned to two groups: those having or not having the susceptibility test before retreatment. For those patients in the susceptibility-test group, the authors used what they considered the best regimen based on susceptibility testing; while for those patients in the group with no susceptibility testing, PPI-amoxicillin-metronidazole was prescribed. The cure rates in the groups with and without susceptibility testing were not different.

SECOND-LINE *H PYLORI* RESCUE THERAPY AFTER FAILURE OF ONE ERADICATION TREATMENT

Rescue regimen after PPI-clarithromycin-amoxicillin failure

PPI, amoxicillin and metronidazole: After failure of a combination of PPI, amoxicillin and clarithromycin, a theoretically correct alternative would be the use, as second option, of other PPI-based triple therapy including amoxicillin (which does not induce resistance) and metronidazole (an antibiotic not used in the first trial), and several authors have reported encouraging results with this strategy[37-44]. However, in our experience, when this therapy has been administered twice-daily for one week, eradication rates lower than 50% have been obtained[45]; the subsequent use of higher (three times per day) antibiotic doses was followed only by a mild increase in eradication rate (58%), which was still unacceptable[45]. However, if ranitidine bismuth citrate (RBC) is used instead of PPI, also plus amoxicillin and nitroimidazole, encouraging results have been reported (81% cure rate), although in this protocol antibiotics were administered for 14 d instead of 7 d[46]. In this same study, the readministration of clarithromycin, even when co-prescribed with RBC, was associated with poor eradication rates. In the same way, Nagahara *et al*[47] studied a group of patients who, after failure of first-line PPI-clarithromycin-amoxicillin therapy, had received second-line therapy with the same regimen (for 14 d) or had received PPI-amoxicillin-metronidazole (for 10 d). The eradication rates for second-line therapy with the same regimen (thus readministering clarithromycin) was only 53%, while it was 81% with PPI-amoxicillin-metronidazole. These observations underlie the idea that antibiotics, and specifically clarithromycin, should not be readministered in successive treatments.

Quadruple therapy: Another alternative, the use of a quadruple regimen (i.e. PPI, bismuth, tetracycline and metronidazole), has been generally used as an optimal second-line therapy after PPI-clarithromycin-amoxicillin failure, and has been the recommended rescue regimen in several guidelines[3,48-50]. Several studies have obtained relatively good results with this quadruple regimen, and the results are summarized in Table 1[45,51-71]. Thus, the weighted mean eradication rate with this rescue therapy, calculated from the studies included in the table, is 77%. In this combination regimen, PPI should be prescribed in the usual dose for twice a day, colloidal bismuth subcitrate 120 mg four times per day, tetracycline 500 mg four times per day, and metronidazole is probably best prescribed at high doses (i.e. 500 mg three times per day). The study with the lowest efficacy[57] administered metronidazole at low doses (250 mg four times per day). Limited experience suggests that quadruple therapy may also be effective when the first (failed) regimen included RBC instead of PPI. Thus, Beales *et al*[72] reported that four of the five patients

Table 1 Eradication rates with quadruple therapy (proton pump inhibitor, bismuth, tetracycline and a nitroimidazole) as "rescue" therapy for proton pump inhibitor-clarithromycin-amoxicillin failure

Author	Number of patients	Duration (d)	Eradication rate (%)
Baena Diez *et al*[51]	31	14	90
Bilardi *et al*[52]	46	7	37
Elizalde *et al*[53]	31	7	87
Choung *et al*[54]	56	7	77
Choung *et al*[54]	99	14	88
Chung *et al*[55]	87	7	84
Gasbarrini *et al*[56]	9	7	88
Gisbert *et al*[57]	30	7	57
Gisbert *et al*[45]	9	7	78
Gomollón *et al*[58]	21	7	95
Lee *et al*[59]	20	7	68
Lee *et al*[60]	63	7	75
Marko *et al*[61]	27	7	63
Michopoulos *et al*[62]	38	14	76
Navarro-Jarabo *et al*[63]	54	7	70
Nista *et al*[64]	70	7	63
Nista *et al*[64]	70	14	68
Orsi *et al*[65]	50	12	88
Perri *et al*[66]	45	10	67
Perri *et al*[67]	60	7	83
Sicilia *et al*[68]	21	10	83
Uygun *et al*[69]	100	14	82
Wong *et al*[70]	53	7	91
Wu *et al*[71]	47	7	77

Eradication rates by intention-to-treat analysis when available. *H pylori* eradication rate (weighted mean) with quadruple therapy is 77%.

initially failing RBC-clarithromycin-amoxicillin therapy were successfully treated with quadruple therapy. Seven-day treatment duration seems to be sufficient when quadruple therapy is used after a failed first regimen, as quite similar eradication rates with 7, 10 and 14 d have been reported (mean figures, calculated from Table 1, of 74%, 72% and 81%, respectively). Furthermore, in a recent retrospective study, patients who failed the standard triple therapy (PPI, amoxicillin, clarithromycin) received 1 or 2 wk quadruple therapy, and the eradication rate was similar between the two regimens[54]. These results are in agreement with those reported previously with quadruple therapy as a first-line regimen, where 1-wk therapy appeared sufficient, and prolonging treatment did not increase efficacy[73]. Finally, although PPIs are generally prescribed as the antisecretors in quadruple therapy, some authors have shown, in a randomized study, that omeprazole 20 mg *b.i.d.* and ranitidine 300 mg *b.i.d.* were equally effective as antisecretory agents combined in a second-line quadruple eradication regimen after failure with previous regimens without metronidazole[62]. Nevertheless, these regimens were administered over 14 d and, therefore, it remains to be demonstrated whether the equivalence between both antisecretors-PPIs and H_2-blockers- is also observable with 7 d regimens.

The question may be suggested whether treatment with PPI-clarithromycin-amoxicillin followed by rescue with quadruple therapy if initial failure occurs is preferable to the inverse strategy. To analyze this interesting aspect, Gomollón *et al*[74] randomized consecutive patients to one of two strategies: (1) treatment during 7 d with quadruple therapy, and if failure occurs then second-line treatment with omeprazole-clarithromycin-amoxicillin during 7 d; and (2) initial treatment with omeprazole-clarithromycin-amoxicillin and if failure occurs then treatment with quadruple therapy. Direct and indirect costs were estimated, and a cost-effectiveness analysis using a decision-tree model was undertaken after real clinical data. Eradication was obtained (intention-to-treat) in 73% with the first strategy, *versus* 92% with the second strategy. Furthermore, cost per case eradicated was lower in the second group (320 *versus* 296 euros). However, in a similar but more recent study, Marko *et al*[61] assessed the usefulness and the cost-effectiveness of these two treatment strategies, performing a decision analysis. The effectiveness of "triple first" and "quadruple first" strategies was similar, although the latter seemed slightly more cost-effective.

RBC, tetracycline and metronidazole: More recently, it has been reported that replacing the PPI and the bismuth compound of the quadruple therapy by RBC also achieves good results as a rescue regimen[57,75-80]. RBC is a compound that has, on the one hand, the antisecretory activity of ranitidine, and, on the other hand, the mucosal protective and anti-*H pylori* effects of certain other bismuth salts[81-83]. To date, several studies have evaluated 7-14 d RBC-based second-line regimens after PPI-based triple therapy failures, achieving encouraging results, with eradication rates of 67%[77], 68%[79], 76%[84], 82%[75], 83%[57], 86%[80], and 96%[76]. Furthermore, one randomized study has demonstrated that triple RBC-based therapy, when prescribed to patients with previous PPI-clarithromycin-amoxicillin failure, achieved an even higher efficacy than quadruple therapy, with additional advantages of a lower number of drugs and a simpler dose scheme[57]. Nevertheless, the eradication rate with a quadruple regimen in this last study was remarkably low, which was explained by the low dose of metronidazole prescribed. The favorable results obtained with RBC in the aforementioned studies were explained, at least in part, by the fact that RBC-based therapies may overcome the impact of metronidazole resistant and clarithromycin resistant strains on *H pylori* eradication treatment[18,81-83]. In summary, due to the aforementioned encouraging results, quadruple therapy (as well as RBC-based regimens) may be considered as the preferred regimen after initial treatment failure with PPI-clarithromycin-amoxicillin[3,20,78,85]. However, bismuth salts, including RBC, are no longer available worldwide, and some National Guidelines have been changed accordingly[86].

PPI, amoxicillin and levofloxacin: As previously mentioned, after failure of a combination of a PPI-based triple regimen, the use of the quadruple therapy has been generally recommended as the optimal second-line therapy based on the relatively good results reported

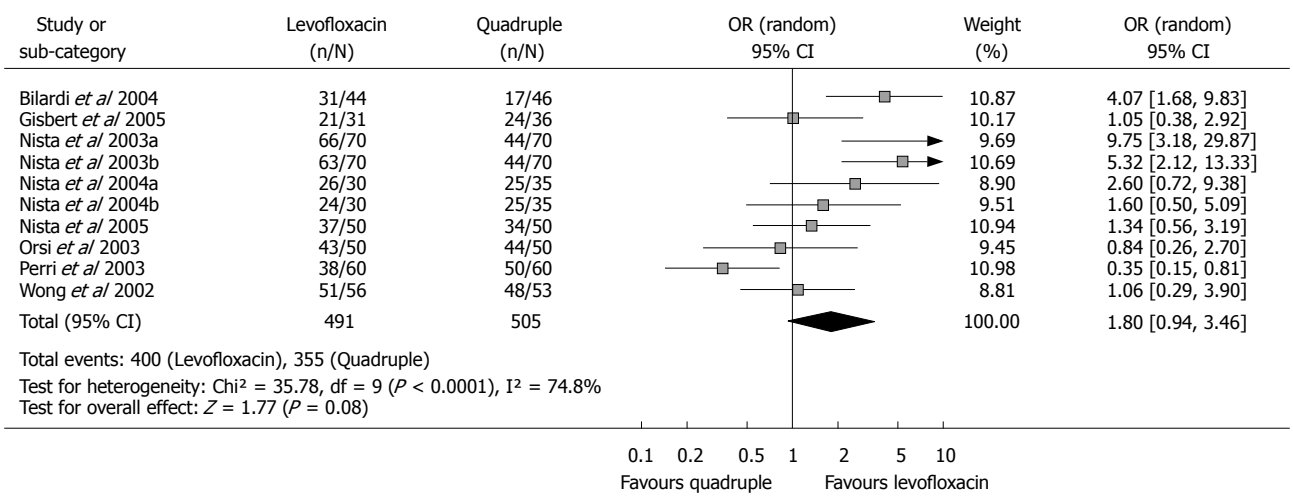

Figure 1 Meta-analysis comparing *H pylori* eradication efficacy with levofloxacin-based triple regimens *versus* quadruple therapy, as second-line "rescue" regimen after failure of a proton pump inhibitor-amoxicillin-clarithromycin.

by several authors[3,20,78,85]. However, this quadruple regimen requires the administration of 4 drugs with a complex scheme (bismuth and tetracycline usually prescribed every 6 h, and metronidazole every 8 h) and is associated with a relatively high incidence of adverse effects[20]. Furthermore, this quadruple regimen still fails to eradicate *H pylori* in approximately 20% to 30% of the patients, and these cases constitute a therapeutic dilemma, as patients who are not cured with two consecutive treatments including clarithromycin and metronidazole will usually have double resistance[20].

Levofloxacin is a fluoroquinolone antibacterial agent with a broad spectrum of activity against Gram-positive and Gram-negative bacteria and atypical respiratory pathogens[87]. Recently, some studies have evaluated the efficacy of new fluoroquinolones, such as levofloxacin, that could prove to be a valid alternative to standard antibiotics, not only as first-line therapies, but more interestingly, as second-line regimens[21,88-90]. In this respect, levofloxacin-based second-line therapies represent an encouraging strategy for eradication failures, as some studies have demonstrated that levofloxacin has, *in vitro*, remarkable activity against *H pylori*[91], and that primary resistances to such an antibiotic are (still) relatively infrequent (when compared with metronidazole or clarithromycin)[92-96]. A recent *in vitro* study also showed a synergistic effect of quinolone antimicrobial agents and PPIs on strains of *H pylori*[97]. Furthermore, it has been shown *in vitro* that levofloxacin retains its activity when *H pylori* strains are resistant to clarithromycin and metronidazole[95,98,99]. These favorable results have been confirmed *in vivo*, indicating that most of the patients with both metronidazole and clarithromycin resistance are cured with the levofloxacin-based regimen[52,94,100].

A combination of a PPI, amoxicillin and levofloxacin, as a first-line regimen, has been associated with favorable results, with mean eradication rates of about 90%[95,101-106]. Subsequently, other authors studied this same regimen in patients with one previous eradication failure, also reporting exciting results, with *H pylori* cure rates ranging from 60% to 94%[52,64,65,67,70,98, 100,106-111]. A recent systematic review showed a mean eradication rate with levofloxacin-based rescue regimens (combined with amoxicillin and a PPI in most studies) of 80%, which represents a relatively high figure when considering that this regimen was evaluated as a rescue therapy[89]. This systematic review found higher *H pylori* cure rates with a 10-d rather than a 7-d regimen, both in general (81% *vs* 73%) and also with the levofloxacin-amoxicillin-PPI combination in particular (80% *vs* 68%), suggesting that the longer (10-d) therapeutic scheme should be chosen.

Furthermore, two recent meta-analyses have suggested that after *H pylori* eradication failure, a levofloxacin-based rescue regimen is more effective than the generally recommended quadruple therapy[88,89]. In one of these meta-analyses[89], higher *H pylori* cure rates with the levofloxacin-based triple regimens than with the quadruple combinations were found (81% *vs* 70%), but with borderline statistical significance (Figure 1). Nevertheless, results were heterogeneous, mainly due to the discordant results of the study by Perri *et al*[67], who reported a cure rate of only 63% with the levofloxacin-regimen, which is the lowest reported in the literature, and is a figure that contrasts with the mean eradication rate of 80% calculated in a systematic review[89]. Nevertheless, when that single outlier study[67] was excluded from the meta-analysis, the difference between cure rates with both regimens reached statistical significance and heterogeneity markedly decreased. Furthermore, when only high-quality studies were considered, the advantage of the levofloxacin regimen over the quadruple regimen increased (88% *vs* 64%), also achieving statistical significance, and heterogeneity among studies almost disappeared[89].

As previously mentioned, the quadruple regimen requires the administration of a complex scheme[20]. On the contrary, levofloxacin-based regimens (with amoxicillin and PPIs administered twice daily, and levofloxacin every 12 or 24 h) represents an encouraging alternative to quadruple therapy, with the advantage of simplicity. Furthermore, the quadruple regimen is associated with a relatively high incidence of adverse

effects[20]. In contrast, levofloxacin is generally well tolerated, and most adverse events associated with its use are mild to moderate in severity and transient[87]. The most frequent adverse effects affect the gastrointestinal tract[87]. Occasional cases of tendinitis and tendon rupture have been reported in the literature with levofloxacin therapy[52,87]. However, data derived from more than 15 million prescriptions in the US indicated the rate is fewer than 4 per million prescriptions[112]. In the aforementioned systematic review[89], adverse effects were reported, overall, by 18% of the patients treated with levofloxacin-based therapies, and these adverse effects were severe (defined so by the authors or explaining treatment discontinuation) in only 3% of the cases. Furthermore, the incidence of adverse effects was not different when levofloxacin-amoxicillin-PPI was administered for 7 d or 10 d, supporting the aforementioned recommendation of prescribing the more effective 10-d regimen. Moreover, two meta-analyses have demonstrated a lower incidence of adverse effects with levofloxacin-based treatments than with the quadruple combinations[88,89].

Unfortunately, it has been shown that resistance to quinolones is easily acquired, and in countries with a high consumption of these drugs, the resistance rate is increasing and is already relatively high[94,103,107,113-123]. More importantly, it has been demonstrated that the presence of levofloxacin resistance significantly reduce the eradication rate following a therapy with this antibiotic[94,103,121,124]. Therefore, it has been suggested to reserve levofloxacin for rescue treatment to avoid the increase of the resistance phenomenon.

Rescue regimen after PPI-amoxicillin-nitroimidazole failure

After PPI-amoxicillin-nitroimidazole failure, retreatment with PPI-amoxicillin-clarithromycin has proved to be very effective, and it seems to be a logical strategy, as while amoxicillin is maintained (which does not induce resistance), clarithromycin is substituted for metronidazole. Furthermore, the absence of cross-resistance among nitroimidazoles and clarithromycin favors this position. With this therapy, some authors[45] have achieved *H pylori* eradication in 85% of cases, while others have reported success rates of 86%[125] or even 100%[126]. In favor of this strategy is the study by Magaret *et al*[127], who studied a group of 48 patients after failure of previous *H pylori* therapy with a metronidazole-containing regimen, and randomized them to either lansoprazole, amoxicillin and clarithromycin twice daily for 14 d, which is the logical approach with triple therapy not repeating metronidazole, or to lansoprazole, bismuth, metronidazole and tetracycline for 14 d, which is the quadruple therapy repeating metronidazole. Intention-to-treat efficacies were 75% for a triple regimen and 71% for a quadruple. Although this difference did not reach statistical significance, the small sample size of this study does not preclude the possibility of a small but clinically significant difference in efficacy between the regimens. Finally, preliminary studies have suggested that RBC may be used instead of PPI in this triple second-line strategy (i.e., RBC-clarithromycin-amoxicillin), with similar or even better results[72].

Rescue regimen after PPI-clarithromycin-nitroimidazole failure

As previously mentioned, acquired bacterial resistance to metronidazole or clarithromycin results primarily from the previous treatment failure[26,32], and therefore the first choice probably should not be a regimen that combines these two antibiotics in the same regimen[23,24,128]. Although this regimen is very effective[4], patients who are not cured will probably have double resistance[26,129], and no logical empirical treatment remains afterwards (although, more recently, the levofloxacin-based regimens may represent an option). Thus, some authors have demonstrated that initial regimens containing both clarithromycin and nitroimidazole are associated with significantly worse results overall, with lower eradication rates after logically chosen second-line therapy and sensitivity-directed third-line therapy; these poor results were due to the emergence of multiple resistant strains as evidenced by the results of culture testing after the second failed course[72]. In summary, due to problems with resistance it could be suggested that both key antibiotics-clarithromycin and metronidazole- should not be used together until a valid empirical back up regimen is available[23].

Nevertheless, if culture is not performed after failure of PPI-clarithromycin-metronidazole, and hence antibiotic susceptibility is unknown, several rescue options may be suggested. Firstly, omeprazole plus amoxicillin, with a high dose of both the antibiotic and the antisecretor, could, in theory, be recommended[128,130]; however, we must remember that this "old-fashioned" dual combination has achieved disappointing results in many countries. Therefore, a second antibiotic should be added, and at this point a difficult decision appears, as both antibiotics used in the first trial (clarithromycin and metronidazole) are capable of inducing secondary resistance to *H pylori*, playing a negative role in future efficacy[25-29,131]. Nevertheless, the following possibilities exist:

Readministering metronidazole: Due to the fact that metronidazole resistance is frequent and clinically relevant[25-28], if this antibiotic is readministered, it should be used within a bismuth-based quadruple regimen (thus PPI might reduce the negative effect of metronidazole resistance[28,58,132,133]). With this regimen, eradication rates up to 80% have been achieved[45]. RBC, which may overcome the impact of resistance to metronidazole[81], may also play a role in this regimen. Thus, some authors have reported an 88% cure rate with a 2-wk regimen or RBC-tetracycline-tinidazole in patients who had previously failed a clarithromycin-tinidazole based triple therapy[76].

Readministering clarithromycin: Several studies have underlined the relevance of clarithromycin

resistance[25-27,29], and advise against readministering this antibiotic. Therefore, a further option which has been proposed, is to add (for example to PPI-amoxicillin-clarithromycin) a fourth medication (such as bismuth) with a bactericidal effect against *H pylori*, with which a 70% eradication rate has been achieved[45].

Readministering no antibiotic: A final alternative, obviously, consists of no readministering of either metronidazole or clarithromycin. Although only published in abstract form, one study has prescribed RBC, tetracycline and amoxicillin for 2 wk and has reported eradication in 89% of the cases which had previously failed PPI, clarithromycin and tinidazole[134]. These encouraging results may be due, at least in part, to the use of RBC instead of bismuth in this regimen, as "classic" triple therapy with bismuth, tetracycline and amoxicillin have been previously considered relatively ineffective. Finally, although not specifically evaluated in PPI-clarithromycin-metronidazole failures, rifabutin or levofloxacin-based regimens (e.g. PPI, amoxicillin and either levofloxacin or rifabutin) could play a role in this difficult situation.

IS IT NECESSARY TO PERFORM CULTURE AFTER FAILURE OF THE SECOND ERADICATION TREATMENT?

As previously mentioned, it has been generally recommended that performing culture after a first eradication failure is not necessary, and therefore assessing *H pylori* sensitivity to antibiotics only after failure of the second treatment may be suggested in clinical practice[3,23,48,135]. However, the utility of the culture (with consequent antibiotic susceptibility testing) and the moment when it must be performed after eradication failure are both controversial[21]. It is evident that, as pretreatment, antibiotic resistance is the most important factor in nonresponse to initial treatment[25-29], and knowledge of the organism's antibiotic susceptibility may represent an aid in selecting the therapy regimen. However, performing culture systematically after the second eradication failure also has some limitations, which are summarized as follows:

(1) Culture implies, obviously, the performance of endoscopic exploration, which has several disadvantages: it is not free of risk, and, since endoscopy centers have been meeting increasing demand, culture usually involves prolonged waiting times.

(2) *H pylori* culture is expensive, due to the cost of the procedure itself, but mainly the costs of the associated endoscopy, which is necessary to obtain biopsy samples.

(3) Culture is time-consuming, as *H pylori* is a rather "fastidious" bacterium at culture, especially when a low bacterial load is present, as generally occurs after eradication failure[22].

(4) Culture is not always available on a routine basis.

(5) The sensitivity of bacterial culture is not 100%, and therefore the antimicrobial susceptibility cannot be obtained in all cases[136]. Indeed, even in the optimal conditions usually encountered in therapeutic trials- when both gastroenterologist and microbiologist are thoroughly motivated- a culture sensitivity of "only" approximately 90% has been achieved in patients not previously treated[22]. Furthermore, in several studies enrolling patients who had failed one or more eradication treatments, the bacterium was isolated in less than 80% of cases[22]. Therefore, an even lower probability of isolating the bacterium is to be expected in routine clinical practice. This indicates that, even in the hands of experts, antimicrobial sensitivity would not be obtained in several eradication failure patients, who had undergone an upper endoscopy solely for bacterial culture[22].

(6) Antibiotic susceptibility testing in clinical practice yields useful information only regarding a few antibiotics. Antibiotics effective and generally used against *H pylori* are mainly the following four: amoxicillin, clarithromycin, metronidazole, and tetracycline. Resistance to amoxicillin has been estimated to be less than 1% in most studies[18,22]. Hence, its role in clinical practice may even be marginalized. Similarly, resistance to tetracycline is also very low, or even absent, in most countries[18,22]. Therefore, it may even be assumed that antibiotic susceptibility testing in clinical practice yields useful information only regarding the latter two antibiotics, namely clarithromycin and metronidazole[22].

(7) *In vitro* antibiotic susceptibility does not necessarily lead to eradication *in vivo*. Even knowing the susceptibility of *H pylori*, eradication rates do not achieve 100%, as the results observed *in vivo* by following *in vitro* susceptibility to anti-*H pylori* antibiotics are often disappointing[137]. Some discrepancies between antibiotic susceptibility and *H pylori* eradication may occur, due for example, to the possibility of co-infection with different *H pylori* strains[138]. Thus, a variable proportion of non-eradicated patients is made of subjects who harbor strains sensitive to the administered drugs, and in these patients the reasons for treatment failure are unclear[139]. For example, Gomollón *et al*[140] reported how third-line treatment often (in 50% of the cases) failed to eradicate *H pylori* infection, in spite of giving a 14-d, full-dose, quadruple culture-guided combination, showing that *in vitro* susceptibility did not predict eradication success. In the same way, Vicente *et al*[141] determined the effectiveness of a third, culture-guided, treatment of *H pylori* infection after two unsuccessful attempts. Patients received a two-week quadruple culture-guided therapy, and overall eradication was achieved in only 60% of the patients. In fact, paradoxically, the lowest eradication rate was obtained in patients with *H pylori* strains sensitive to all antibiotics. In summary, it seems that despite the use of culture-guided combinations of drugs, a third treatment is frequently unsuccessful, indicating that other factors, different from *in vitro* antibiotic susceptibility, influence eradication rates. On the other hand, the reverse situation is also possible, as *H pylori* eradication may, nonetheless, be achieved in the presence of metronidazole- or clarithromycin-

resistant strains, even with a drug combination including these antibiotics. Therefore, *in vitro* resistance to either clarithromycin or metronidazole could be overcome *in vivo* in a significant proportion of patients by prescribing the same antibiotics[22].

(8) When a repeat (rescue) therapy must be selected, we have several data that will aid us in suspecting resistance to a particular antibiotic, without the necessity of a culture, based on the observation that acquired bacterial resistance to metronidazole or clarithromycin results primarily from previous treatment failure[26,32]. Thus, when a therapy with clarithromycin fails, resistance to this antibiotic appears in most cases, and the same is true when a nitroimidazole is the antibiotic first used[18,29,131,142]. Even if resistance to these antibiotics does not appear, it remains uncertain whether their readministration is adequate, as they were not efficacious (for unknown reasons) for the first time. Some studies suggest that retreatment of *H pylori* infection with the same combination is still a choice when the status of bacterial resistance to antibiotics is unknown, however, full doses and a longer treatment duration must be used and a poor eradication rate has usually been reported[143]. Therefore, the position in the case of therapy failure would be clear: do not readminister any of the antibiotics against which *H pylori* has probably become resistant[1,49].

(9) Finally, relatively high eradication rates have been obtained with *empirical* third-line treatment after two consecutive failures in several studies[76,144-156].

However, limited experience suggests that endoscopy with culture and susceptibility testing may be appropriate after failure of two eradication therapies; in this situation, a non-randomized retrospective study suggests that third-line therapy directed by the results of sensitivity testing improve eradication compared to further empirical antibiotics, demonstrating that the success rate of sensitivity-directed therapy is superior to PPI-amoxicillin-rifabutin triple therapy, and therefore suggesting that endoscopy and sensitivity testing at this point may be worthwhile rather than more widespread use of rifabutin-based regimens[72]. Cammarota *et al*[122] assessed the efficacy of a third-line, culture-guided treatment approach for the eradication of *H pylori*. After the first two eradication attempts, all patients were resistant to metronidazole, and 95% were resistant to clarithromycin. Consequently, most patients (89 out of 94) received a quadruple regimen including PPI, bismuth, tetracycline and amoxicillin, and *H pylori* eradication was obtained in 90% of the cases. Although the authors concluded that a culture-guided, third-line therapeutic approach is effective for the eradication of *H pylori*, it would seem more appropriate to conclude, in fact, that the tetracycline- and amoxicillin-based quadruple regimen may be a good *empirical* third-line rescue treatment option (as to choose such a regimen, which implies not readministering metronidazole or clarithromycin, it would not be necessary to know antibiotic susceptibilities).

In summary, when critically reviewing the role of culture in the management of *H pylori* infection in clinical practice it may be concluded, in coincidence with other authors, that *H pylori* culture is an invasive, time-consuming method, offering quite low sensitivity, requiring significant cost, and which, in practice, tests very few antibiotics, with a questionable contribution to the management of non-responder patients[22,157]. Obviously, the importance of *H pylori* culture remains unaltered both in epidemiological and pharmacological research fields. However, whether patients should undergo an upper endoscopy for bacterial culture after second-line therapy failure remains a debatable matter, and the role of culture in clinical practice requires a critical reappraisal[22,157]. As it has been brilliantly expressed by Zullo *et al*, regrettably, gastroenterologists need to accept that gastric biopsy culture is not as simple as filling a sample bottle![22]

Nevertheless, it is recommended that those prescribing *H pylori* eradication therapies continually assess their success rate and adjust the relevant local practices and policies in line with the results and local bacterial resistance patterns. Thus, it would be recommendable that culture should be routinely performed after eradication failure in some specialized centers with special interest in *H pylori* research and treatment, with the intention to study the incidence of resistances after failures and also to evaluate the influence of such resistances on the efficacy of rescue regimens[158]. Data coming from this experience on *H pylori* resistance will be used as a reference for the corresponding population. This preventive approach has been recommended to avoid an increase in refractory *H pylori* infection in the future[158].

EMPIRICAL THIRD-LINE *H PYLORI* RESCUE THERAPY AFTER FAILURE OF TWO ERADICATION TREATMENTS

If it is decided, finally, not to perform culture before the administration of a third-line rescue treatment after failure of the first two trials (generally including clarithromycin and metronidazole), different possibilities for *empirical* treatment may be suggested. As eradication regimens may be less efficacious for retreatment, as compared to their efficacy when used as primary treatment, it may be suggested that the course of the rescue therapy should be extended to 10-14 d, at least when rescue therapy fails and third-line regimens are therefore prescribed[159]. As several studies have underlined the relevance of metronidazole[25-28] and clarithromycin[25-27,29] resistance, these two antibiotics should not be readministered, and several regimens have been evaluated in this scenario.

Amoxicillin ± tetracycline-based regimens

In a recent study, patients with at least one treatment failure who were infected with *H pylori* resistant to both metronidazole and clarithromycin, were treated with high doses of omeprazole (4 × 40 mg) and amoxicillin (4 × 750 mg) for 14 d, and the infection was cured

Table 2 Rifabutin-based "rescue" therapies (rifabutin-amoxicillin-proton pump inhibitor) in patients with previously failed eradication treatments and/or resistance to clarithromycin and nitroimidazoles

Author	Number of patients	Drugs and doses	Duration of treatment (d)	Eradication rate (%)
Beales *et al*[72]	10	Rifabutin 300 mg *o.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	14	60
Bock *et al*[152]	25	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Lansoprazole 30 mg *b.i.d.*	7	72
Borody *et al*[169]	67	Rifabutin 150 mg *b.i.d.* Amoxicillin 1-1.5 g *t.i.d.* Pantoprazole 60 mg *t.i.d*	12	90
Canducci *et al*[153]	10	Rifabutin 300 mg *o.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	10	70
Gisbert *et al*[149]	14	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	14	79
Gisbert *et al*[155]	20	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	10	45
Gonzalez Carro[170]	92	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Pantoprazole 40 mg *b.i.d*	10	61
Miehlke *et al*[130]	73	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Esomeprazole 20 mg *b.i.d.*	7	74
Navarro-Jarabo *et al*[63]	45	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Omeprazole 20 mg *b.i.d.*	7	44
Perri *et al*[151]	41	Rifabutin 300 mg *o.d.* Amoxicillin 1 g *b.i.d.* Pantoprazole 40 mg *b.i.d.*	7	71
Toracchio *et al*[171]	65	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* Pantoprazole 40 mg *b.i.d.*	10	78
Van der Poorten *et al*[172]	44	Rifabutin 150 mg *b.i.d.* Amoxicillin 1 g *b.i.d.* PPI *b.i.d.*	10	68

PPI: Proton pump inhibitor (omeprazole, pantoprazole, rabeprazole or esomeprazole) at the usual dose. Eradication rates by intention-to-treat analysis when available. *H pylori* eradication rate (weighted mean) with rifabutin-based "rescue" therapy is 69%.

in 76% of the cases[160]. This study suggests that, although the "old-fashioned" dual combination of omeprazole plus amoxicillin is generally considered quite ineffective as a first-line regimen, it may be associated with relatively good results if prescribed at high doses, even for *H pylori* resistant to both metronidazole and clarithromycin, in patients who experienced previous treatment failures. Another possibility to avoid retreatment with clarithromycin or metronidazole is to prescribe a quadruple combination of PPI, bismuth, tetracycline and amoxicillin (instead of metronidazole), which has been used by some authors with favorable results[161]. Nevertheless, this regimen has been tested only as second-line (and not third-line) therapy, and only after failure of PPI-clarithromycin-amoxicillin (and not after metronidazole-based therapy), emphasizing that the experience should be extended to patients with two previous eradication failures containing both clarithromycin and metronidazole. Finally, as previously mentioned, one study prescribed RBC, tetracycline and amoxicillin for 2 wk and achieved eradication in 89% of the cases which had previously failed PPI, clarithromycin and tinidazole[134].

Levofloxacin-based rescue regimens

It has been suggested that levofloxacin-based therapies may also represent an alternative when two (or more) consecutive eradication treatments have failed to eradicate the infection[52,94,118,147,154,155,162-164]. As an example, a recent study by Zullo *et al*[147] aimed to evaluate the efficacy of a levofloxacin-amoxicillin-PPI combination in patients who previously had failed two or more therapeutic attempts, and they found the eradication rate was 83% (intention-to-treat analysis). More recently, Gisbert *et al*[155] evaluated, in a multicenter study including 100 patients, the efficacy of a third-line levofloxacin-based regimen in patients with two consecutive *H pylori* eradication failures. An intention-to-treat eradication rate was 66%, which represents a relatively high figure when considering that this regimen was evaluated as a third-line therapy. Other alternative rescue therapies, different from levofloxacin-based regimens, have been suggested. Rifabutin-based rescue therapy, as will be reviewed in the following section, also constitutes a possible strategy after previous eradication failures, although it has been recently shown that a 10 d triple levofloxacin-based regimen is more effective than the same combination with rifabutin as a rescue regimen[155]. In summary, levofloxacin-based rescue therapy constitutes an encouraging empirical third-line strategy after multiple previous *H pylori* eradication failures with key antibiotics (such as amoxicillin, clarithromycin, metronidazole and tetracycline).

Rifabutin-based rescue regimens

As previously mentioned, the evaluation of drugs without cross-resistance to nitroimidazole or macrolides as components of retreatment combination therapies seem to be worthwhile. *H pylori* has been proved to be highly susceptible *in vitro* to rifabutin, a rifamycin derivate of the established tuberculostatic drug[165-167]. Moreover, rifabutin is chemically stable at a wide pH range and its antibacterial activity is likely not to be hampered by the acidic environment of the stomach[168]. Furthermore, selection of resistant *H pylori* strains has been low in experimental conditions. Thus, until now, no rifabutin resistant strain has been isolated from patients who were either treated or untreated for *H pylori* infection[166].

As summarized Table 2, rifabutin-based rescue therapy constitutes an encouraging strategy after multiple previous eradication failures[63,72,130,149,151-153,155,169-172]. As an example, Perri *et al*[151,173] used a 1-wk regimen of PPI, amoxicillin and rifabutin in patients who were still *H pylori* infected after two or more courses of PPI-based triple therapies, and achieved an eradication rate of 71% by intention-to-treat analysis. Gisbert *et al*[149], in a prospective multicenter study, included patients in whom a first eradication trial with PPI, clarithromycin and amoxicil-

lin and a second trial with PPI, bismuth, tetracycline and metronidazole had failed. A third 14 d eradication regimen with rifabutin, amoxicillin and a PPI was effective in 79% of the patients (intention-to-treat analysis). However, these encouraging results were not confirmed in a more recent study by these same authors[155]. In the largest study on rifabutin[170], 92 consecutive patients diagnosed with *H pylori* infection resistant to two previous treatment regimens were treated with a PPI, rifabutin and amoxicillin for 10 d and the intention-to-treat eradication rate was 61%. In summary, the weighted mean eradication rate with rifabutin-based rescue therapy, calculated from the studies included in the Table 2, is 69%.

These findings suggest that new rifabutin-based combinations are effective for *H pylori* strains resistant to antibiotics, and specifically to clarithromycin or metronidazole[174]. Furthermore, rifabutin-based therapies have been compared with the widely used "classic" quadruple therapy. Perri *et al*[66] performed a randomized study where three groups of patients were treated for 10 d with pantoprazole, amoxicillin, and rifabutin 150 mg *o.d.*, or 300 mg *o.d.*, and quadruple therapy. On intention-to-treat analysis, eradication rates were 67% in the rifabutin 150 mg and quadruple groups, and higher (87%) in the rifabutin 300 mg group. Finally, in this comparative study, side-effects were less frequent in rifabutin-treated patients than in those on quadruple therapy[66].

Several concerns still remain, however, regarding rifabutin treatment. Firstly, this drug is very expensive. Secondly, severe leucopoenia and trombocytopenia have been reported in one patient treated with rifabutin, with myelotoxicity demonstrated by bone marrow aspirate[153]. Although blood cell count returned to normal at day 15 after discontinuation of therapy, physicians should be aware of the risk of major side-effects arising during a rifabutin-based regimen[149,155]. Finally, there is some concern about wide-spread use of rifabutin, a member of a class of established antimycobacterial drugs, in patients with *H pylori* infection. Because multiresistant strains of *Mycobacterium tuberculosis* increase in numbers, indications for these drugs should be chosen very carefully to avoid further acceleration of development of resistance[152]. At present, therefore, rifabutin should be considered only as the last option (e.g. restricted to infected patients even after several eradication regimens including, among them, levofloxacin).

Furazolidone-based rescue regimens

Furazolidone is an antimicrobial drug that belongs to synthetic nitrofurans and is active against a broad spectrum of gram-negative and gram-positive bacteria and protozoa. This antibiotic has demonstrated a high antimicrobial activity against *H pylori* if given as a single drug[175], and the majority of first-line furazolidone-based combination therapies revealed eradication rates above 80%[92]. Primary resistance to furazolidone is virtually absent[158,176,177], and its potential to develop resistance is as low as for bismuth compounds or amoxicillin[178]. Moreover, this drug has no cross-resistance potential to metronidazole[176]. Triple therapy in which furazolidone is used instead of metronidazole achieves high eradication rates, even in populations with a high prevalence of nitroimidazole resistance[179-182]. In this respect, a recent study has evaluated furazolidone-based triple therapy (combined with bismuth and tetracycline) in the eradication of *H pylori* resistant to metronidazole, with favorable results (86% eradication rate)[183]. A few years ago, some authors tested a quadruple combination of furazolidone, bismuth, tetracycline and PPI as a second-line eradication therapy, and reported encouraging results[184]. More recently, Treiber *et al*[145] investigated whether this quadruple regimen containing furazolidone could be effective as a third-line therapy in patients with *H pylori* treatment failure after first-line (clarithromycin-metronidazole ± amoxicillin) and second-line (PPI-bismuth-tetracycline-metronidazole) regimens, and *H pylori* infection was cured in up to 90% of the cases. Furthermore, a 7 d triple-regimen comprising furazolidone, amoxicillin and a PPI achieved an eradication rate of 60% in 10 patients who failed first-line, second-line and even rifabutin-based triple therapy[185].

A recent systematic review and meta-analysis of the effect of furazolidone- and nitrofurantoin-based regimens in the eradication of *H pylori* infection has been performed[186]. The pooled eradication rate of primary PPI-based regimens containing furazolidone was 76%. Second-line schedules containing furazolidone obtained eradication rates of 76%. Finally, third-line rescue therapies were effective in 65% of the cases. In summary, a quadruple regimen including furazolidone, bismuth, tetracycline and PPI seems to represent a promising alternative after two consecutive failures with regimens including both metronidazole and clarithromycin.

CUMULATIVE ERADICATION RATES WITH THREE (OR MORE) CONSECUTIVE ERADICATION TREATMENTS

In patients with conditions where the indication for *H pylori* eradication is definitively accepted, as is the case of peptic ulcer disease (or gastric MALT lymphoma), rescue treatment after first-line failure is clearly advisable. Furthermore, if the second therapy fails, a third or even a fourth regimen should be prescribed, as infected patients continue to have high risk of ulcer recurrence and ulcer complications and are in an obviously disadvantageous situation in view of the enormous benefits that follow *H pylori* eradication in peptic ulcer disease: increased ulcer healing, less ulcer recurrence, and less ulcer bleeding. However, multiple repeated antibiotic treatment of patients where benefits of *H pylori* eradication has not been so clearly established, such as those with functional dyspepsia[174,187], may not be completely justified.

Some authors have evaluated, in the same study,

different regimens after failure of two eradication treatments, which provide interesting information about cumulative, and not only absolute, eradication rates[21]. For example, in the study by Gasbarrini *et al*[188], a total of 2606 patients were administered a PPI, tinidazole and clarithromycin for 1 wk. Patients with continuing infection were then given a second 1-wk course of amoxicillin, clarithromycin and RBC. Finally, patients still infected after the second course underwent upper gastrointestinal endoscopy with *H pylori* culture, and then received a 1-wk quadruple scheme established on antibiotic sensitivity. Eradication rates after the first, second and third treatment, were, respectively, 79%, 77%, and 52%. This algorithm led to overall per-protocol eradication rates of 99%. Chan *et al*[146] prescribed quadruple therapy to a group of patients who had failed to respond to RBC-based regimens (as first regimen) and PPI-clarithromycin-amoxicillin combination (as second regimen), and achieved successful eradication in 83% of the cases receiving a quadruple regimen, finally achieving a 99% cumulative eradication rate. Beales *et al*[72] evaluated 469 patients receiving eradication therapy in routine clinical practice. Second-line therapy was chosen empirically, using whichever of clarithromycin or metronidazole was not used initially. All patients requiring third-line therapy underwent endoscopy, choice of therapy being guided by sensitivities. Overall success after one, two and three courses of therapy were 73%, 94% and 98%, respectively. Zullo *et al*[76] reported 83% cure rate in patients who had previously failed two courses of clarithromycin-amoxicillin and clarithromycin-tinidazole based triple therapies. Gomollón *et al*[140] studied the effectiveness of third-line treatment of *H pylori* infection with two-week quadruple, culture-guided regimens. The combination of omeprazole, tetracycline, bismuth and clarithromycin showed an eradication rate of only 36%, but if amoxicillin was used the rate was 67%. In the study by Vicente *et al*[141], after two unsuccessful attempts at eradication, all patients underwent endoscopy and culture, and patients received a quadruple culture-guided therapy. Cumulative *H pylori* eradication rate with this strategy was as high as 99.6%. Treiber *et al*[145] investigated whether a quadruple regimen containing furazolidone could be effective as a third-line therapy in patients with two previous *H pylori* treatment failures. Cure of *H pylori* was achieved in 90% of the patients nonresponsive to a second eradication trial, which gave a final eradication rate of 99%. In the study by Qasim *et al*[185], 3280 patients received standard first-line eradication therapy, which was successful in 77% of the cases. Second-line therapy (bismuth-based quadruple) or triple therapy (altering constituent antibiotics) was successful in 56% of treated patients. Subsequent eradication attempts using rifabutin-based regimen was successful in 38% of patients, giving a cumulative eradication rate of 94%. Gisbert *et al*[150] included consecutive patients in whom two eradication regimens had failed to eradicate *H pylori*, prescribed empirical third-line rescue regimens, and achieved *H pylori* eradication in 71% of the cases (intention-to-treat analysis). Based on these results, with estimated efficacy of 85%, 75% and 71%, respectively with first, second and third regimens, *H pylori* eradication could finally be achieved in 99% of the patients. Finally, Gisbert *et al*[156] evaluated the efficacy of different rescue therapies empirically prescribed during 10 years to 500 patients in whom at least one eradication regimen had failed to cure *H pylori* infection. Antibiotic susceptibility was unknown (therefore rescue regimens were chosen empirically). Overall, *H pylori* cure rates with the second and third-line rescue regimens were 70% and 74%, giving a cumulative eradication rate as high as 98%.

Therefore, a wider perspective of the benefits of retreating *H pylori* infection can be obtained if cumulative eradication rates with successive treatments are taken into account. Thus, as represented in Figure 2, it can be concluded that *H pylori* eradication can finally be achieved in almost 100% of the patients if three rescue therapies are consecutively given[72,76,140,141,144-146,150,156,185,188].

Furthermore, these encouraging (cumulative) results have been obtained when more than three consecutive treatments have been prescribed[21]. As an example, Seppälä *et al*[144] reported a cumulative eradication rate of 93% (intention-to-treat analysis) and even 100% (per-protocol analysis) after four empirical retreatments. We have recently confirmed that a levofloxacin-based regimen can also be administered with good results after three previous eradication failures with antibiotics, such as amoxicillin, clarithromycin, metronidazole, tetracycline, and even rifabutin[163]. Thus, we prospectively evaluated 10 patients with three consecutive *H pylori* eradication failures (1st treatment with PPI-clarithromycin-amoxicillin, 2nd treatment with RBC-tetracycline-metronidazole, and 3rd treatment with PPI-amoxicillin-rifabutin). A fourth eradication regimen with 10 d levofloxacin, amoxicillin and PPI was prescribed, and intention-to-treat eradication rates were 70%. When we reviewed our experience with different rescue therapies empirically prescribed during 10 years to 500 patients, the cumulative *H pylori* eradication rate with 4 successive treatments was 99.5%[156].

Finally, reports of "ineradicable" *H pylori* infection after more than four eradicating treatments failed have been recently published. Dore *et al*[148] prescribed a quadruple combination of PPI, bismuth, tetracycline, and metronidazole to patients who had failed two or more treatment courses of *H pylori* eradication therapy (33 patients had failed prior treatment twice, 19 had failed three times, and 16 had failed four or more times); despite this *a priori* difficult task, *H pylori* eradication was finally achieved in 93% of the patients. Tucci *et al*[189] reported their experience of 13 patients with at least 5 eradication failures and *H pylori* strains resistant to both clarithromycin and nitroimidazoles. The treatment was organized into three sequential schedules employing partially different drug combinations (to face the various resistant strains), suspension formulations were preferred to tablets (to improve the dispersal of the drugs into the stomach), antibiotics were administered after meals and

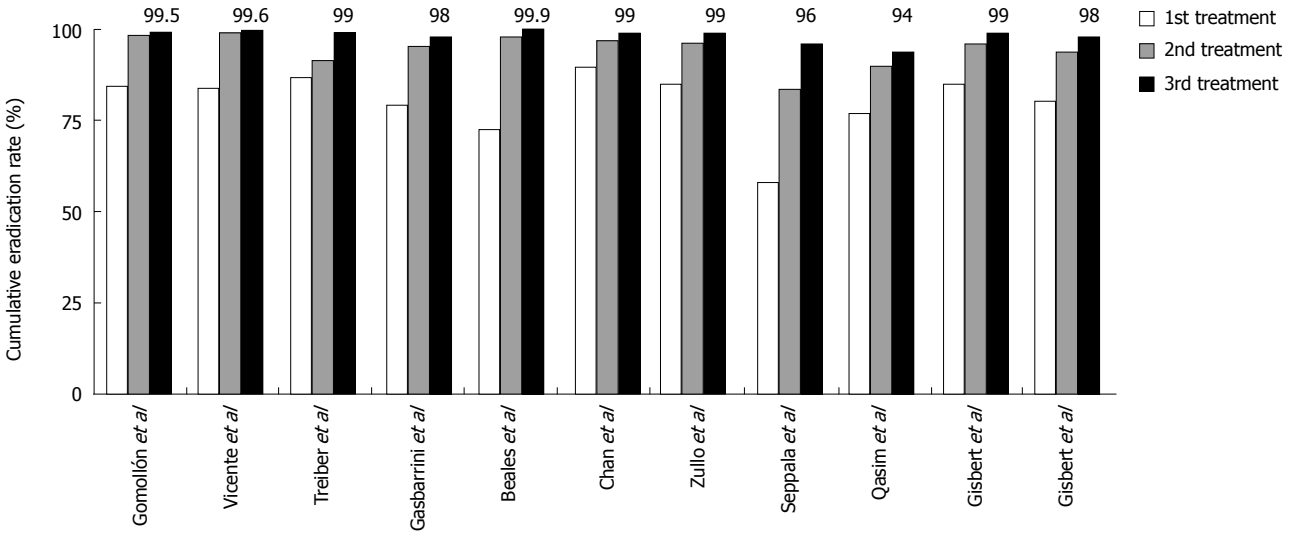

Figure 2 Cumulative *H pylori* eradication rates with three consecutive eradication treatments.

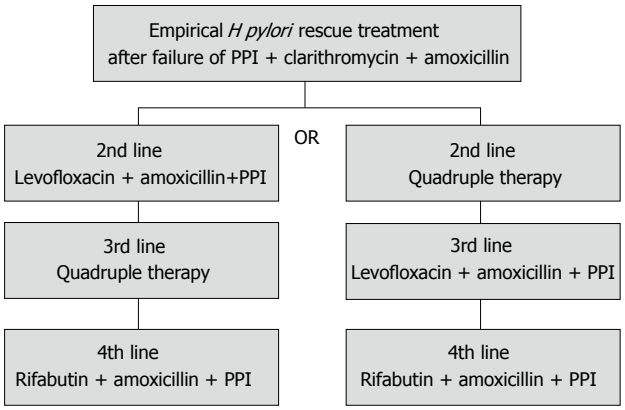

Figure 3 Choice of a empirical retreatment regimen, without culture and antimicrobial sensitivity testing, after failure of proton pump inhibitor (PPI), amoxicillin and clarithromycin combination. Quadruple therapy: Combination of PPI, bismuth, tetracycline and nitroimidazole (metronidazole or tinidazole).

a variation on a standard diet exceeding the normal fat composition was given (to increase the time of contact of the antimicrobials with gastric mucosa), and patients were invited to lie down after the meals, changing their position every 5 min (to facilitate the penetration of drugs amid the anfractuosities of fundic mucosa). With this particular therapy, eradication was successful in 70% of the patients. In another example of "ineradicable" *H pylori* infection, levofloxacin-amoxicillin combination was successfully employed in a patient with a clarithromycin- and metronidazole-resistant strain, who previously failed eight consecutive therapeutic attempts[162].

CONCLUSION

Even with the current most effective treatment regimens, ⩾ 20% of patients will fail to eradicate *H pylori* infection. This issue seems important at the present time, as therapy for *H pylori* infection is becoming more and more frequently prescribed. Currently, apart from having to know first-line eradication regimens well, we must also be prepared to face treatment failures. Therefore, in designing a treatment strategy we should not focus on the results of primary therapy alone, but also on the final (overall) eradication rate.

The choice of a rescue treatment depends on which treatment is used initially. If a first-line clarithromycin-based regimen was used, a second-line metronidazole-based treatment (such as the quadruple therapy) may be used afterwards, and then a levofloxacin-based combination would be a third-line rescue option. Alternatively, it has recently been suggested that levofloxacin-based rescue therapy constitutes an encouraging second-line strategy, representing an alternative to quadruple therapy in patients with previous PPI-clarithromycin-amoxicillin failure, with the advantage of efficacy, simplicity and safety. In this case, quadruple regimen may be reserved as a third-line rescue option. Finally, rifabutin-based rescue therapy constitutes an encouraging empirical fourth-line strategy after multiple previous eradication failures with key antibiotics such as amoxicillin, clarithromycin, metronidazole, tetracycline, and levofloxacin (Figure 3).

Even after two consecutive failures, several studies have demonstrated that *H pylori* eradication can finally be achieved in almost all patients if several rescue therapies are consecutively given. As a final conclusion, therefore, the attitude in *H pylori* eradication therapy failure, even after two or more unsuccessful attempts, should be to fight and not to surrender[190].

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BASIC RESEARCH

Anti-tumor activity of erlotinib in the BxPC-3 pancreatic cancer cell line

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Abstract

AIM: To investigate the effect and mechanism of action of erlotinib, an epidermal growth factor receptor (EGFR) small molecule tyrosine kinase inhibitor (TKI), in the human pancreatic cancer cell line BxPC-3 both *in vitro* and *in vivo*.

METHODS: *In vitro*, human pancreatic cancer cell line BxPC-3 was exposed to varying concentrations of erlotinib, and its effects on proliferation, cell cycle distribution, apoptosis and the expression of pro- and antiapoptotic factors such as bcl-2, bcl-xl, bax and bak, and the expression of vascular endothelial cell growth factor (VEGF) were measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, flow cytometric analysis, terminal deoxynucleotidyl transferase-mediated nick end labeling assay (TUNEL), and reverse transcription-polymerase chain reaction (RT-PCR). Potential effect of erlotinib on angiogenesis was examined by tube formation assay. Tumor growth suppression was observed in xenografted nude mice with pancreatic cancer *in vivo*. Immunohistochemical (IHC) staining for EGFR and factor Ⅷ-related antigen was undertaken to detect the microvessel density and VEGF expression in tumor tissue in xenograft nude mice.

RESULTS: Erlotinib, as a single agent, repressed BxPC-3 cell growth in a dose-dependent manner, triggered G_1 arrest and induced cell apoptosis, and suppressed capillary formation of endothelium *in vitro*. Expressions of VEGF were significantly down-regulated at a high concentration of 200 μmol/L, however, the expressions of bcl-2 and bcl-xl were decreased at 50 μmol/L. *In vivo*, Erlotinib-treated mice demonstrated a reduced tumor volume, weight and microvessel density as compared to the control. IHC staining showed decreased expression of EGFR and RT-PCR had lower VEGF expression in treated mice.

CONCLUSION: The *in vitro* and *in vivo* findings provide evidence that BxPC-3 cells are inhibited with erlotinib treatment. Inhibition of EGFR may be a promising adjuvant chemotherapy strategy in pancreatic cancer treatment.

Key words: Pancreatic cancer; Erlotinib; Epidermal growth factor receptor; Human xenograft model; Angiogenesis

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INTRODUCTION

Pancreatic cancer is one of the most lethal human cancers and continues to be a major unsolved health problem[1,2]. Recently, several orally bioavailable compounds aimed at specific molecular targets have been developed in hopes of improving survival in this dismal disease. Tumor development and progression depend on cellular changes like overexpression of oncogenic tyrosine kinase receptors. Many gastrointestinal tumors, including pancreatic cancer, have been shown to overexpress the epidermal growth factor receptor (EGFR)[3,4]. The overexpression of the EGFR and its ligands correlates with rapidly progressive disease and resistance to chemotherapy.

EGFR is a 170-kDa transmembrane protein with intrinsic tyrosine kinase activity. Stimulation of the EGFR results in activation of multiple intracellular signaling cascades that increase cellular proliferation and prevent programmed cell death[5]. Multiple therapeutic strategies designed to manipulate this receptor have been developed, including specific antibodies and low molecular EGFR tyrosine kinase inhibitors (TKIs). Erlotinib is a small molecule TKI that efficiently blocks EGFR. Preliminary results of a phase Ⅲ trial of gemcitabine with or without erlotinib in pancreatic cancer revealed a modest improvement in survival with the addition of erlotinib. Treatment with anti-EGFR agents is used as a potential therapeutic strategy for pancreatic cancer, but the mechanisms are not yet precisely understood.

The aim of this study was to investigate the growth inhibitory effects of erlotinib in pancreatic cancer cells *in vitro* and *in vivo*, to determine the mechanisms involved and to examine the effects of erlotinib on the regulation of angiogenesis.

MATERIALS AND METHODS

Cell culture and reagents

Human pancreatic cancer cell lines BxPC-3, obtained from Shanghai Institute of Biochemistry and Cell Biology, and ECV 304, a cell line derived from human umbilical vein endothelial cells from ATCC were maintained in RPMI-1640 (Gibco) medium supplemented with 10% fetal calf serum in a humidified atmosphere containing 5% CO_2 at 37℃. The EGFR-selective TKI erlotinib was provided by DeBioChem (Nanjing, China). The agent was dissolved in DMSO (Sigma) or carboxymethylcellulose sodium at appropriate concentrations for *in vitro* or *in vivo* studies, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted with TRIzol (Life Technologies, INC) following the manufacturer's instructions. Reverse transcription was performed starting with 2 μg of total RNA, using oligo (dT) primer and other reagents, and procedures contained in the MMulv RT-PCR kit (Promega) to form cDNA. cDNA (2 μL), 2 μL of 50 pmol/L of each primer, 10 mmol/L dNTP Mix 1 μL, 1 μL of Taq DNA polymerase (Sangon, China) were used for PCR analysis. The PCR amplification cycles consisted of denaturation at 94℃ for 5 min, 35 cycles of denaturation at 94℃ for 60 s, annealing [54℃ for *bcl-2*, *bcl-xl*, *bax*, *bak*, vascular endothelial cell growth factor (VEGF), 56℃ for GAPDH] for 60 s, extension at 72℃ for 60 s, and a final elongation at 72℃ for 10 min. The PCR products were separated on a 1.5% agarose gel, stained with 0.5 mg/mL ethidium bromide, and visualized by UV light. The primer sequences are listed in Table 1.

Tube formation assay

A well established method was used for the process of *in vitro* angiogenesis assay[6] with a kit from Chemicon (Temecula, California, USA). A 96-well tissue culture plate was coated with Matrigel (50 μL/well). After matrix solution gelled, ECV304 cells were premixed with RPMI-1640 (control), erlotinib (100 μmol/L) and then seed at a concentration of 1×10^4 per well onto the surface of the polymerized gel. Four wells were used for each treatment. After 18 h of incubation at 37℃ and 5% CO_2, the status of capillary tube formation by ECV304 cells was recorded using a CCD camera attached to an inverted light microscope (40 × objective lens).

Cell viability assay

The viability of BxPC-3 cells treated with erlotinib was determined by the standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. BxPC-3 cells were plated (5×10^3 per well) in 96-well plates and incubated overnight at 37℃. Erlotinib was dissolved in DMSO and added to the cell culture medium at a concentration not exceeding 0.1% (v/v). The effects of erlotinib on cell proliferation were studied at various concentrations (0, 1, 5, 10, 50 and 100 μmol/L) and at different time points (24, 48, 72 and 96 h) with a certain concentration (50 μmol/L). The MTT assay was done in quadruplicate for each drug concentration used. At appropriate intervals, 100 μg MTT solution was added to each well and incubated for 4 h at 37℃, 5% CO_2. The supernatant was removed, and 150 μL of DMSO was then added. Plates were then read at 490 nm wavelength using a microplate reader (BIO-RAD550, USA). Percentage of inhibition was determined by comparing the cell density in the drug-treated cells with that in the untreated cell controls in the same incubation period [percentage of inhibition = (1-cell density of a treated group)/cell density of the control group]. All experiments were repeated three times.

Cell cycle analysis and apoptosis assays

The effects of EGFR TKIs erlotinib on both cell cycle and apoptosis in BxPC-3 cells were analyzed using flow cytometry. Cells were plated into 12-well plates and the following day, erlotinib (50 μmol/L) was added and kept for 48 h. Cell floating in the medium combined with adherent layer were trypsinized and fixed with 2 mL of Citrate buffer for 1 h. Cells were then incubated with RNase A (1500 μL) and stained with propidium iodide (1500 μL). Samples were immediately analyzed by flow cytometry for cell cycle and apoptosis assays. Immunocytochemical (ICC) detection of apoptotic cells was carried out with terminal deoxynucleotidyl transferase-mediated nick end labeling assay (TUNEL), in which residues of digoxigenin-labeled dUTP were catalytically incorporated into the DNA by terminal deoxynucleotidyl transferase Ⅱ. After treatment with erlotinib (50 μmol/L) for 48 h, slides were fixed and washed thrice in 0.01 mol/L PBS, the following procedures were performed according to the manufacturer instructions (Boster, Wuhan, China). The positive particles of DAB staining were viewed under microscope (Olympus Japan). The number of apoptotic cells was viewed and counted

Table 1 PCR primers

Target genes		Primer sequence	Size (bp)	Annealing temperature (℃)	Cycles
Bcl-2	Sense	5'-GGTGCCACCTGTGGTCCACCT-3'	458	54	35
	Antisense	5'-CCTCACTTGTGGCCCAGATAGG-3'			
Bax	Sense	5'-CTGACATGTTTTCTGACGGC-3'	289	54	35
	Antisense	5'-TCAGCCCATCTTCTTCCAGA-3'			
Bcl-xl	Sense	5'-TTGGACAATGGACTGGTTG-3'	765	54	35
	Antisense	5'-GTAGAGTGGATGGTCAGTG-3'			
Bak	Sense	5'-TGAAAAATGGCTTCGGGGCAAGGC-3'	642	54	35
	Antisense	5'-TCATGATTTGAAGAATCTTCGTACC-3'			
GAPDH	Sense	5'-CATGCCAGTGAGCTTCCCGTT-3'	408	56	35
	Antisense	5'-GTGGAGTCTACTGGCGTCTTC-3'			
VEGF	Sense	5'-ATGAACTTTCTGCTGTCTTG-3'	382	54	35
	Antisense	5'-TGCATGGTGATGTTGGAC-3'			

under microscope (40 × objective lens, Olympus Japan) and expressed as the Apoptotic Index (AI = number of apoptotic body/1000 cells).

Development of nude mice xenografts of pancreatic cancer

BALB/C nu/nu female mice, aged 4-6 wk, weighing about 20 g, were maintained pathogen free at the Shanghai Experimental Animals Centre of Chinese Academy of Sciences. BxPC-3 cells (1×10^7, suspended in 200 μL of PBS) were implanted *s.c.* in the hind flank of each mouse. Once palpable tumors were established, animals were randomly divided into two groups so that all groups had similar starting mean tumor volumes of 100-150 mm^3. The mice in each group were orally gavaged with the vehicle control (0.5% CMC-Na, $n = 6$) and erlotinib (100 mg/kg, $n = 6$) for 4 wk. The tumor size was measured with a linear caliper twice a week up to 4 wk, and the volume was estimated using the equation $V = (a \times b^2)/2$, where a is the large dimension and b the perpendicular diameter. After all the mice were sacrificed, part of the tissue was fixed in formalin and embedded in paraffin, and some parts were frozen in liquid nitrogen. Hematoxylin and eosin staining confirmed the presence of tumors. Total mRNA was prepared and RT-PCR analyses were performed as described previously.

Immunohistochemistry (IHC) of tumor xenografts

To assess EGFR expression and microvessel density (MVD) in xenograft tumors, rabbit polyclonal anti-EGFR antibody (diluted to 1:100, Boster, Wuhan) and rabbit polyclonal factor Ⅷ antibody (Boster, Wuhan) were used in IHC. Paraffin-embedded tissue sections (4 μm) were dried, deparaffinized, and rehydrated. Endogenous peroxidase was blocked with 3% hydrogenperoxide in ion free water for 30 min. After nonspecific binding sites were blocked with 10% goat serum, slides were incubated at 4℃ overnight with 1:100 dilution of primary antibody directed against EGFR and factor VIII followed by a 30-min incubation in a Horseradish peroxidase (HRP)-conjugated sheep anti-rabbit IgG secondary antibody. Sections were rinsed with PBS and developed with the DAB kit (Boster, Wuhan) and then counterstained with haematoxylin. Each slide was scanned at a low power (× 100) and the area with a higher number of new vessels was identified (hotspot). This region was then scanned at × 400 magnification. For individual tumors, microvessel count was scored by averaging the five field counts[7].

Statistical analysis

Quantitative results were expressed as mean ± SEM. Statistical analysis was performed using a two-tailed unpaired *t* test (between two groups) or a one way analysis of variance (ANOVA) (among three or more groups) with the computer software SAS 8.02. $P < 0.05$ was considered statistically significant.

RESULTS

Growth inhibition of BxPC-3 pancreatic cancer cells by erlotinib

Overall cell growth of BxPC-3 pancreatic cancer cells treated with erlotinib at different concentrations ranging from 1 to 100 μmol/L was determined by MTT assay. The results showed that erlotinib inhibited cell growth in a dose-dependent manner and significant growth inhibition was demonstrated at 72 h ($P < 0.01$). A similar result was obtained after erlotinib treatment for 96 h. We also treated BxPC-3 cells with erlotinib at 24 and 48 h. The results showed a trend toward growth inhibition, but it was not statistically significant among different concentrations ($P > 0.05$). Cell proliferation was again determined by MTT at 24, 48, 72 and 96 h. No significant difference in cell growth was noted between 24 h and 48 h, 72 h and 96 h at a concentration of 50 μmol/L. Percentage of survival cells is illustrated in Figure 1.

Effect of erlotinib treatment on cell cycle progression and apoptosis in BxPC-3 cells

To elucidate potential mechanisms of erlotinib-induced growth inhibition previously shown by MTT assay, we examined the distribution of cell cycle and apoptotic cells by flow cytometry and TUNEL. The concentration of the drug was 50 μmol/L which inhibited BxPC-3 cell growth by approximately 53.5% after 3 d of exposure. The results showed that erlotinib led to the accumulation of BxPC-3

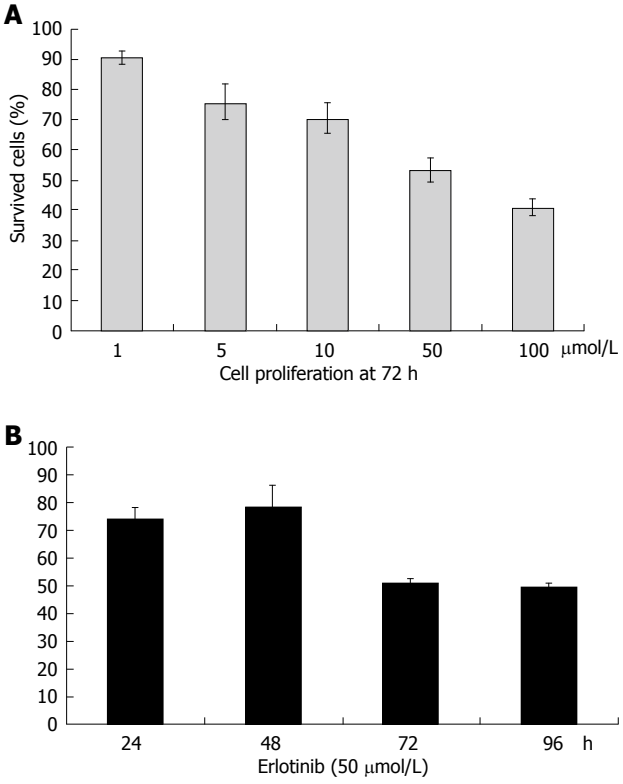

Figure 1 **A**: Growth percentage of BxPC-3 cell line after 72 h of exposure to erlotinib at varying concentrations ranging from 1-100 μmol/L. BxPC cell proliferation was significantly suppressed by erlotinib at different concentrations; **B**: Growth percentage of BxPC-3 cell line after exposure to erlotinib at a concentration of 50 μmol/L at different time points.

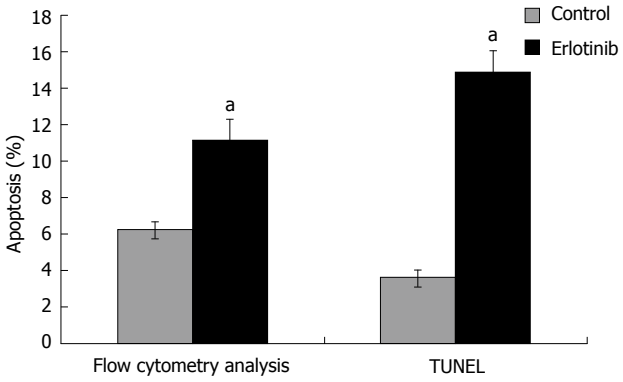

Figure 2 Induction of apoptosis in BxPC-3 cell line. There was a significant apoptosis in erlotinib treated group both in flow cytometry analysis and TUNEL assay after 48 h treatment ($^aP < 0.05$).

cells in G_0/G_1 phase, thereby decreasing the proportion of cells in the S phase (Table 2). To assess the effects of erlotinib on induction of cell apoptosis of pancreatic cancer cells, we performed a PI apoptosis and TUNEL assay after 48 h. Flow cytometric analysis showed an induction of apoptosis (11%) compared with the control (6%) ($P < 0.05$), which was further confirmed by TUNEL (AI 14.86 ± 1.20 to 3.60 ± 0.45) ($P < 0.05$) (Figure 2). The cell cycle alterations and cell apoptosis increase indicated that cell cycle arrest and increase in apoptosis are one of the mechanisms responsible for the antiproliferative action of erlotinib in BxPC-3 cells *in vitro*.

Table 2 Cell cycle analysis (%, mean ± SD)

Group	G_0/G_1	S	G_2/M
Control	63.31 ± 0.99	25.28 ± 0.88	11.40 ± 1.68
Erlotinib	73.40 ± 1.34[b]	14.15 ± 0.99[b]	12.44 ± 1.95

$^bP < 0.01$ *vs* control group.

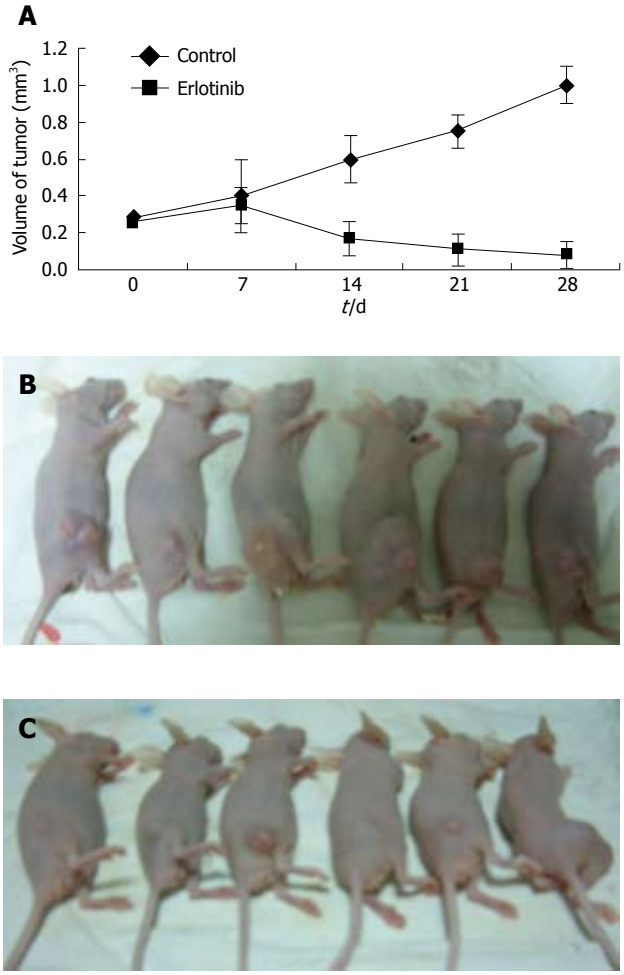

Figure 3 **A**: Effect of erlotinib on mean tumor volume in the BxPC-3 xenograft model. Mice were implanted with BxPC-3 cells. When palpable tumors were established, animals were randomly divided into two groups. The animals were continuously gavaged with the agents for 4 wk as described in Matericals and Methods. Tumor size was measured twice per week. Values are means, $n = 6$. **B**: Mice of control group; **C**: Mice of erlotinb group.

Effect of treatment with erlotinib on the growth of mouse xenografts

A nude mouse model of pancreatic cancer was used for the *in vivo* portion of the study to assess the *in vivo* antitumor activity of erlotinib. Heterotopic murine pancreatic carcinoma was successfully established in the flank of BALB/C nude mice. Erlotinib at a dose of 100 mg/kg per day was administered to mice bearing established BxPC-3 tumors. The results indicated that erlotinib significantly inhibited tumor growth. As shown in Figure 3, at day 14, the control group of mice showed an increased tumor volume by 114.3% of the initial tumor while the group treated with erlotinib showed a decreased tumor volume by 34.6%. At the end of the

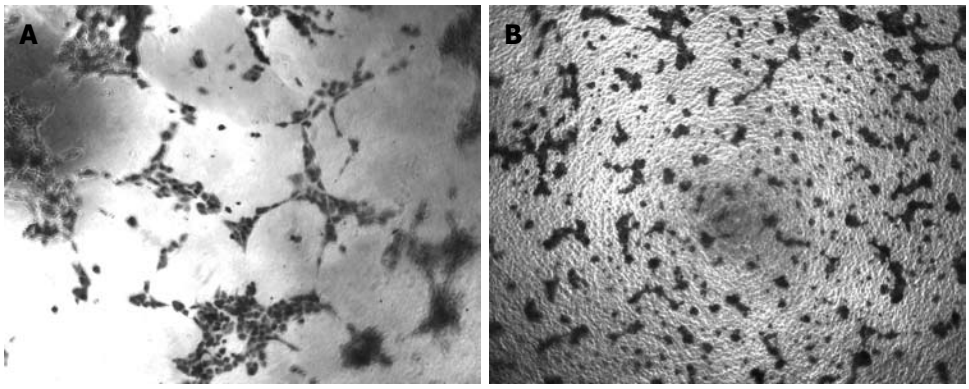

Figure 4 Effects of erlotinib on capillary formation by ECV304 were examined *in vitro*. **A**: When cultured on ECMatrix, ECV304 cells rapidly aligned with hollow tube-like structures; **B**: ECV304 cells were treated with erlotinib (100 μmol/L), and significant inhibition of tube formation was achieved compared with the control.

study in the BxPC-3 xenograft (day 28), the growth inhibition rate was 74.5% ($P < 0.05$) in the erlotinib treated group.

In vitro angiogenesis

EGFR TKIs have been reported to inhibit angiogenesis of pancreatic carcinoma. To determine whether erlotinib suppressed tumor vessel formation *in vitro*, we examined the effects of erlotinib on the ability of ECV304 to form capillary tube structures. ECMatrix is a solid gel of basement proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumor and consists of laminin, collagen type Ⅳ, heparin sulphate proteoglycans, entactin, and nidogen. It also contains various growth and proteolytic enzymes that occur normally in EHS tumors. When cultured on ECMatrix, ECV304 cells rapidly aligned and formed tube-like structures (Figure 4A), however, the length of the tubes in the erlotinib treated cells was markedly reduced compared with the control which demonstrated that erlotinib significantly inhibited ECV304 tube formation (Figure 4B).

In vivo inhibition of EGFR and MVD after treatment with erlotinib

The EGFR expression pattern in the BxPC-3 tumors was examined by IHC staining. IHC analysis confirmed that the tumor xenograft tissues maintained both cell membrane and cytoplasmic EGFR expression. The expression of EGFR was decreased in the erlotinib treated groups (2.45 ± 0.81) compared with the control (10.65 ± 1.26) ($P < 0.05$). MVD was also assessed by immunostaining with factor Ⅷ-related antigen in the most intense areas of neovascularization. Representative images of the two groups are shown in Figure 5. MVD of erlotinib treated tumors (1.86 ± 0.43) was significantly lower than that of the control (5.98 ± 1.27) ($P < 0.05$).

Modulation of apoptosis-associated gene and VEGF expression in BxPC-3 cells and xenografts treated with erlotinib

The effect of erlotinib on the expression of EGFR, apoptosis-associated factors such as *bcl-2*, *bak*, *bax*, *bcl-xl* and *VEGF* mRNA was determined by RT-PCR. BxPC-3 cells were treated with erlotinib at different concentrations ranging from 5-200 μmol/L for 48 h. The results showed that the expression of *EGFR* and *VEGF* mRNA in the BxPC-3 cell line seemed to be down-regulated, the highest suppression was detected at the highest concentration used, 200 μmol/L. Densitometric analysis revealed the relative expression of VEGF at 200 μmol/L concentration of erlotinib (1.2% ± 0.68%) was significantly lower than that of the control (2.67% ± 0.13%) ($P < 0.05$). Apoptosis-associated gene including *bcl-xl* and *bcl-2* was suppressed by erlotinib in a concentration dependent manner; however, bax appeared to be up-regulated following erlotinib treatment at various concentrations; but it did not apparently affect bak expression. In the tumor xenograft tissues, the expression of *VEGF* mRNA was significantly lower (almost disappeared) in the erlotinib-treated group compared with that in the control ($P < 0.01$) (Figure 6).

DISCUSSION

Treatment options of pancreatic adenocarcinoma are unsatisfactory, and the prognosis of patients with pancreatic cancer is poor. Considering that the cure rate for these patients with surgery alone is low[8], development of potentially effective treatment is urgently needed. Many features of the pancreatic malignant phenotype are associated with the signaling networks that involve the EGFR. EGF and its receptor, EGFR, are over-expression in many human pancreatic cancers[9,10]. Recently, there is increasing evidence demonstrating the therapeutic potential of EGFR blockade in the management of pancreatic cancer and other malignancies[11-13]. EGFR inhibitors have shown activity in clinical trials in pancreatic, colorectal and non-small cell lung cancers[14-16]. Erlotinib is an orally available low-molecular-weight quinazolinamine that acts as a potent and reversible inhibitor of EGFR-TK activity. Single agent activity was observed in patients with non-small cell lung cancer, head and neck carcinoma and ovarian cancer[17-20]. A randomized phase Ⅲ placebo-controlled trial has shown that the combination of gemcitabine and erlotinib is associated with a modest but statistically significant survival benefit compared with gemcitabine and placebo and this represents an EGFR-targeted agent conferred benefit in addition to chemotherapy[21]. In this study, we evaluated the efficacy of erlotinib, as a single agent, on pancreatic cancer cells grown *in vitro* and *in vivo*

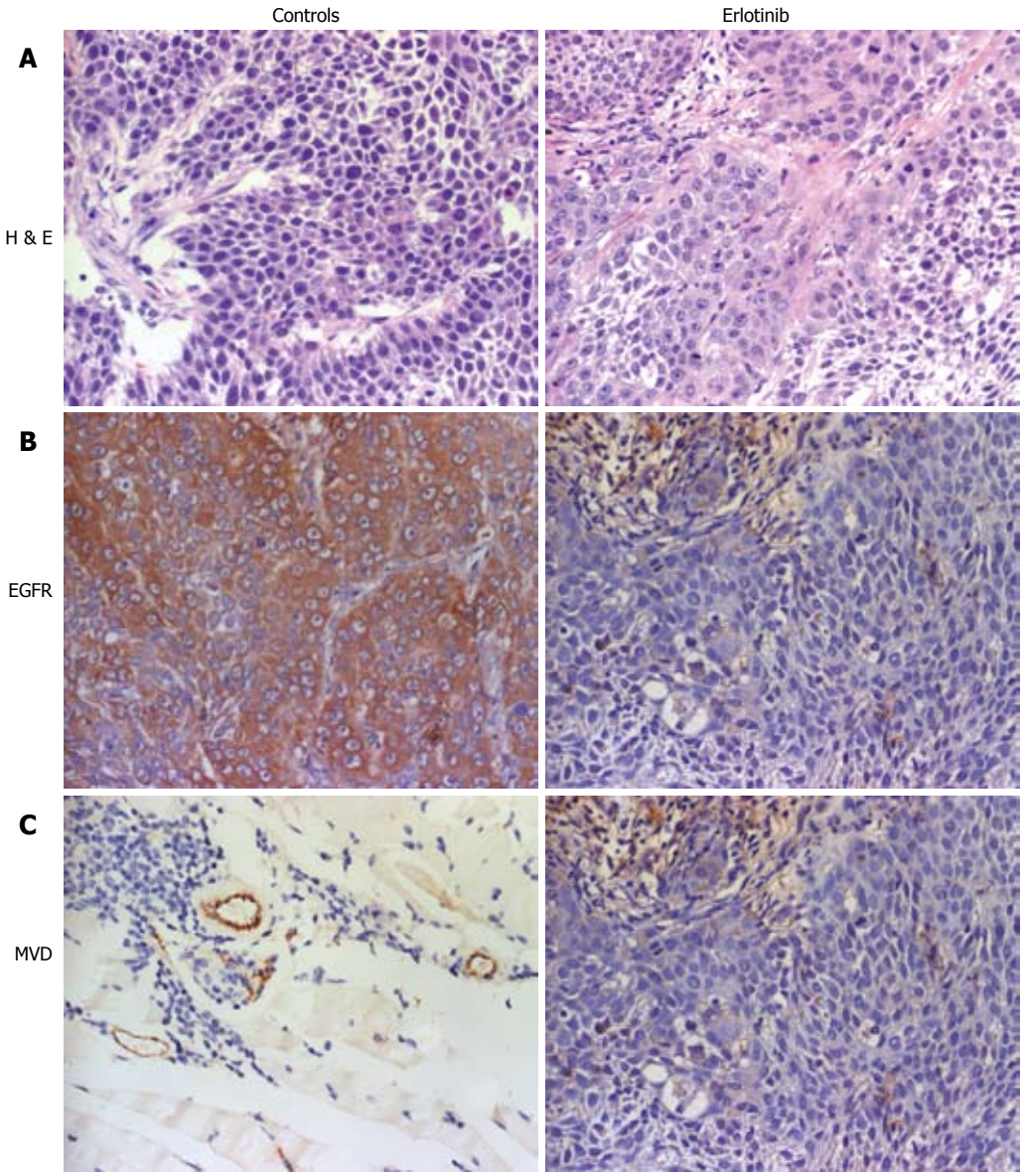

Figure 5 Expression of EGFR and the blood vessel endothelial cells in different treatment group in BxPC-3 mouse xenograft tissues. IHC was used to determine expression levels of EGFR and evaluate tumor microvessel density. **A**: HE staining for each sample (x 400); **B**: Expression of EGFR in treatment group was decreased compared with the control (x 400); **C**: Microvessel density of erlotinib treated group was lower than that of the control group.

using a nude mice xenograft model and explored the mechanisms involved as well. *In vitro* results showed an inhibition efficiency of 53.5% by erlotinib at the concentration of 50 μmol/L in the growth of cultured BxPC-3 pancreatic carcinoma cells as determined by MTT assay. The cell viability of BxPC-3 pancreatic cells was 53.5% with erlotinib treatment at a 50 μmol/L concentration by the MTT assay *in vitro*. There was no difference between 50 μmol/L and 100 μmol/L concentrations of erlotinib when cells were treated for 48 h (data not shown). We performed cell cycle analyses to characterize the underlying mechanisms of erlotinib's mode of action. Upon erlotinib treatment for 48 h, the proportion of cells in the G_0/G_1 phase increased to 73.4%, being significantly higher than that of the control. The antineoplastic effect of erlotinib is not solely due to cell cycle arrest. Induction of apoptosis by EGFR-TK inhibition has recently been reported[22,23]. In our study, we also observed cell apoptosis induced by erlotinib following cell cycle arrest. As we know, Bcl-2 members are crucial regulators of apoptotic cell death. We also checked the expression of anti-apoptotic factors such as bcl-2, bcl-xl as well as pro-apoptotic factors bax and bak. Erlotinib induced a moderate decrease in the expression of bcl-2 and bcl-xl at a concentration of $\geqslant$ 50 μmol/L. Our study *in vitro* provides evidence that the growth of BxPC-3 cells can be suppressed by erlotinib and at least two mechanisms are involved, i.e. cell cycle arrest and apoptosis. We used a nude mouse xenograft model to further evaluate the antitumor efficacy of erlotinib in pancreatic cancer *in vivo*. Erlotinib was very effective in suppressing the growth of BxPC-3 tumors in a subcutaneous tumor model. Tumor inhibition of approximately 74.5% was observed after 4 wk of treatment with erlotinib at a dose of 100 mg/kg per day. IHC staining of sections of subcutaneously implanted tumors showed constitutively high EGFR expression, however, the mice treated with 100 mg/kg erlotinib daily showed lower levels of EGFR. These results showed that erlotinib as monotherapy has strong antitumor activity in human BxPC-3 xenograft models expressing EGF receptors.

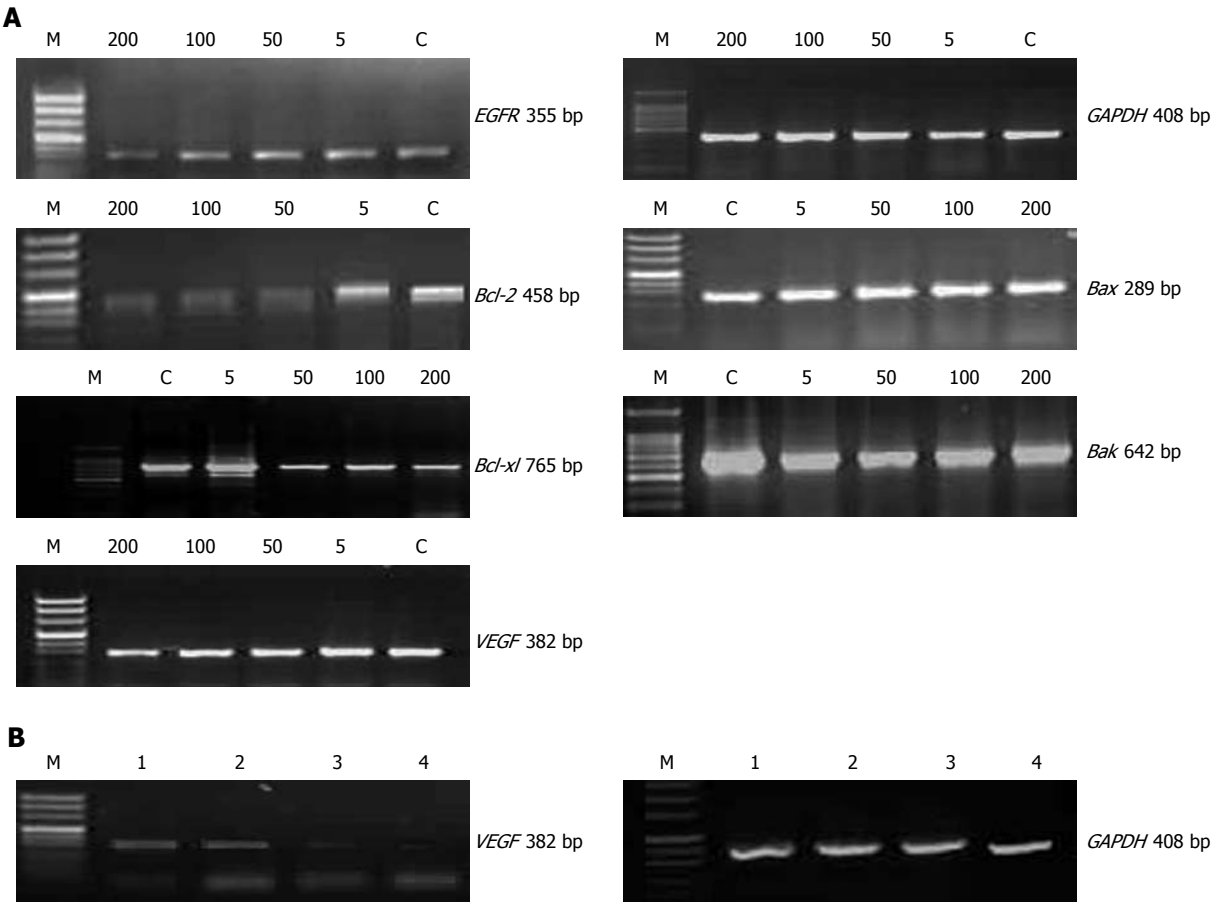

Figure 6 Expressions of *EGFR*, *Bcl-2*, *Bcl-xl*, *Bax*, *Bak* and *VEGF* mRNA in BxPC-3 cells treated with different concentrations (μmol/L) of erlotinib for 48 h and xenograft tissues were detected by RT-PCR. **A**: Effects of erlotinib on the expressions of EGFR, apoptosis-associated factors and *VEGF*. Lane M: DNA marker; Lane C: Control; other lanes: Different concentrations of erlotinib. **B**: Effects of erlotinib (100 mg/kg daily) on expression of VEGF in tumor tissues. Lane M: DNA marker; Lane 1, 2: Control group; Lane 3, 4: Treatment group. RT-PCR for *GAPDH* was performed in parallel and showed an equal amount of total RNA in the sample.

Numerous lines of evidence have shown that angiogenesis plays a significant clinicopathological role in tumors. Although pancreatic cancer is not a grossly vascular tumor, it often exhibits enhanced foli of endothelial cell proliferation. Moreover, several studies have reported a positive correlation between blood vessel density and disease progression in cases of pancreatic cancer, supporting the important role of angiogenesis in this disease[24-26]. EGFR signaling has previously been shown to play a role in angiogenesis[27]. In this study, we used the expression of VEGF, the MVD and tube formation assay to investigate the effects of erlotinib on angiogenesis both *in vitro* and *in vivo*. At the molecular level, VEGF is believed to be critical for pancreatic cancer angiogenesis[28]. Erlotinib exhibited a moderate decrease in VEGF expression *in vitro*, especially at a high concentration (200 μmol/L). Similarly, in xenograft tissues, there were marked reductions in the amounts of VEGF present in the treated group as compared with the control. As a unique tool, an *in vitro* capillary formation assay has been used to verify specific antiangiogenic activities of many agents with a good correlation to blood vessel formation *in vivo*[29]. Using this method, we observed that erlotinib as a single agent inhibited capillary tube formation by ECV304. Our immunohistochemical analysis of tumor specimens revealed that the treatment of mice with erlotinib produced a significant decrease in the number of tumor-associated blood vessels (MVD). The decrease in MVD could be attributable to a decrease in endothelial cell proliferation, as we proved *in vitro*.

In summary, our study showed that blockade of the EGFR signaling pathway by erlotinib has provided significant treatment in the BxPC-3 cell line *in vitro* and in nude mice and it may have a potential for the treatment of human pancreatic carcinoma. However, it is apparent that the use of a single signal transduction inhibitor cannot antagonize all the potentially relevant survival pathways in pancreatic cancers. As an adjuvant use in chemotherapy, targeting EGFR pathway seems to be a promising approach in the prevention and/or treatment of pancreatic cancer.

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COMMENTS

Background

The activity of the epidermal growth factor receptor (EGFR), a tyrosine kinase

receptor of the ErbB family, is abnormally elevated in most human solid tumors, including pancreatic cancer, and is associated with progression and poor prognosis. There are two main categories of therapeutic strategy for targeting the EGFR pathway, specific anti-EGFR monoclonal antibodies and EGFR tyrosine kinase inhibitors (TKIs). Blockage of EGFR activity may lead to growth inhibition in pancreatic cancer.

Research frontiers

Molecularly targeted therapies have recently expanded the options available for patients with gastrointestinal tumors. Low weight EGFR TKIs such as erlotinib may play an important role in the treatment of human pancreatic carcinoma. Administration of erlotinib inhibites the BxPC-3 human pancreatic cancer cell line growth and induces antiangiogenic effect both *in vitro* and *in vivo*.

Innovations and breakthroughs

Small-molecule inhibitors of the EGFR tyrosine kinase, such as erlotinib, have shown promise in phase Ⅲ trials in non-small-cell lung cancer (NSCLC). But the anticancer mechanism has not been clearly elucidated especially in gastrointestinal tumors, including pancreatic cancer. The results indicate that the blockade of EGFR may provide a rationale for translating this therapeutic strategy into a clinical setting in some pancreatic cancer patients.

Applications

This study showed that blockade of the EGFR signaling pathway by erlotinib suppressed the BxPC-3 human pancreatic cancer cell line growth and induced antiangiogenic effects both *in vitro* and *in vivo*. As an adjuvant used in chemotherapy, targeting the EGFR pathway seems to be a promising approach in the prevention and or treatment of pancreatic cancer.

Terminology

The epidermal growth factor (EGF) receptor (or ErbB1) and the related ErbB4 are transmembrane receptor protein tyrosine kinases which bind extracellular ligands of the EGF family. ErbB2 and ErbB3 are "co-receptors" structurally related to ErbB1/ ErbB4, but ErbB2 is an "orphan" receptor and ErbB3 lacks tyrosine kinase activity. They transduced biological signals from the extracellular to the intracellular compartment. These families of ligands and receptors have been firmly linked to proliferative signaling and oncogenesis.

Peer review

In this paper, the effect of erlotinib on the proliferation and apoptosis of the human pancreatic cancer cell line BxPC-3 was studied. A dose-dependent inhibition of BxPC-3 cell growth *in vitro*, with a block of G_1-S transition, and rise in apoptosis were observed in erlotinib-treated cells. A decrease in tumor volume and microvessel density was also found in erlotinib-treated nude mice carrying BxPC-3 cell xerographs. This is a very interesting manuscript with some minor points needing further addressing.

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BASIC RESEARCH

Biological impact of hepatitis B virus X-hepatitis C virus core fusion gene on human hepatocytes

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Abstract

AIM: To investigate the biological impact of hepatitis B virus X- hepatitis C virus core (HBV X-HCV C) fusion gene on hepatoma cells.

METHODS: The recombinant adenoviruses Ad-XC, Ad-X and Ad-C expressing HBV X-HCV C fusion gene, *HBV X* gene and *HCV C* gene were constructed, respectively. Hepatoma cells were infected with different recombinant adenoviruses. MTT, colony-forming experiment, FCM, TUNEL assay were performed to observe the biological impact of the HBV X-HCV C fusion gene on liver cells.

RESULTS: MTT showed that the Ad-XC group cells grew faster than the other group cells. Colony-forming experiment showed that the colony-forming rate for the Ad-XC group cells was significantly higher than that for the other group cells. FCM analysis showed that Ad-XC/Ad-X/Ad-C infection enhanced the progression of G1→S phase in the HepG2 cell cycle. The apoptosis index of the Ad-XC, Ad-X, Ad-C group cells was significantly lower than that of the Ad0 and control group cells. Semi-quantitative RT-PCR showed that the expression level of c-myc was the highest in Ad-XC infected cells. Tumor formation was found at the injected site of mice inoculated with Ad-XC-infected LO2 cells, but not in control mice.

CONCLUSION: Ad-XC, Ad-X and Ad-C facilitate the proliferation activity of HepG2 cells and inhibit their apoptosis *in vitro*. The effect of Ad-XC is significantly stronger than that of Ad-X and Ad-C. Up-regulation of c-myc may be one of the mechanisms underlying the synergism of *HBV X* and *HCV C* genes on hepatocarcinogenesis in athymic nude mice.

Key words: Hepatitis B virus X gene; Hepatitis C virus core gene; Hepatocellular carcinoma; Proliferation; Apoptosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the second most common cancer in China. Many etiological factors are related with HCC development. Chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and prolonged dietary exposure to aflatoxin are responsible for about 80% of all HCCs in human beings. Chronic HBV and HCV infection often results in cirrhosis and enhances the probability of developing HCC. The underlying mechanisms leading to malignant transformation of infected cells, however, remain unclear. Based on epidemic data, super-infection with HBV and HCV is associated with the increased frequency in the development of HCC, but the relative mechanism remains elusive. It has been reported that both *HBV X* gene and *HCV core* (*HCV C*) gene play an important role in hepatocarcinogenesis. The fact that *HBV X* and *HCV C* genes induce HCC in transgenic mice offers more evidence for the relationship between these genes and HCC.

Imbalance between proliferation and apoptosis may contribute to hepatocarcinogenesis. HBV X and HCV C proteins are multiple-functional proteins, and can deregulate cell cycle check points, transactivate or activate cellular and viral genes, which are involved in transcription regulation, signal transduction pathway, cell cycle regulation, *etc*. Therefore, they may deregulate cell cycle and apoptosis and may have a common target

point. However, if there is synergism of HBV X and HCV C proteins on hepatocarcinogenesis still remains unclear. In the present study, recombinant adenoviruses expressing HBV X-HCV C fusion protein, HBV X protein and HCV C protein were constructed, and their effect on the biological behavior and expression of c-myc in hepatocytes were investigated.

MATERIALS AND METHODS

Materials

Pyrobest™ DNA polymerase, S*al* I , E*co*RV, H*ind*Ⅲ and T4 DNA ligase were purchased from TaKaRa Company (Dalian, China). Plasmid pecob6 was constructed by professor Ren Hong in our institute, plasmid pcDNA3.1/HCV-C was constructed by Dr. Wei-xian Chen in our institute. Lipofectamine was purchased from Invitrogen Company (California, USA). P*ac* I and P*me* I were from New England Biolabs (Beijing, China). Primers were synthesized by Shanghai Sangon Company (Shanghai, China). AdEasy system was a gift from professor Tong-Chuan He, University of Chicago Medical Center. DH5α was kept in our laboratory. Fetal bovine serum and calf serum were purchased from Hyclone (Utah, USA). RPMI 1640 was provided by GIBCO (New York, USA). 293 cells, LO2 and HepG2 cells were purchased from Shanghai Cytobiology Research institute of Chinese Academy of Science (Shanghai, China). BALB/c nude mice were from Shanghai Experimental Animal Centre of Chinese Academy of Science. Mouse anti-human hepatitis B virus X-protein monoclonal antibody was purchased from CHEMICON (California, USA). Monoclonal antibody to hepatitis C virus core protein was purchased from BIODESIGN (Maine, USA).

Construction, identification and amplification of recombinant adenoviruses

The recombinant adenoviruses Ad-XC, Ad-X and Ad-C containing *HBV X-HCV C* fusion gene, *HBV X* gene and *HCV C* gene, were constructed using the AdEasy system[1]. *HBV X* and *HCV C* genes were amplified by PCR from pecob6 or pcDNA3.1-HCV C. Using gene SOEing method[2], *HBV X* and *HCV C* genes were fused through a linker coding for a sequence rich in glycine. The sequences of gene primers used in this study are listed in Table 1. The fragment was bi-mold-cut with S*al* I and E*co*RV and inserted into two spots of S*al* I and E*co*RV of the pAdTrack-CMV. The recombinant shuttle plasmid was confirmed by PCR, double restriction nuclease digestion and sequencing. Mini preparations were performed using the conventional alkaline lysis method. The linearized shuttle plasmid was co-transformed with adenoviral backbone plasmid pAdEasy-1 to *E. coli* BJ5183 by electroporation. The cloned candidate was further tested by restriction nuclease digestion with P*ac* I and PCR. After digested with P*ac* I , the recombinant adenoviral plasmid was transfected into 293 cells for package. Generation of recombinant adenoviruses was monitored by GFP expression. Transfected cells were collected 12-15 d after transfection by scraping cells off flasks and pelleting them with 1 mL PBS. After three cycles of freezing and rapid thawing at 37℃, 10 μL proteinase K was added into 5 μL of viral lysate at 55℃ for 1 h and boiled for 5 min and 2 μL of which was used as a model to identify the correct recombinant adenovirues (*HBV X*, *HCV C* and *HBV X-HCV C* fusion gene). The upstream sequence of adenovirus primer is 5'-CTGTGGACCGTGAGGATA-3', the downstream sequence of adenovirus primer of adenovirus primer is 5'-TGTTGGGCATAGATTGTT-3' (Table 1). PCR system contained 2 μL viral DNA, 2 μL 10 μmol/L primer, 5 μL 10 × PCR buffer, 3 μL $MgCl_2$, 4 μL 2 mmol/L dNTP and 1 μL Taq enzyme. Water was added until the final volume of PCR reached 50 μL. Thirty-five cycles of PCR were carried out, each consisting of 94℃ for 30 s, 51℃ for 40 s, 72℃ for 40 s, and a final extension at 72℃ for 10 min. Electrophoresis on 1.0% agarose gel was performed. The identified positive recombinant adenoviruses were amplified in 293 cells for further experiment. The virus titer was tested with GFP expression and limited dilution method.

Table 1 Sequences of gene primers

Target gene	Primer sequences	Product (bp)
HBV X gene	P1: 5′-ATCTGTCGACATGGCTG CTAGGCTGTGCT G-3′	465
	P2: 5′-CGCGGATATCTTAGGC AGAGGTGAAAAAGT TG-3′	
HCV C gene	P3: 5′-ACTGGTCGACATGAGC ACGAATCCTAAACCT C-3′	576
	P4: 5′-ACTGGATATCTTAGGC TGAAGCGGGCAC AG-3′	
HBV X-Linker fragment	P1: 5′-ATCTGTCGACATGGCT GCTAGGCTGTGCT G-3′	510
	P2′: 5′-GCTGCCGCCACCGCCG CTTCCGCCACCGCCGCTTGCC ACCGGCAGAGGTGAAAAAGTTGCA-3′	
Linker-HCV C fragment	P3′: 5′-GGTGGCGGTGGAAGCG GCGGTGGCGGCGGAAGCGGC GGTGGCGGCAGCATGAGCACG AATCCTAAACCTC -3′	621
	P4: 5′-ACTGGATATCTTAGGCT GAAGCGGGCAC AG-3′	
HBV X-HCV C fusion gene	P1: 5′-ATCTGTCGACATGGCTG CTAGGCTGTGCT G-3′	1086
	P4: 5′-ACTGGATATCTTAGGCT GAAGCGGGCAC AG-3′	
c-myc	5′-TTCGGGTAGTGGAAAACCAG-3′ 5′-CAGCAGCTCGAATTTCTTCC-3′	203
β-actin	5′-GTGGGGCGCCCCAGGCACCA-3′ 5′CTTCCTTAATGTCACGCACGATTTC-3′	540

Infective efficiency of recombinant adenoviruses

Two hundred and ninety three, NIH3T3 and HepG2 cells were seeded in six-well plates in the appropriate medium at a density of 2×10^5 cells/well, infected with various adenoviruses at a multiplicity of infection (MOI) from 5 to 100, and incubated at 37℃ for 48 h. The number of cells expressing GFP was recorded under an inversion difference fluorescence microscope.

The percentage of cells with GFP expression was calculated.

Infection with recombinant adenoviruses and expression of fusion protein

The human liver cancer cell line HepG2 was incubated in an atmosphere containing 50 mL/L CO_2 at 37℃. When the density reached 90%, the experimental groups were infected with Ad-XC, Ad-X, Ad-C or Ad0, respectively, at different MOI. During the process, the culture fluid was shaken every 30 min, 4 h later. Another 6 mL 10% NCS RPMI 1640 was added. The control group was a non-virus group. After HepG2 cells were incubated for 48 h, total protein was extracted using RIPA. The expression of HBV X-HCV C fusion protein, HBV X and HCV C protein was detected by Western blot.

MTT assay

HepG2 cells (1.5×10^3 cells/well) were plated in 96-well plates (16 wells for each group) and infected with various MOI of Ad0, Ad-C, Ad-X or Ad-XC, respectively. Cell proliferation was determined by MTT assay. After 1-7 d, 20 μL of MTT solution (5 mg/mL) was added to each well. After incubation for 4 h at 37℃, MTT was removed and 200 μL dimethyl sulfoxide (Sigma) was added. The mixture was shaken and the crystals were fully dissolved for about 10 min. The A value of each well was detected at a test wavelength of 490 nm. Cell growth curve was plotted according to the A values.

Colony-forming experiment

HepG2 cells of five groups were digested into a single-cell suspension and inoculated into a six-well plate. Each well was inoculated with 1.0×10^3 cells. The cells were incubated for 12 d and fixed with methanol, stained with Giemsa stain fluid. Then the number of colonies with more than 50 cells was recorded. The experiment was repeated five times.

Flow cytometry

When the density of HepG2 cells reached 90%, the experimental groups were infected with Ad-XC, Ad-X, Ad-C and Ad0, respectively. Forty-eight hours after infection, the cells were collected (using trypsin digestion) and centrifuged at 1000 r/min for 5 min. The upper clear fluid was discarded, PBS was added to adjust the cell density to 10^6/mL. One hundred microliters of cell suspension was put into a tube, into which 200 μL DNA-PREPTMLPR was added and mixed. Two microliters of DNA-PREPTM stain reagent (PI stain) was added and mixed after 30 s. After 30 min, the cell cycle was detected by flow cytometry (FCM).

Cell apoptosis assay

Cell apoptosis was estimated by TUNEL staining. HepG2 cells were planted into 24-well plates at a density of 1×10^5 cells/well and infected with Ad-XC, Ad-X, Ad-C and Ad0 respectively. At the same time, 1 mL TNF-α (100 ng/mL) was added into each well. After incubation at 37℃ for 48 h, the cells were fixed with 4% paraformaldehyde and chilled in ice bath for 4 min with permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate). Then, 50 μL of TUNEL mixture was added, incubated in a humidified chamber at 37℃ for 90 min. The TUNEL mixture was removed, 50 μL POD was added and incubated for another 40 min. The cells were rinsed with PBS, stained with DAB, and detected by optic microscopy.

Effect of Ad-XC infection on c-myc mRNA expression in HepG2 cells

Expression of c-myc mRNA in each group was assayed by semi-quantitative RT-PCR. β-actin was used as an internal control. Total RNA was extracted with TRIZOL reagent. RT-PCR was performed using an access RT-PCR system (Promega). The reaction volume was 50 μL containing 10 μL AMV/Tf1 buffer, 2 μL $MgSO_4$, 1 μL dNTP, 1 μL target gene sense and anti-sense primers, 1 μL β-actin primer pair, 1 μL AMV reverse transcriptase, 1 μL Tf1 DNA polymerase, 2 μg RNA and nuclease-free water. The sequences of gene primers are listed in Table 1. Thirty cycles of amplification were performed, each consisting of denaturation at 94℃ for 45 s, annealing at 58℃ for 30 s, extension at 37℃ for 1 min, an initial denaturation at 45℃ 45 min and at 94℃ for 2 min, and a final extension at 72℃ for 10 min. About 5 μL PCR products were separated by electrophoresis on 10 g/L agarose gel and detected by ultraviolet radiography. The densities of bands were analyzed using a Bio imaging system, the ratio of c-myc density to β-actin density was represented as the relative expression level of mRNA. The semi-quantitative detection was analyzed five times.

Nude mice experiment

LO2 cells were infected with Ad0 or Ad-XC. The infected cells were collected and resuspended in 200 μL PBS after 48 h. Three BALB/c nude mice were subcutaneously inoculated with the infected LO2 cells randomly (Ad0, $n = 1$; Ad-XC, $n = 2$). Tumors were observed every 2 d for 6 wk.

Statistical analysis

All data were expressed as mean ± SE. The significance for the difference between groups was assessed with SPSS 12.0 by one-way ANOVA. $P < 0.05$ was considered statistically significant.

RESULTS

Identification of recombinant adenoviruses by PCR co-amplification method

The PCR product depending on the viral DNA model was evaluated by 1.0% agarose electrophoresis. The recombinant adenoviruses that could amplify 465 bp HBV X cDNA fragment/576 bp HCV C cDNA fragment/HBV X-HCV C fusion gene fragment and a 759 bp virus gene frame fragment at the same time were obtained (Figure 1). Recombinant adenoviruses

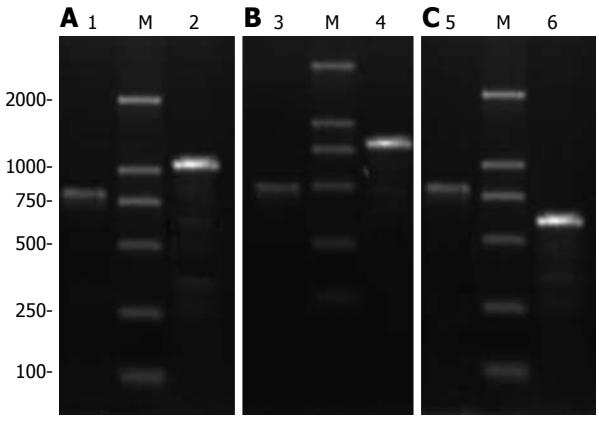

Figure 1 PCR verifying recombinant adenoviruses of Ad-XC (**A**), Ad-X (**B**), and Ad-C (**C**). M: DL2000; lanes 1, 4 and 5: PAdEasy-1; lane 2: HBV X-HCV C fusion gene; lane 3: X gene; lane 6: C gene.

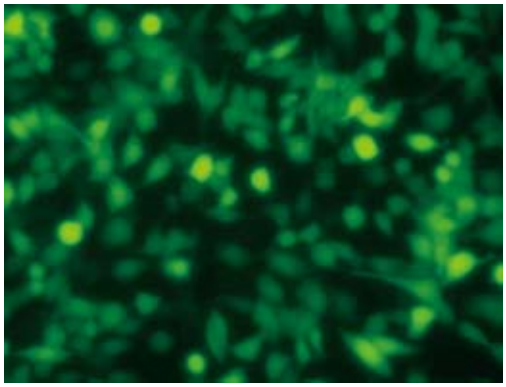

Figure 2 HepG2 cells infected with recombinant adenoviruses (x 200).

containing HBV X cDNA fragment, HCV C cDNA fragment and HBV X-HCV C fusion gene fragment, respectively, were produced.

Titer and transfection rate of recombinant adenoviruses

The titer of amplified recombinant adenoviruses Ad-XC, Ad-X, Ad-C and Ad0 was 1.9×10^9 pfu/mL, 2.0×10^9 pfu/mL, 2.2×10^9 pfu/mL, 1.7×10^9 pfu/mL, respectively. When the MOI was 20 or greater, the infection rate of HepG2 cells reached 100% (Figure 2).

Expression of different proteins in HepG2 cells

Forty-eight hours after infection, Western blot revealed the expression of HBV X-HCV C fusion protein, HBV X and HCV C proteins in HepG2 cells infected with recombinant adenovirus (Figure 3).

Effect of recombinant adenoviruses on growth curve of HepG2 cells

The expression of HBV X-HCV C fusion gene, *HBV X* and *HCV C* genes improved cell proliferation significantly compared with that of control HepG2 cells. The A values were higher in HepG2 cells infected with Ad-XC, Ad-X and Ad-C than in control HepG2 cells (Figure 4). These results indicate that HBV X-HCV C fusion gene, HBV X and HCV C genes, especially Ad-XC, stimulated the metabolic activity and the viability of HepG2 cells.

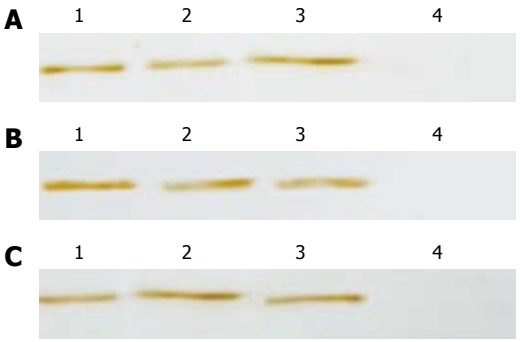

Figure 3 Western blotting displaying expression of fusion protein (**A**), HBV X protein (**B**), and HCV C protein (**C**). Lanes 1-3: Infected HepG2 cells; lane 4: Uninfected HepG2 cells.

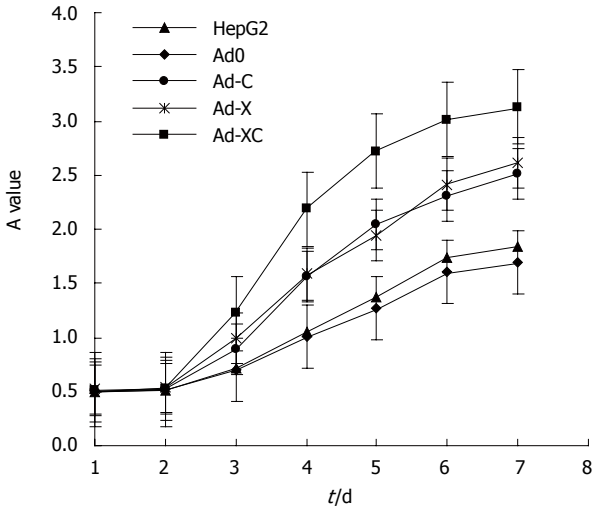

Figure 4 Growth status change in HepG2 cells after infection.

Table 2 Cell cycle of HepG2 cells infected with recombinant adenovirus (%, mean ± SE)

	G_0/G_1	S	G_2/M
HepG2	71.57 ± 0.79	19.18 ± 0.77	9.25 ± 0.76
Ad0	72.94 ± 1.84	18.38 ± 0.9	8.68 ± 1.12
Ad-C	50.21 ± 1.37	32.15 ± 0.15[b]	17.65 ± 1.22[b]
Ad-X	48.17 ± 1.13	33.19 ± 1.47[b]	18.64 ± 0.56[b]
Ad-XC	36.49 ± 0.84	42.06 ± 0.24[a,b]	21.45 ± 0.89[a,b]

[a]$P < 0.005$ *vs* Ad-C and Ad-X cells; [b]$P < 0.001$ *vs* HepG2 and Ad0 cells.

Effect of recombinant adenoviruses on colony-forming ability of HepG2 cells

The colony-forming rate of Ad-XC infected HepG2 cells was 82.2% ± 6.1%, significantly higher than that of the Ad-X, Ad-C, Ad0 infected HepG2 cells and control cells (53% ± 4.1%, 49% ± 7.1%, 27.6% ± 5.1%, 30.2% ± 4.4%, respectively, $P < 0.0001$, $n = 5$).

Effect of recombinant adenoviruses on cell cycle

Cell cycles from FCM are listed in Table 2. Compared

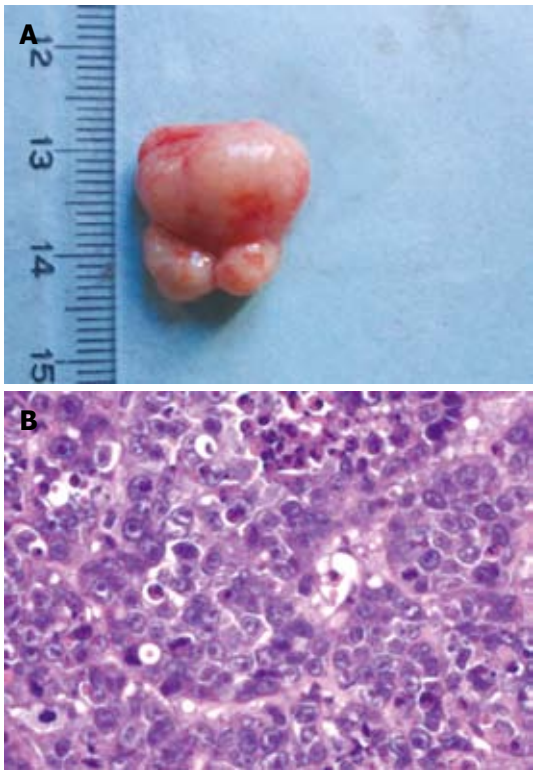

Figure 5 Surgical specimens of tumor tissue (**A**) and histological examination (**B**) showing a 1.5 cm tumor (HE staining, × 400).

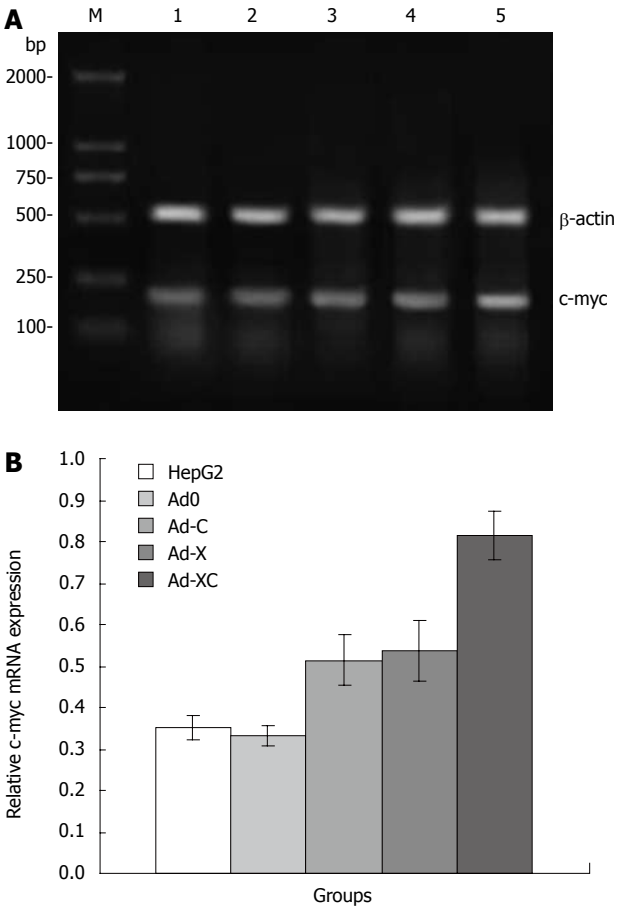

Figure 6 RT-PCR revealing mRNA expression of c-myc (**A**) and relative mRNA expression level of c-myc (**B**) in HepG2 cells. M: DL2000; lanes 1-5: HepG2, Ad0, Ad-C, Ad-X and Ad-XC cells ($P < 0.05$, Ad-XC group *vs* other groups).

with Ad0 and non-virus group cells, the number of cells in G_0/G_1 phase was decreased, but increased in Ad-XC, Ad-X, Ad-C groups at S phase, indicating that proliferation of the cells was active. A significant difference was observed in cell proliferation between the Ad-XC group and other groups ($P < 0.0001$, $n = 3$).

Cell apoptosis assay

TUNEL showed that the apoptosis rate of HepG2, Ad0, Ad-C, Ad-X and Ad-XC cells was 20.7% ± 0.6%, 21.8% ± 0.9%, 12.6% ± 0.8%, 11.7% ± 0.9% and 5.1% ± 0.8%, respectively. The apoptosis rate of the experimental group decreased obviously in comparison to the control group. The apoptosis rate of Ad-XC was the lowest. The apoptosis rate of these five groups of cells differed sharply when compared to each other ($P < 0.0001$, $n = 3$).

Nude mice experiment

Tumor formation was observed at the injection site of mice inoculated with Ad-XC infected LO2 cells (Figure 5), but not in control mice.

Effect of Ad-XC infection on c-myc mRNA expression in HepG2 cells

The mRNA level of c-myc in Ad-XC cells was the highest (Figure 6), indicating that transient expression of *HBV X-HCV C* fusion gene obviously induced expression of c-myc in HepG2 cells.

DISCUSSION

HCC is one of the most common malignant tumors in the world. Chronic hepatitis B and C are responsible for the great majority of cases of HCC worldwide. Both HBV and HCV are parenterally transmitted and superinfection is not uncommon in intravenous drug users and in countries with a high prevalence of HBV[3]. The risk of developing HCC in subjects with both HBV and HCV infections has been investigated in two meta-analyses[4,5], showing that there is a synergistic hepatocarcinogenic interaction between HBV and HCV infections and that the increased risk is super-additive but not multiplicative.

It was reported that transgenic mice with hepatitis B and C have the oncogenic potential of *HBV X* and *HCV C* genes in the liver[6,7]. It was also reported that HBV X and HCV C proteins have an oncogenic potential[8-14], but the involvement of their synergisms in hepatocarcinogenesis remains unclear. HBV X and HCV C proteins additively repress the universal cyclin-dependent kinase inhibitor p21 gene at the transcription level and additively stimulate cell growth, suggesting that additive repression of p21 is important to understand the cooperative development of HCC due to these two proteins[15]. When HBV X and HCV C proteins transform mouse fibroblast NIH3T3 cells in cooperation, they additively stimulate cell growth, especially in the absence of serum growth factors. Cells expressing these two viral proteins exhibit a higher tumorigenicity, as demonstrated in athymic nude mice[16]. HBV X protein

increases liver pathogenesis in HCV transgenic mice by a mechanism involving an imbalance between hepatocyte death and regeneration[17]. In the present study, Ad-XC, Ad-X and Ad-C could facilitate the proliferation activity of HepG2 cells and inhibit their apoptosis *in vitro*. The effect of Ad-XC was significantly stronger than that of Ad-X and Ad-C, suggesting a more than additive but less than multiplicative effect of *HBV X* and *HCV C* genes on hepatocarcinogenesis as demonstrated in athymic nude mice[16].

The increased expression of oncogene is thought to be a major cause for tumor formation/progression. C-myc, an oncogene located on 8q24, may be important in hepatocarcinogenesis. The expression level of c-myc in the cells transiently transfected with the HBV X gene was much higher than that in the control cells[18]. c-myc protein expression above its basal level significantly increased c-myc stability, as revealed by its prolonged intracellular half-life in HepG2 expressing HCV core protein, suggesting that HCV core protein may promote cell cycle progression in HepG2 cells by increasing the stability of c-myc oncoprotein[19]. The present study aimed to evaluate the expression level of c-myc in cells infected with different recombinant adenoviruses by RT-PCR. The highest expression level of c-myc was observed in Ad-XC infected cells, suggesting that up-regulation of c-myc expression may be one of the mechanisms underlying the synergism of *HBV X* and *HCV C* genes in hepatocarcinogenesis.

In conclusion, HBV X and HCV C genes have a synergism in hepatocarcinogenesis. The reasons for the interaction are uncertain, although the increased c-myc expression in the presence of both genes with tumor promoting effects, including the enhanced up-regulation of c-myc expression, may play a role in hepatocarcinogenesis. Interaction between hepatocarcinogenic effects of the two genes remains to be investigated.

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COMMENTS

Background

Chronic hepatic B virus (HBV) and hepatic C virus (HCV) infection often results in cirrhosis and enhances the risk of developing HCC. The underlying mechanism leading to malignant transformation of infected cells, however, remains unclear. Based on epidemic data, Super-infection with HBV and HCV is associated with an increased frequency in the development of HCC, but the relative mechanism remains to be elucidated. It was reported that both HBV X and HCV core (HCV C) genes play an important role in hepatocarcinogenesis.

Research frontiers

The fact that HBV X and HCV C genes induce HCC in transgenic mice offers more evidence for the relationship between these genes and HCC. However, whether there is a synergism of HBV X and HCV C proteins on hepatocarcinogenesis is still unclear.

Innovations and breakthroughs

In the present study, recombinant adenoviruses expressing HBV X-HCV C fusion protein were constructed, and their effects on biological behavior and c-myc expression level in hepatocytes were investigated.

Applications

HBV X and HCV C genes may have a synergism in hepatocarcinogenesis. The reasons for the interaction are uncertain, although increased c-myc expression in the presence of both genes with tumor promoting effects, including the enhanced up-regulation of c-myc expression, may play a role in hepatocarcinogenesis. Interaction between hepatocarcinogenic effects of the two genes remains to be investigated.

Peer review

This is an elegant, well designed study investigating the combined effect of adenoviruses expressing HBV-X and HCV-C genes on the proliferation and apoptosis of HepG2 cells. The major finding of the study was that the combined effect of the two genes on cell proliferation and apoptosis was superior over that of HBV-X or HCV-C gene alone. The authors also stressed that the underlying mechanism may be, at least, partly explained by the increased c-myc expression.

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RAPID COMMUNICATION

Liver *insulin-like growth factor 2* methylation in hepatitis C virus cirrhosis and further occurrence of hepatocellular carcinoma

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Abstract

AIM: To assess the predictive value of the insulin-like growth factor 2 (*Igf2*) methylation profile for the occurrence of Hepatocellular Carcinoma (HCC) in hepatitis C (HCV) cirrhosis.

METHODS: Patients with: (1) biopsy-proven compensated HCV cirrhosis; (2) available baseline frozen liver sample; (3) absence of detectable HCC; (4) regular screening for HCC; (5) informed consent for genetic analysis were studied. After DNA extraction from liver samples and bisulfite treatment, unbiased PCR and DHPLC analysis were performed for methylation analysis at the *Igf2* locus. The predictive value of the *Igf2* methylation profile for HCC was assessed by Kaplan-Meier and Cox methods.

RESULTS: Among 94 included patients, 20 developed an HCC during follow-up (6.9 ± 3.2 years). The methylation profile was hypomethylated, intermediate and hypermethylated in 13, 64 and 17 cases, respectively. In univariate analysis, two baseline parameters were associated with the occurrence of HCC: age (P = 0.01) and prothrombin (P = 0.04). The test of linear tendency between the three ordered levels of *Igf2* methylation and probability of HCC occurrence was significant (Log Rank, P = 0.043; Breslow, P = 0.037; Tarone-Ware, P = 0.039).

CONCLUSION: These results suggest that hypomethylation at the *Igf2* locus in the liver could be predictive for HCC occurrence in HCV cirrhosis.

Key words: Liver cancer; Cirrhosis; Insulin-growth factor 2, DNA methylation

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignant neoplasms worldwide[1], and its incidence has increased in the past decade in Europe and the USA[2-5]. In Western countries, HCC mostly develops in the presence of cirrhosis. Chronic Hepatitis C virus (HCV) infection plays an important role in the increased incidence of HCC in the western world[6] where HCC is presently the leading cause of death

of patients with HCV related cirrhosis[7]. In these patients, the annual incidence of HCC varies from 2% to 6%[8-13]. The main predictive factors of HCC in patients with HCV-cirrhosis are age over 50[14-17], male gender[12,14,15,17], increased serum alpha-fetoprotein (AFP) baseline levels[12,18,19], symptoms of portal hypertension, such as thrombopenia[17,18] or esophageal varices[17,19], obesity[20,21] and diabetes[22,23]. Identification of molecular abnormalities associated with an increased risk of HCC is particularly important to improve knowledge of both the pathways of liver carcinogenesis and the outcomes.

Insulin-like growth factor 2 (IGF2) is a fetal growth peptide produced by the liver which is structurally and functionally closely related to insulin[24]. It is over expressed in a wide variety of neoplasms[25,26] and is involved in experimental liver carcinogenesis. *In vitro*, a pathophysiological link between IGF2 over expression and hepatocyte proliferation was demonstrated by Lin *et al*[27], who found high concentrations of IGF2 in human hepatoma cell lines HuH7 and HepG2 and showed that antisense oligonucleotides complementary to *Igf2* mRNA reduced both *Igf2* mRNA and protein, in association with decreased cell proliferative activity. *In vivo*, Rogler *et al*[28] reported an increased frequency of HCC in *Igf2* transgenic mice and serum IGF2 has been recently proposed as a marker for human HCC to improve the diagnostic accuracy and sensitivity in patients with low serum AFP level[29].

Various epigenetic alterations have been reported in human cancers, including global DNA hypomethylation, gene hypomethylation and promoter hypermethylation, and *Igf2* loss of imprinting[30]. The *Igf2* gene is controlled by genomic imprinting, a non-Mendelian inherited epigenetic process that leads to the silencing of either a maternal or paternal allele[31,32]. In the liver, unlike in other tissues, its expression is monoallelic (maternally imprinted) during the fetal period and becomes biallelic thereafter. Early observations showed over expression of the *Igf2* gene in liver tumors and preneoplastic hepatic foci in different animal models as well as in human HCC[33,34]. This over expression is associated with re-expression of the fetal pattern of *Igf2* transcripts and restoration of monoallelic *Igf2* expression in preneoplastic hepatic foci[35] as well as in HCC[36], and with re-expression of monoallelic fetal promoters P2-P4[37] and loss of activity of the adult biallelic promoter P1[38]. One key factor of these epigenetic changes is the alteration of the genomic methylation pattern within regulatory Differentially Methylated Regions (DMRs) of imprinted genes, which inappropriately leads to loss of imprinting in the *Igf2* gene[39] and to transcriptional activation of the normally silent maternal allele. Hypomethylation at the *Igf2* locus has been found in many type of cancers, including ovarian, lung and colon[40]. In a previous study analyzing the methylation status of *Igf2* DMR2 in 71 liver samples from mostly viral HCC compared to 6 normal liver samples, we observed a hypomethylated profile at the *Igf2* locus in 89% of cases of HCC in contrast with the pattern observed in normal livers[41]. In addition, Cui *et al*[42] showed that hypomethylation of the *Igf2* gene in peripheral blood lymphocytes (PBL) is associated with a predisposition to colorectal cancer, suggesting that the epigenetic alteration of *Igf2* could be an early event in colorectal carcinogenesis.

The aim of the present study was to investigate whether hypomethylation at the *Igf2* locus in the liver is a predisposing factor for HCC in patients with HCV-related cirrhosis. Thus, we analyzed the methylation status of the *Igf2* gene spanning the 11p15 imprinted domain in patients with compensated HCV-related cirrhosis who were prospectively followed-up with periodic HCC screening.

MATERIALS AND METHODS

Patients

Ninety-four patients were retrospectively selected for this study among all patients hospitalized for liver biopsy between January, 1989 and December, 2000 in our department, based on the following criteria: (1) compensated (Child-Pugh A) HCV-related cirrhosis with presence of serum HCV RNA; (2) absence of viral co-infection by hepatitis B virus or human immunodeficiency virus; (3) regular follow-up until death with periodic HCC screening by liver ultrasonography and test of serum AFP levels every 6 mo at least; (4) absence of detectable HCC at enrollment; (5) available baseline frozen (-80℃) liver biopsy specimen for genetic study; (6) informed consent for genetic analysis obtained from the patient according to French guidelines.

Baseline demographic, clinical, biological and histological data (at time of liver biopsy) were recorded. All patients were prospectively followed-up. Complete physical examination, standard biochemical tests, serum AFP determination and abdominal ultrasonography were repeated every 6 mo. When a focal liver lesion or increased AFP levels were detected, tomodensitometry and, whenever possible, fine needle guided liver biopsy were performed. Diagnostic criteria for HCC were: (1) histological and (2) clinical, in patients with AFP value greater than 400 ng/mL and evidence of focal liver lesion at imaging techniques. After 2002, the HCC diagnosis was based on the guidelines of the European Association for the Study of the Liver[43].

Twenty-five histopathologically normal liver samples were also studied as control cases.

DNA extraction, bisulfite treatment of DNA and methylation analysis

DNA from frozen liver biopsies was extracted and treated with sodium bisulfite. Unbiased PCR amplification and Denaturing High Performance Liquid Chromatography (DHPLC) analysis were

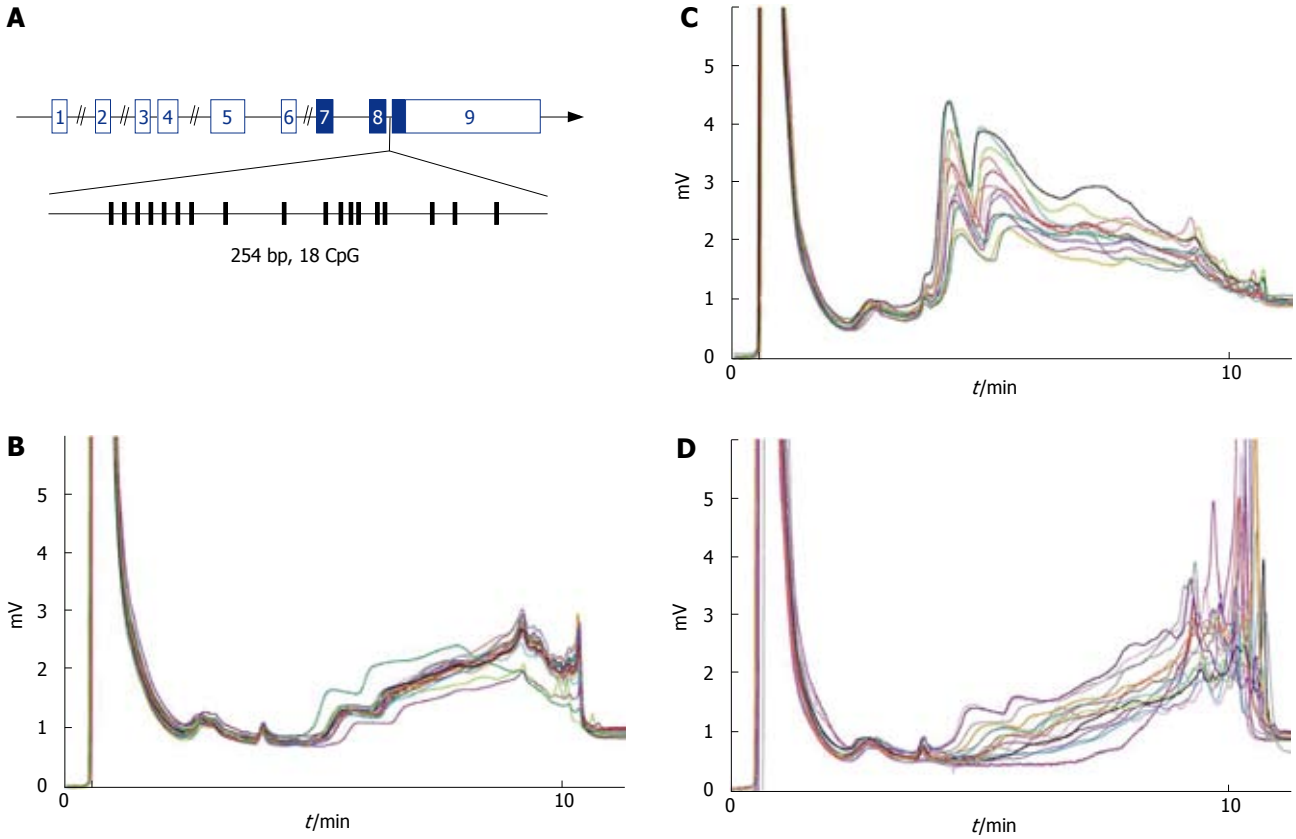

Figure 1 Methylation analysis of *Igf2* DMR2 in normal and HCV related cirrhosis livers. **A**: Exon-intron structure of *Igf2* gene. Exons are shown as numbered boxes (plain are coding). The 254bp fragment of *Igf2* DMR2 amplified for methylation analysis is enlarged below. Vertical lines indicate CpG positions. **B**: DHLPLC chromatograms of PCR products from normal liver samples. Twenty-two out of 25 are superimposable, and this major profile was used to assess hypermethylated profiles (ie more methylated than normal liver). **C** and **D**: DHPLC chromatograms of PCR products from HCV-related cirrhosis. Among 94 samples, 13 (**C**) and 17 (**D**) samples show respectively hypomethylated and hypermethylated profiles.

used for methylation analysis at the DMR of exon 8 and 9 of the *Igf2* imprinted gene (Figure 1A), as previously reported[42]. We amplified a DNA fragment encompassing 18 CpGs of the *Igf2* gene (Accession number AC005809; nt 43058-43312; 254 bp) by nested-PCR using the following primers: forward external 5'-GTTAAGAGGTTTTAGAGGTTATAGG-3', reverse external 5'-CCTTCCAAAACCTAACCTAAAAACA-3', forward internal 5'-GGGAAAGGGGTTTAGGAT-TTTTAT-3', reverse internal 5'-ATAATTTACTCCCCC-TTCAACCTC-3'. PCRs were performed in 3 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.5 μmol/L of each primer and 1.25 U of AmpliTaq Gold® DNA polymerase (Perkin Elmer, Norwalk, CT) under the following conditions: 94℃ for 10 min followed by 40 cycles of 94℃ for 45 s, 62℃ for 45 s, 72℃ for 1 min and a final extension step of 10 min at 72℃. After the first round of DNA amplification, a 1 μL aliquot of the PCR solution was used for the nested PCR.

Methylation profiles were studied by a newly developed DHPLC-based method, as previously described[44]. Briefly, DHPLC scanning was performed on an automated DHPLC instrument (WAVE®, Hitachi model D-7000. Chromatography Data Station Software, Transgenomic LTD Cheshire, UK); the column used was a DNAsep® Cartridge (Transgenomic, Santa Clara, CA) and the running temperature experimentally evaluated was 57℃. In a second step, methylation profiles of PCR products from liver biopsies were analyzed in comparison with PCR products from reference 100% methylated and 0% methylated control alleles, and with PCR product from normal liver biopsies. Fifteen microliter aliquots of the PCR products were eluted within a linear acetonitrile gradient. Because of the difference in retention times, the methylation patterns could be assessed by DHPLC independently of sequencing information by overlaying the DHPLC profiles with those of reference fragments. Methylation profiles were objectively classified in three categories as follows: samples displaying a higher proportion of methylated alleles than normal liver were considered as hypermethylated (M); samples which showed less methylation than normal liver were sorted according to the maximal absorbances of their first (4 min < retention time < 5 min, demethylated alleles) and last (10 min < retention time < 11 min, methylated alleles) elution specific peaks, by calculating R = [Abs (first peak) - Abs (baseline)]/[Abs (last peaks) - Abs (baseline)]; samples with R>2 (high proportion of demethylated alleles) were considered hypomethylated (U), the others being intermediate (UM). Reproducibility of the method was checked by double testing of randomly chosen

Table 1 Baseline characteristics in 94 patients with Child-Pugh A hepatitis C-related cirrhosis and defined methylation profile at the *insulin growth factor 2* gene: distribution and prognostic value for the occurrence of hepatocellular carcinoma at 10 years in univariate analysis

		Patients (n = 94)	No HCC (n = 74)	HCC (n = 20)	HR (95% CI)	P
Gender	Female	39	32	7	1	
	Male	55	42	13	1.38 (0.55-3.48)	0.49
Age (yr)		57.7 ± 13.7	56.0 ± 14.4	63.9 ± 8.0	1.05 (1.01-1.09)	0.01
BMI (kg/m^2)		25.0 ± 4.8	25.1 ± 4.8	24.7 ± 4.8	1.00 (0.90-1.11)	0.96
BMI ⩾ 30 kg/m^2	No	80	64	16	1	
	Yes	14	10	4	2.16 (0.72-6.51)	0.17
Diabetes	No	73	57	16	1	
	Yes	21	17	4	1.00 (0.33-2.98)	0.99
Oesophageal	0 or Ⅰ	78	63	15	1	
Varices grade	Ⅱ or Ⅲ	16	11	5	2.47 (0.89-6.83)	0.08
Bilirubin (μmol/L)		15.1 ± 8.8	14.6 ± 8.3	17.2 ± 10.4	1.05 (1.00-1.10)	0.05
Albumin (g/L)		41.1 ± 5.7	41.1 ± 5.6	41.0 ± 6.3	0.98 (0.90-1.06)	0.59
Prothrombin (%)		84.1 ± 16.2	85.2 ± 15.9	79.7 ± 16.9	0.97 (0.95-1.00)	0.04
Platelets (× 10^3/mm^3)		151 ± 61	155 ± 64	138 ± 51	1.00 (0.99-1.00)	0.29
ALAT (× ULN)		3.2 ± 2.5	3.2 ± 2.6	3.2 ± 1.8	0.97 (0.80-1.19)	0.80
ASAT (× ULN)		2.9 ± 2.1	2.8 ± 2.0	3.2 ± 2.5	1.10 (0.91-1.33)	0.33
GGT (× ULN)		2.8 ± 2.7	2.8 ± 2.9	2.7 ± 1.7	0.99 (0.84-1.16)	0.86
AFP (ng/mL)		16.4 ± 26.9	17.2 ± 29.2	13.3 ± 15.7	1.01 (0.99-1.02)	0.54
Knodell score		10.8 ± 2.2	10.6 ± 2.4	11.7 ± 2.4	1.23 (1.00-1.51)	0.06
Serum IGF2 (ng/mL)[1]		279.6 ± 114.3	291.4 ± 117.3	234.1 ± 92.1	0.99 (0.98-1.01)	0.08
Liver Igf2	U	13	9	4	7.64 (0.85-68.62)	0.07
Methylation profile	UM	64	49	15	3.98 (0.53-30.14)	0.18
(3 classes)	M	17	16	1	1	

Continuous values are used for quantitative parameters. HR: Hazard ratio; CI: Confidence interval; HCC: Hepatocellular carcinoma; BMI: Body mass index; ALAT: Alanine amino-transferase; GGT: Gamma glutamyl transferase; ULN: Upper limit of normal; AFP: Alpha-foetoprotein; IGF2: Insulin-growth factor 2. [1]Performed in 63 patients.

samples. To rule out interpretation bias, clinical database including outcome of the patients, especially in relation to the occurrence of HCC, was kept by clinicians (NG, PN, JCT, MB) and not available for molecular biologists (PC, AK, AM, JC).

IGF2 serum quantification

Frozen serum collected at enrollment and stored at -25℃ was available in 63 (67%) of the 94 included patients. In these patients, serum IGF2 was quantified using an enzymatic amplified "two step" sandwich-type immunoassay (active IGF2 ELISA, Diagnostic Systems Laboratories, Webster, USA). Each sample was duplicated and tested blindly.

Statistical analysis

Data were expressed as mean ± SEM and percentages. All means were compared using the Mann-Whitney rank-sum test or the Kruskal-Wallis nonparametric analysis of variance. Furthermore, continuous variables were transformed into binary information according to median and cut-off points. Associations were tested in 2 × 2 cross tabulations using the Fisher's exact test. In case of larger cross tabulations, and as appropriate according to the validity conditions, liaisons were tested by the Pearson's Chi-square, or by computing either the exact probability value or the Monte Carlo estimate of the exact probability value. The basic non parametric Kaplan-Meier method[45] was used to search for heterogeneity of time-dependent cumulative probabilities of HCC according to levels of methylation and a linear trend between HCC probability and ordered methylation levels. From then on and practically, we used a series of tests, the Log Rank (Mantel-Cox) test, the Breslow test (Generalized Wilcoxon), and Tarone-Ware test in the two situations in which we attempted to test the heterogeneity of HCC occurrence or a linear trend between HCC probability and ordered methylation levels[46]. As regards to heterogeneity of risk according to *Igf2* levels, the Cox regression[47] was used for the estimation of the Hazard Ratios and 95% CI intervals. The 0.05 probability level was used for all statistical significance. Statistical analyses were performed using SPSS software (SPSS 10.05, SPSS Inc., Chicago, IL) and STATXACT (StatXact, CYTEL Software Corporation, Cambridge, MA).

In Table 1, the expression HR = 1.05 (1.01-1.09) is linguistically awkward, henceforth, 1.05 indicates that with each extra year in age the estimated hazard is 1.05 times that for subjects one year younger. Another way to express this variation is to convert it into a percentage difference in hazard by using the expression 100 × (HR - 1). Then, 100 × (1.05 - 1) = 5% tells us that the HR of HCC is 5% higher for each additional year of age.

RESULTS

Characterization and interpretation of methylation profiles

Methylation profiles of normal liver samples were highly

Table 2 Baseline characteristics according to the methylation profile at the *Igf2* locus (U, UM, and M, respectively, for hypomethylated, normal and hypermethylated patterns) in patients with Child-Pugh A hepatitis C-related cirrhosis

	U (*n* = 13)	UM (*n* = 64)	M (*n* = 17)	Asymptotic global *P*-value
Male gender (%)	7 (53.8%)	38 (59.4%)	10 (58.8%)	0.934
Age (yr)	58.15 (16.71)	57.55 (13.00)	57.89 (14.41)	0.985
Alcohol (g/d)	43.08 (64.21)	22.28 (45.97)	38.24 (77.48)	0.551
Tobacco (Pack, yr)	3.8 (7.1)	6.1 (13.1)	5.7 (11.5)	0.981
BMI (kg/m^2)	26.32 (5.18)	25.41 (4.97)	23.86 (4.04)	0.244
Diabetes (%)	2 (15.4%)	14 (21.9%)	4 (23.5%)	0.883
Platelets (× 10^3/mm^3)	147.28 (64.46)	152.32 (63.71)	177.30 (127.43)	0.958
Prothrombin (%)	79.46 (17.81)	84.58 (16.59)	85.59 (13.28)	0.433
Albumin (g/L)	40.45 (4.96)	40.77 (6.27)	42.76 (3.40)	0.437
Bilirubin (μmol/L)	14.58 (9.66)	15.16 (8.85)	15.50 (8.45)	0.731
ALAT (× ULN)	3.23 (2.13)	3.23 (2.61)	3.14 (2.16)	0.961
AFP (ng/mL)	13.58 (11.24)	13.88 (18.57)	28.21 (51.52)	0.530
Serum IGF2 (ng/mL)[1]	249.64 (81.23)	276.32 (116.97)	321.55 (129.24)	0.393
OV grade Ⅱ or Ⅲ (%)	5 (38.5%)	9 (14.1%)	2 (11.8%)	0.084
Knodell score (mean, SD)	11.6 (1.8)	10.6 (2.3)	10.7 (2.5)	0.414
HCV genotype 1[2] (%)	7 (63.6%)	41 (78.8%)	8 (53.3%)	0.125

Quantitative variables are expressed as means (SD). BMI: Body mass index; ALAT: Alanine amino-transferase; ULN: Upper limit of normal; AFP: Alpha-foetoprotein; IGF2: Insulin growth factor 2; OV: Esophageal varices; HCV: hepatitis C virus. [1]Performed in 63 patients, [2]known in 78 patients (11, 52, 15 patients respectively in U, UM and M groups).

similar (Figure 1B) and 22 of 25 were superimposable. Among 94 tested patients, 13 (14%) were considered as hypomethylated (U), 64 (68%) as intermediate (UM) and 17 (18%) as hypermethylated (M) (Figure 1C). All double tested samples showed similar results in both experiments (data not shown).

Baseline patient characteristics

The main characteristics of patients at enrollment were not significantly different according to the methylation profile at the *Igf2* locus as shown in Table 2. All patients but 2 were Caucasians (1 from Africa with M profile and 1 from Asia with UM profile). In addition, the proportion of patients who received antiviral treatment during the study (72.7% in U, 58.8% in UM and 61.7% in M; $P = 0.738$), the proportion of sustained responders (22.2% in U, 31.4% in UM and 40.0% in M; $P = 0.707$), the mean follow-up (5.20 ± 3.63 years in U, 7.21 ± 3.09 years in UM, 7.22 ± 3.04 years in M; $P = 0.198$), were not statistically different between the three groups.

IGF2 serum quantification

Each IGF2 serum level measurement was duplicated and results were reproducible in 98% of cases. The mean serum value was 279.6 ng/mL (range, 36-640) without any significant difference between patients with U, UM or M methylation profiles at the *Igf2* locus (Table 2).

Predictive value for HCC

During a mean follow-up of 6.9 ± 3.2 years, 20 patients developed an HCC (4, 15 and 1 cases, in patients with U, UM and M methylation profile, respectively). The cumulative incidence of HCC at 10 years reaches 30.8% in patients with a U profile, 23.4% in patients with a UM profile, and 24.7% in patients with either a U or a UM profile in contrast with 5.9% only in patients with a M profile.

In the Cox analysis testing successively each of the 17 baseline studied variables, two were predictive for the occurrence of HCC: age at liver biopsy ($P = 0.01$; HR, 1.05; 95% CI, 1.01-1.09) and prothrombin time ($P = 0.04$, HR, 0.97; 95% CI, 0.95-1.00; Table 1). Moreover, a clear trend ($0.05 < P < 0.1$) was observed for 5 baseline variables: bilirubin, esophageal varices, Knodell score, serum IGF2 level and liver *Igf2* hypomethylation.

When patients with a U profile were compared in a paired way to those with M profile, the Log-Rank test was significant ($P = 0.047$). Moreover, the test for linear tendency between the 3 ordered levels of *Igf2* methylation and cumulative probability of HCC occurrence was significant (Log Rank, $P = 0.043$; Breslow, $P = 0.037$; Tarone-Ware, $P = 0.039$; Figure 2).

DISCUSSION

A growing body of evidence underlines that both DNA hypomethylation, leading to genomic instability, and regional CpG hypermethylation, leading to silence tumor suppressor gene, are dominant events during HCC development[48]. Calvisi *et al* recently showed that the extent of genome-wide hypomethylation progressively increased from non-neoplastic surrounding liver to fully malignant HCC[49], indicating that genomic hypomethylation is an important prognostic factor in HCC and opens the possibility of using molecular targets for chemoprevention or

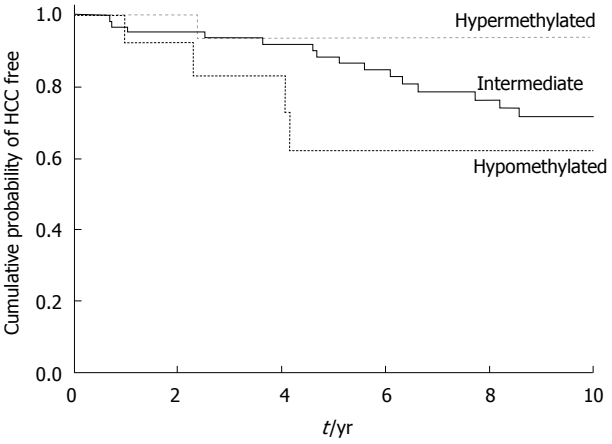

Figure 2 Occurrence of hepatocellular carcinoma at 10 years according to the methylation profile at the *Igf2* gene in 94 patients with Child-Pugh A hepatitis C-related cirrhosis (Kaplan-Meier method). Test of heterogeneity of HCC distributions: (log-rank test) $P = 0.13$. Test of the linear trend between levels of *Igf2* methylation and corresponding survival functions: Breslow (Generalized Wilcoxon) $P = 0.037$.

treatment of HCC. Regarding *Igf2* locus, we have observed hypomethylation at *Igf2* exon 8-9 in 90% (28/31) of HCV associated HCC, in contrast to the normal methylation pattern of two other genes located in the same area, the 11p15 locus[41]. This indicates that alterations in the IGF2 pathway are a pivotal event in hepatocarcinogenesis, at least in patients with HCV-related cirrhosis.

Our results suggest a possible link between *Igf2* hypomethylation in the liver of caucasians with uncomplicated and compensated HCV cirrhosis and the further occurrence of HCC. We observed a significant increased cumulative incidence of HCC at 10 years in patients with a hypomethylated pattern compared with those with a hypermethylated profile (30.8% *versus* 5.9%, $P = 0.047$) and a significant linear tendency between the ordered levels of *Igf2* methylation and the probability of HCC occurrence. The other variables identified in our patients were 2 well-known predictive factors for the occurrence of HCC: age[14-17] and prothrombin time related to liver failure[12,16-18] (Table 1). Due to a low number of patients, we could not show significant link with other known predictive factors (esophageal varices and bilirubin serum levels $0.05 < P < 0.08$). Conversely, as previous studies in patients with HCV-cirrhosis, we did not identify male gender[16,19], high AFP serum level[14,17], low platelet count[14,16,19] and diabetes[21] as predictive factors for the occurrence of HCC. These results, observed in Caucasians, may be different in other ethnic groups. However, IGF2 overexpression in HCC, which is mainly due to aberrant activation of the epigenetically regulated *Igf2* promoters, seems to be independent of ethnic origins. In addition to hypermethylation of promoters of several tumor suppressing genes found even in premalignant conditions, *Igf2* hypomethylation could thus contribute to the multistep process leading to malignant transformation[50].

This link between *Igf2* methylation and HCC occurrence should be validated in an external independent cohort. It may be underestimated in this study for several reasons. First, although the cumulative incidence was as high as previously reported in HCV-cirrhosis, the relative number of patients who further developed HCC ($n = 20$) is low. Secondly, being given cirrhotic liver heterogeneity and the small size of liver samples obtained by fine needle percutaneous biopsy, the extent of *Igf2* hypomethylation could have been underestimated. In addition, as samples were not microdissected, we analyzed not only hepatocytes, but also a variable amount of other minority hepatic cell types, such as sinusoidal cells and Kupffer cells, which may not share the same methylation pattern. Lastly, *Igf2* hypomethylation could be a late event in hepatocarcinogenesis, present to a low extent in uncomplicated cirrhosis and occurring later with the onset of liver failure and/or portal hypertension.

If there is a true link, whether these altered methylation patterns at *Igf2* locus lead to significant changes in expression profile and the function of genetic networks, or whether these changes just indicate severe epigenetic disturbances, remains to be investigated. A link between increased IGF2 expression and HCV infection has already been reported, showing that IGF2 over expression is significantly associated with HCV replication in patients with HCV-related cirrhosis[51]. The persistent process of hepatocyte damage and regeneration in HCV chronic hepatitis could provoke uncontrolled growth of hepatocytes and lead to malignant transformations due to disruption of growth regulation or mitogenic factors. However, whether HCV plays a direct or indirect role in IGF2 deregulation remains unknown. One could wonder if the link between Igf2 and HCC could be mediated by diabetes and metabolic syndrome. Indeed, epidemiological association between diabetes mellitus and HCC has been corroborated by molecular studies related to IGF1 or Igf Binding Protein 3. However, conversely to IGF1, IGF2 is mainly a fetal protein and its insulin-like metabolic effects in the post-natal period remains uncertain[52].

In these experiments, epigenetic changes in *Igf2*, potentially leading to re-expression of its fetal pattern, could be considered in parallel with AFP over expression as the hallmark of some fetal characteristics in the cirrhotic liver. The lack of correlation between the *Igf2* intron 8-9 methylation profile and IGF2 serum levels may be explained by an IGF2 local over expression leading to an autocrine effect, as previously suggested by Cariani *et al*[53].

Our observation that hypomethylation at the *Igf2* exon 8-9 is present in 14% of patients (13/94) with uncomplicated HCV-related cirrhosis and associated with a trend of overrisk of cancer are comparable to recent studies in the field of colorectal cancer[42,54]. Cui

et al[42] observed *Igf2* hypomethylation in normal colonic mucosa in 30% of patients with colorectal cancer in contrast to 10% in healthy patients. Moreover, *Igf2* hypomethylation is present in mesoderm-derived PBL and abnormal methylation profiles in this tissue are also highly correlated with both familial and personal histories of colorectal cancer. The prevalence of abnormal methylation patterns in PBL increases from 6.5% in patients with no personal history of colorectal cancer to 23% and 28%, respectively in patients with a personal history of adenoma or a family history of colorectal cancer, and to 56% in patients with colorectal cancer. These facts support a possible role of epigenetic changes of *Igf2* in the early steps of colorectal carcinogenesis. The most obvious unanswered question is whether the *Igf2* hypomethylation profile in PBL could be constitutive, resulting from inherited genetic mutations, or due to environmental events leading to epigenetic alterations. To try to answer this question in the field of HCC, further studies are ongoing in the PBL of a large cohort of patients with HCV-related cirrhosis screened for HCC and in healthy controls. If the results are comparable to those in patients with colorectal cancer, this may have clinical implications for defining high risk patients for HCC eligible for intensive screening and/or to reduce their risk with the use of dietary and/or therapeutic agents developed to reverse the epigenetic alterations such as methylated-oligonucleotides[55-57].

COMMENTS

Background

Hepatocellular carcinoma (HCC) is considered the fifth most frequent malignant neoplasm worldwide. In high incidence areas, it is strongly associated with viral hepatitis B and C and liver cirrhosis. Identification of molecular abnormalities associated with an increased risk of HCC is particularly important to improve knowledge of both the pathways of liver carcinogenesis and the outcomes.

Research frontiers

Insulin-like growth factor 2 (IGF2) is a fetal growth peptide produced by the liver, which is over expressed in a wide variety of neoplasms including HCC and is involved in experimental liver carcinogenesis. In a previous work analyzing the methylation status of *Igf2* in 71 HCC liver samples, we observed an hypomethylated profile in 89% of HCC.

Innovation and breakthroughs

Not only can *Igf2* hypomethylation be observed in HCC liver samples, but also in premalignant hepatitis C cirrhotic livers. In this case, *Igf2* hypomethylation is associated with a higher risk of HCC occurrence than *Igf2* hypermethylation.

Applications

Studies examining the Igf2 methylation status in hepatitis C cirrhotic liver could help identify patients with a high risk and patients with a low risk of HCC occurrence.

Peer review

This is a well designed study with interesting results. These results suggest that hypomethylation at the *Igf2* locus in the liver could be predictive for HCC occurrence in HCV cirrhosis.

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RAPID COMMUNICATION

Distribution of secretory inhibitor of platelet microbicidal protein among anaerobic bacteria isolated from stool of children with diarrhea

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Abstract

AIM: To study the secretory inhibitor of platelet microbicidal protein (SIPMP) phenotypes of faecal anaerobic isolates from patients with diarrhea.

METHODS: Faecal isolates of anaerobic bacteria (*B. fragilis*, n = 42; *B. longum*, n = 70; *A. israelii*, n = 21; *E. lentum*, n = 12) from children with diarrhea were tested. SIPMP production was tested by inhibition of platelet microbicidal protein (PMP) bioactivity against *B. subtilis* and was expressed as percentage of inhibition of PMP bactericidal activity.

RESULTS: Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of 13.8% ± 0.7%, 14.7% ± 1.8%, 3.9% ± 0.9% (P < 0.05) and 26.8% ± 7.5% (P < 0.05) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis,* respectively.

CONCLUSION: Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in the intestine, as well as for future improvement in the prevention and therapy of anaerobe-associated infections.

Key words: Platelet microbicidal protein; Secretory inhibitor; Anaerobic bacteria; Intestine

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INTRODUCTION

Anaerobic microorganisms are important constituents of human intestinal microbiota[1]. Enzymes produced by these bacteria provide nutrients for growth, participate in the pathogenesis of infections involving these bacteria, *etc.* Infections caused by anaerobic bacteria are increasingly being recognized as a major problem in clinical medicine[2,3]. The commensal anaerobic bacterial flora of the colon may undergo changes during diarrhea, owing to colonization of the intestine by pathogens and to rapid intestinal transit[4]. As it is difficult to establish exactly the significance of various anaerobic microorganisms in the pathogenesis of infections, it is imperative to delineate both microbial and host factors that contribute to its development. Identifying such a factor(s) produced by anaerobes is important for understanding and possibly modulating interactions between these bacteria and the host.

The intestinal mucosa forms a primary barrier providing both barrier function and immediate effective recognition of bacterial products invading the mucosa. This is of great importance for the prevention of permanent and chronic inflammation as a reaction to the commensal intestinal flora and the multitude of antigens present in the intestinal lumen[5].

The major role of endogenous cationic antimicrobial peptides in preventing the onset of infection has been emphasized recently[6,7]. In mammals, these peptides have evolved to have a central function in the host defense properties of granulocytic leukocytes, mucosal surfaces, skin and other epithelia[7]. Antibacterial protection of intestinal mucosa is provided in part by Paneth cell-derived antibacterial peptides[8-10]. Such peptides have also been found by several authors in human platelets

and are designated platelet microbicidal proteins (PMPs)[11,12]. These peptides are secreted at sites of infection and exert microbicidal activity against many pathogens[11]. Bukharin *et al* showed that an enhanced level of PMP in coprofiltrates of patients is associated with *Salmonella* gastroenteritis[13]. However, we suspect that successful pathogens (especially anaerobes) would have involved mechanisms to resist or degrade the inhibitory and microbicidal activities presented by the host. For example, in a recent publication[14], we reported the detection of an extracellular staphylococcal product, designated secretory inhibitor of platelet microbicidal protein (SIPMP), which causes local inhibition of the bactericidal action of PMP in the fluid phase. We also demonstrated that SIPMP represents a hitherto unrecognized determinant of staphylococcal pathogenicity and SIPMP production is associated with a prostatitis source.

At the same time, it is surprising that no extracellular product of anaerobic microorganisms with remarkable anti-PMP potential has been described. Thus, in this communication we report on *in vitro* detection of SIPMP phenotypes of faecal anaerobic isolates from patients with diarrhea.

MATERIALS AND METHODS

Clinical isolates were obtained from diarrhea stool samples collected from April to December 2000 in Orenburg Regional Child Hospital. A total of 145 strains of anaerobic bacteria (*B. fragilis*, $n = 42$; *B. longum*, $n = 70$; *A. israelii*, $n = 21$; *E. lentum*, $n = 12$) were kindly provided by Natalia Elagina (Department of Dysbiosis, Institute of Cellular and Intracellular Symbiosis). Bacteria were isolated from children with diarrhea (ranging from 10 mo old to 6 year old) and identified at the Anaerobe Laboratory, Department of Dysbiosis, Institute of Cellular and Intracellular Symbiosis, Russian Academy of Sciences, Orenburg.

PMP was prepared and standardized as described previsiously[12].

SIPMP production was performed by viable counting according to the recently proposed procedures[14]. Strains were grown in brain heart infusion broth (BHI, Oxoid), supplemented with yeast extract (0.5%) under anaerobiosis conditions (90% N_2/10% CO_2), at 37℃, for 48 h and cell-free supernatants were obtained by centrifugation. Bacterial supernatants were sterilized by filtration *via* 0.45 μm pore-size membranes (Millipore). Each culture supernatant (0.6 mL) (an equal volume of BHI was loaded in the control tubes) was combined with 0.3 mL of PMP at 3.0 μg/mL and incubated at 37℃. After 1 h, 100 μL *B. subtilis* suspension at 10^4 CFU/mL was added to each of the tubes. The tubes were incubated on a rotary shaker (300 r/min) at 37℃. After 1 h, aliquots of 200 μL were plated on blood agar plates. Colonies were counted after incubating overnight at 37℃ and numbers of surviving microorganisms were calculated. The SIPMP production as expressed in percentage of inhibition of PMP bactericidal activity and calculated by using the formula: % inhibition = (No. - Nk1) × 100/(Nk2 - Nk1), where No. was the number of surviving *B. subtilis* cells in the presence of bacterial supernatant and PMP, Nk1 was the number of surviving *B. subtilis* cells in the presence of PMP alone, and Nk2 was the number of surviving *B. subtilis* cells in BHI.

All of the experiments were carried out in triplicate and mean values and SEM were calculated. The differences between groups of microorganisms were assessed by using Student's *t*-test. $P \leqslant 0.05$ was considered significant.

Table 1 SIPMP production of fecal isolates of anaerobic bacteria *n* (%)

Organism	No. of SIPMP-producing strains (total/%) with different levels of SIPMP[1]			
	0	0.1-10.0	10.1-20.0	> 20
B. longum ($n = 70$)	14 (20)	0 (0)	56 (80)	0 (0)
A. israelii ($n = 21$)	3 (14.3)	0 (0)	18 (85.7)	0 (0)
E. lentum ($n = 12$)	6 (50)	6 (50)	0 (0)	0 (0)
B. fragilis ($n = 42$)	3 (7.14)	0 (0)	2 (4.76)	37 (88.1)

[1]SIPMP was expressed in percentage of inhibition of PMP bactericidal activity.

RESULTS

For exclusion the cooperative inhibitory effect of PMP and culture supernatants on *B. subtilis*, each culture supernatant was combined with *B. subtilis* suspension. After coincubation for 1 h, aliquots were plated on blood agar plates. Colonies were counted after incubating overnight at 37℃ and numbers of surviving microorganisms were calculated. None of the supernatants tested inhibited growth of *B. subtilis* cells. The stability of SIPMP was tested by subjecting culture supernatants to boiling for 30 min. This treatment completely destroyed the biological activity of SIPMP. Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive (Table 1). The extracellular products of bacteria reduced the PMP-induced killing of *B. subtilis*. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of 13.8% ± 0.7%, 14.7% ± 1.8%, 3.9% ± 0.9% ($P < 0.05$) and 26.8% ± 7.5% ($P < 0.05$) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis* respectively.

DISCUSSION

At local sites of microbial infections, epithelial cells, platelets, neutrophils, or macrophages release large amounts of different bactericidal peptides[7]. However, most infections are the result of contamination of host tissues with anaerobic flora from the gut[15] despite the presence of multiple antibacterial peptides in intestinal cells and mucus[7,16,17]. There is an urgent need to understand the virulence properties of anaerobic

organisms that may take part in their resistance to cationic antimicrobial peptides; identifying such a factor(s) would be helpful in devising effective treatment strategies.

In the present work, we detected an extracellular bacterial product of anaerobic microorganisms with remarkable anti-PMP potential which, to our knowledge, has not been described before. We anticipate that SIPMP serves to protect invading bacteria by inducing local consumption of PMP in the fluid phase. The strategy underlying this process would be straightforward and effective. We believe that SIPMP represents a widespread and hitherto unrecognized determinant of bacterial pathogenicity. Similarly, in a study of distribution of streptococcal inhibitor of complement variants in pharyngitis and invasive isolates by Hoe *et al*[18], 62% of group A streptococci from patients with pharyngitis produced this extracellular protein. Collectively, our study and the results of several studies[19-21] suggest that the inactivation of components of innate immunity may be important for bacterial pathogens to induce and perpetuate infections of different localization by surviving or avoiding microbicidal proteins mediated clearance. Bacteria-derived proteases may contribute to mucosal surface destruction, and are likely to impair host defense by degrading antimicrobial peptides[22]. It was confirmed by the fact that the lowest level of SIPMP production was observed with the non-protease producing species *E. lentum*. On the other hand, proteases of anaerobic microorganisms caused platelet aggregation with followed by release of a number of antibacterial proteins[11,23].

In contrast to *B. fragilis*, normal microflora have low levels of SIPMP. Hypothetically, the constituents of normal flora must have basal levels of resistance to the antimicrobial host defense factors. It is possible that low levels of inactivation of PMP activity by normal organisms are sufficient to protect them from PMP-dependent killing, thus providing stability of intestinal microflora. We believe that SIPMP is the stable characteristic and the same strains express more SIPMP in case of infection. On the other hand, our results suggest that the normal microflora was replaced by other organisms with pronounced pathogenic properties in patients with persistent infection[24].

At the same time, in the presence of infections, properties of normal microflora probably could change. The constituents of normal microflora, receiving signs of pathogenicity, are capable of causing diseases, as has been shown for lactobacilli and staphylococci[24,25].

The predominantly anaerobic microbiota of the distal ileum and colon contain an extraordinarily complex variety of metabolically active bacteria that intimately interact with the host's epithelial cells and mucosal immune system[26]. Crohn's disease, ulcerative colitis, and pouchitis are the result of continuous microbial antigenic stimulation of pathogenic immune responses as a consequence of host genetic defects in mucosal barrier function, innate bacterial killing, or immunoregulation. Identification of these host and microbial alterations in individual patients should lead to selective targeted interventions that correct underlying abnormalities and induce sustained and predictable therapeutic responses[27]. New treatment strategies aim at neutralization of such pathogenic properties of microorganisms as pronounced resistance to the cationic antimicrobial peptides and/or ability to inhibit the antimicrobial host defense factors and thereby improve the quality of life in patients[28-30].

Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in intestine, as well as for future improvement in the prevention of and therapy for anaerobe-associated infections. However, the exact mechanism of PMP inhibition in anaerobic bacteria remains to be determined, as does its molecular characteristics, occurrence and possible significance *in vivo*.

COMMENTS

Background

Anaerobic microorganisms are important constituents of human intestinal microbiota. Infections caused by anaerobic bacteria are increasingly being recognized as a major problem in clinical medicine. The commensal anaerobic bacterial flora of the colon may undergo changes during diarrhea, owing to colonization of the intestine by pathogens and to rapid intestinal transit. The major role of endogenous cationic antimicrobial peptides in preventing the onset of infection has been emphasized recently. Such peptides have been found in platelets and are designated platelet microbicidal proteins (PMPs). It is shown that an enhanced level of PMP in coprofiltrates of patients is associated with *Salmonella* gastroenteritis. Here we made an attempt to *in vitro* detection of secretory inhibitor of platelet microbicidal protein (SIPMP) phenotypes of faecal anaerobic isolates from patients with diarrhea.

Research frontiers

The article focuses on inhibition of PMP by extracellular bacterial products of faecal anaerobic microorganisms isolated from stool of children with diarrhea. Among anaerobic isolates 80% of *B. longum* strains, 85.7% of *A. israelii* strains, 50% of *E. lentum* strains and 92.86% of *B. fragilis* strains were SIPMP-positive. The isolated anaerobic organisms demonstrated SIPMP production at a mean level of 13.8% ± 0.7%, 14.7% ± 1.8%, 3.9% ± 0.9% ($P < 0.05$) and 26.8% ± 7.5% ($P < 0.05$) for bifidobacteria, *A. israelii*, *E. lentum* and *B. fragilis*, respectively.

Innovations and breakthroughs

In the present work, the authors detected an extracellular bacterial product of anaerobic microorganisms with remarkable anti-PMP potential that has not been described before. SIPMP represents a widespread and hitherto unrecognized determinant of bacterial pathogenicity.

Applications

Data from the present study may have significant implications in understanding the pathogenesis of microecological disorders in intestine, as well as for future improvement in the prevention and therapy of anaerobe-associated infections.

Terminology

PMP is a group of small cationic peptides isolated from rabbit and human platelets after stimulation by acid or thrombin; the secretory inhibitor of PMP is an extracellular bacterial product with anti-PMP activity.

Peer review

In this manuscript, the authors reported the detection of SIPMP phenotypes of faecal anaerobic isolates from patients with diarrhea. The study was well performed and interesting.

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RAPID COMMUNICATION

Is there a role for Tc-99m (V) DMSA scintigraphy in ischemic colitis?

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Author contributions: Stathaki MI, Koukouraki SI, Karkavitsas NS performed and evaluated the scintigraphic images; Koutroubakis IE, Kouroumalis EA collected all clinical data; Stathaki MI, Koutroubakis IE analyzed the data and wrote the paper.
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Abstract

AIM: To evaluate the role of pentavalent Tc-99m dimercaptosuccinic acid [Tc-99m (V) DMSA] in the diagnosis of ischemic colitis.

METHODS: Fourteen patients with endoscopically and histologically confirmed ischemic colitis were included in the study. Tc-99m (V) DMSA scintigraphy was performed within 2 d after colonoscopy. Images were considered positive when an area of increased activity was observed in the region of interest and negative when no abnormal tracer uptake was detected.

RESULTS: In 3 out of the 14 patients, Tc-99m (V) DMSA images showed moderate activity in the bowel. The scintigraphic results corresponded with the endoscopic findings. In the other 11 patients, no abnormal tracer uptake was detected in the abdomen.

CONCLUSION: Besides the limited number of patients, Tc-99m (V) DMSA could not be considered as a useful imaging modality for the evaluation of ischemic colitis.

Key words: Scintigraphy; Technetium-99m pentavalent dimercaptosuccinic acid; Ischemic colitis; Intestinal ischemia; Diagnosis

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INTRODUCTION

Ischemic colitis (IC) initially was described by Boley *et al* in 1963[1] and represents the most common form of gastrointestinal ischemia[2]. It is presented either as an occlusive or a nonocclusive form, usually seen in the elderly population with associated co-morbid factors[2-4]. Its pathophysiologic characteristic is the sudden loss of blood flow and the extent of damage is proportional to the degree and the duration of tissue hypoxia[5,6]. Moreover the disruption of the mucosal barrier may lead to inflow of intraluminal bacteria and toxins from the gut[3]. The clinical spectrum ranges from transient self-limited ischemia with brief episodes of abdominal pain and rectal bleeding to fulminant transmural necrosis, perforation and death[5,6]. The histological findings include mucosal necrosis and ulcerations, submucosal edema and haemorrhage or transmural infarction[2,3,5].

The identification of colonic ischemia is highly dependent upon clinical suspicion[2,7]. Although invasive, colonoscopy and colonic biopsies have become the standard for diagnosing ischemic colitis[6,8].

The radionuclide imaging in IC is an area under investigation. Since mucosal inflammatory changes often coexist with bowel ischemia, radiotracers used to localise inflammation could probably play an important role in the diagnosis of IC. A few reports of the scintigraphic findings using radionuclide labelled leukocytes have been published[9-12]. Moreover, recently pentavalent Tc-99m dimercaptosuccinic acid [Tc-99m (V) DMSA] has been successfully used in the identification of intestinal inflammation[13-15]. To our knowledge, its role in the diagnosis of intestinal ischemia has not been yet reported in the literature.

The aim of the present study was to determine

whether Tc-99m (V) DMSA scintigraphy could provide an alternative non-invasive imaging modality in the diagnosis of IC.

MATERIALS AND METHODS

Patients

We examined fourteen patients, (5 males and 9 females, mean age 70.6 years) with clinically, endoscopically and histologically confirmed IC. All patients included were admitted at the Department of Gastroenterology of the University Hospital Heraklion, Crete, Greece, within two days after the onset of symptoms. The patients were non-surgically treated and non-received medical therapy that would interfere with scintigraphic results. Half of the patients reported daily tobacco use. Endoscopic assessment was performed the day following the hospital admission in all cases.

Concerning the disease type, transient IC was the most frequent presenting in 9 patients (62.4%) followed by reversible ischemic colopathy in 3 patients (21.4%), chronic ulcerative IC in 1 patient (7.1%) and ischemic colonic stricture in 1 patient (7.1%).

The lesions were distributed depending on their locations: splenic flexure in 8 patients (57.1%), rectosigmoid in 3 patients (21.4%), right colon in 2 patients (14.2% and extensive IC in 1 patient (7.1%)

All patients were subjected to standard laboratory tests such as red and white blood cell counts, haemoglobin and hematocrit level, platelet count, albumin level, erythrocyte sedimentation rate and C-reactive protein level. Colonoscopy with biopsies was performed in all patients. The endoscopic findings for each bowel segment were evaluated by blinded specialists.

The study was approved by the ethics committee and patients were provided with an informed consent.

Scintigraphic imaging

Tc-99m (V) DMSA scintigraphy was performed in fourteen patients with IC. In all patients scintigraphy was performed within 2 d after colonoscopy, in order to avoid any variation in disease activity. Imaging was performed after intravenous administration of 555 MBq (15 mCi) of Tc-99m (V) DMSA. A gamma-camera (Millenium; GE Medical Systems, Milwaukee, Wis) equipped with a low energy all purpose collimator was used. With the patient in the supine position, planar views of the abdomen were obtained 4 hours after radiotracer injection. Before scanning patients were asked to void their bladders to avoid false results.

The bowel was divided to five segments: small bowel (A), ascending colon (B), transverse colon (C), descending colon (D) and rectosigmoid (E). Images were considered positive when an area of increased uptake was observed and negative when no abnormal tracer uptake was detected in any of the five segments. In the event of a positive result, semi quantitative measurements were included with reference to the uptake in the iliac crest and was graded as: 0: No uptake in the region of interest, 1: Faint uptake less than the iliac crest bone marrow, 2: Moderate uptake similar to that of the iliac crest, 3: Severe uptake greater than the iliac crest bone marrow.

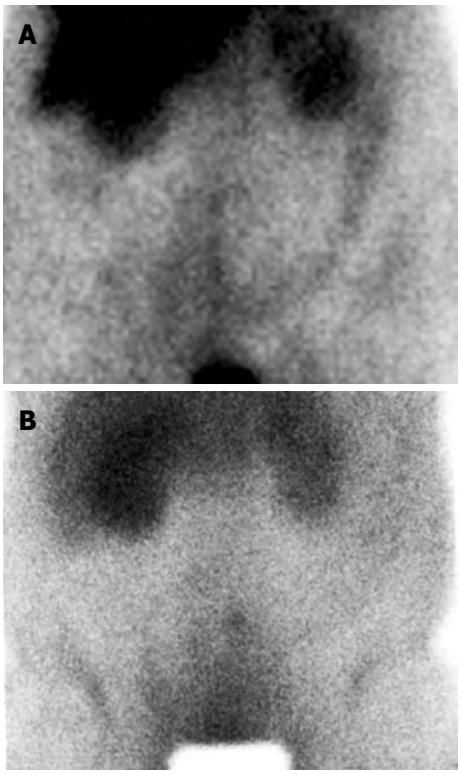

Figure 1 Anterior Tc-99m (V) DMSA scintigram in a patient with ischemic colitis: Moderate uptake of the radiotracer in the splenic flexure and the descending colon (**A**) and no uptake of the radiotracer in the bowel (**B**).

Scintigrams were blindly evaluated by three nuclear medicine physicians (MIS, SIK, NSK) and the results were compared with endoscopic and clinical data.

RESULTS

No patients showed adverse events during or after scintigraphy. A total of fourteen patients were included in this study. In all patients, endoscopy revealed characteristic findings suggestive of IC, which was histologically confirmed. In three out of the fourteen patients, the Tc-99m (V) DMSA scintigraphy demonstrated moderate uptake in the bowel (Figure 1A). Compared to endoscopic findings, radionuclide images were in agreement with the segments concerned. In the other eleven patients, Tc-99m (V) DMSA revealed no increased uptake in the bowel at all (Figure 1B).

Positive cases included one case with localization in the splenic flexure, one with ischemic rectosigmoiditis and one with extensive IC. Concerning the disease type among positive cases there were two cases with transient IC and one with chronic ulcerative IC. The small number of cases does not permit further statistical analysis.

The calculated sensitivity was only 21.4%. Owing to the low sensitivity and the false-negative results the study was stopped.

DISCUSSION

The most important finding in our study was that the majority of the patients with IC, who underwent Tc-99m (V) DMSA scintigraphy, yielded false- negative results. Only three of them showed increased tracer uptake in the regions of interest which corresponded with the findings at colonoscopy.

The pathophysiologic basis of IC is the sudden loss of blood supply. Localized low flow states involve mostly the splenic flexure and the rectosigmoid junction while systemic low flow states involve mostly the right colon, follow a relatively benign course and may affect younger patients[4,5,7,8].

Colonic ischemia may be precipitated by several conditions, such as shock, colon cancer, surgical intervention on the aorta or the mesenteric vessels, autoimmune disease, coagulopathies, long-distance running, constipation, illicit drug use and medications[2,4,6]. Recent studies have suggested a role of prothrombotic disorders in the development of IC[16,17].

No test specific for IC has yet been developed. The diagnosis of IC depends on the clinical evaluation of the patient in association with the biochemical, radiological, endoscopic and histologic assessment[18]. Most laboratory tests will be normal usually, yet in the event of abnormal results they have been found nonspecific[3,5,8].

Radiological evidence includes a wide spectrum of findings, which are frequently nonspecific, insensitive, and often they cannot easily differentiate ischemic from other forms of colitis[5,7,8].

Endoscopic assessment is the most sensitive and specific method of evaluating the colon for ischemic injury. The visual inspection and the ability to biopsy the mucosa allow the clinician in the majority of cases to make a firm diagnosis[6,8].

CT imaging has been employed in the evaluation of patients with abdominal pain of unknown etiology. Besides its limitations, it may be used to detect abnormalities and suggest the diagnosis, exclude other serious medical conditions and narrow the differential diagnosis[19-21].

Non-invasive Doppler sonography has been used as well. Although a high specificity has been reported, it is limited by overlying bowel gas, operator dependent quality and poor sensitivity for low flow vessel disease[3,5,8].

More recently, scintigraphic methods have been used in the diagnosis of IC. In-111 or Tc-99m labeled leukocyte scintigraphy has been studied and demonstrated successful imaging of bowel infarction, yet the localization mechanism still remains unclear. It is suggested that the presence of polymorphonuclear leukocytes in the inflammatory response to tissue ischemia, as a result of reperfusion injury may play the primary role[9-12]. However, the time-consuming preparation procedure, the handling and the reinjection of blood constitute shortcomings of radiotracer labeled leukocyte imaging.

Tc-99m (V) DMSA is a low-molecular weight complex that has been used successfully in the scintigraphic diagnosis of inflammation[22,23]. The suggested mechanisms of uptake by inflammatory lesions is either the infiltration into the interstitial space caused by increased capillary permeability or its similar behavior to phosphate ion since it seems to accumulate in areas where calcification is present[22,23].

Its role in the evaluation of intestinal inflammation has been already reported[13,14], moreover when compared to Tc-99m HMPAO labeled leukocytes, it seems to provide a useful, non-invasive, practical, easy to prepare and accurate alternative method for the assessment of disease activity in patients with IBD[15].

Based on the simultaneous presence of inflammatory response to tissue ischemia, we assumed that Tc-99m (V) DMSA could localize successfully the ischemic bowel and assist in the detection and diagnosis of IC.

The results of our study stand in contrast with the aforesaid assumption. The expected abnormally increased uptake was detected only in three out of the fourteen patients with IC while all other cases yielded false-negative scintigraphic results which were probably due to the presence of a milder degree of inflammatory response compared to the positive ones. It is possible that in the false negative cases of our study there was mainly a transient mucosal congestion and the chronic inflammation was not sufficient to provide successful bowel uptake of the radiotracer.

In conclusion, our data suggest that Tc-99m (V) DMSA has no possible role in the detection and diagnosis of IC.

COMMENTS

Background

Pentavalent Tc-99m dimercaptosuccinic acid [Tc-99m (V) DMSA] has been proved advantageous in the imaging of various inflammatory lesions, intestinal inflammation included.

Research frontiers

Tc-99m (V) DMSA could successfully localize the ischemic bowel and assist in the diagnosis of IC due to the simultaneous presence of inflammatory response to tissue ischemia.

Innovations and breakthroughs

We examined fourteen patients, (5 males and 9 females, mean age 70.6 years) with clinically, endoscopically and histologically confirmed IC. In all patients scintigraphy was performed within 2 d after colonoscopy, in order to avoid any variation in disease activity. In three out of the fourteen patients, the Tc-99m (V) DMSA scintigraphy demonstrated moderate uptake in the bowel however in the other eleven patients, Tc-99m (V) DMSA revealed no increased uptake in the bowel at all (false negative results).

Applications

Despite the fact that Tc-99m (V) DMSA scintigraphy has been used successfully in the evaluation of intestinal inflammation it seems to have no role in the diagnosis of ischemic colitis.

Peer review

The present study is relevant as it emphasizes the role of radionuclide imaging in IC and the focus of interest in future studies.

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RAPID COMMUNICATION

Direct hemoperfusion with a polymyxin B-immobilized cartridge in intestinal warm ischemia reperfusion

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Abstract

AIM: To investigate the effectiveness of direct hemoperfusion with polymyxin B-immobilized fibers (DHP-PMX therapy) on warm ischemia-reperfusion (I/R) injury of the small intestine.

METHODS: The proximal jejunum and distal ileum of mongrel dogs were resected. Warm ischemia was performed by clamping the superior mesenteric artery (SMA) and vein (SMV) for 2 h. Blood flow to the proximal small intestine was restored 1 h after reperfusion, and the distal small intestine was used as a stoma. The experiment was discontinued 6 h after reperfusion. The dogs were divided into two groups: the DHP-PMX group (n = 6, DHP-PMX was performed for 180 min; from 10 min prior to reperfusion to 170 min after reperfusion) and the control group (n = 5). The rate pressure product (RPP), SMA blood flow, mucosal tissue blood flow, and intramucosal pH (pHi) were compared between the two groups. The serum interleukin (IL)-10 levels measured 170 min after reperfusion were also compared.

RESULTS: The RPP at 6 h after reperfusion was significantly higher in the PMX group than in the control group (12 174 ± 1832 mmHg/min *vs* 8929 ± 1797 mmHg/min, $P < 0.05$). The recovery rates of the SMA blood flow at 1 and 6 h after reperfusion were significantly better in the PMX group than in the control group (61% ± 7% *vs* 44% ± 4%, $P < 0.05$, and 59% ± 5% *vs* 35% ± 5%, $P < 0.05$, respectively). The recovery rate of the mucosal tissue blood flow and the pHi levels at 6 h after reperfusion were significantly higher in the PMX group (61% ± 8% *vs* 31% ± 3%, $P < 0.05$ and 7.91 ± 0.06 *vs* 7.69 ± 0.08, $P < 0.05$, respectively). In addition, the serum IL-10 levels just before DHP-PMX removal were significantly higher in the PMX group than in the control group (1 569 ± 253 pg/mL *vs* 211 ± 40 pg/mL, $P < 0.05$).

CONCLUSION: DHP-PMX therapy reduced warm I/R injury of the small intestine. IL-10 may play a role in inhibiting I/R injury during DHP-PMX therapy.

Key words: Ischemia-reperfusion injury; Interleukin-10; Polymyxin B-immobilized hemoperfusion cartridge; PMX

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INTRODUCTION

The small intestinal villi are extremely sensitive to ischemia-reperfusion (I/R) injury, and many microcirculatory disturbances contribute to structural and functional changes[1]. I/R injury of the small intestine is consequently a critical problem that is important in situations such as the interruption of blood flow to the intestines due to abdominal aortic aneurysm surgery, small intestinal transplantation, surgery involving cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis[2]. Intestinal I/R injury produces injury in both the intestines and distant organs including the lungs, kidneys, and liver[3]; therefore, it is associated with

high rates of morbidity and mortality in both surgical and trauma patients[4].

A polymyxin B-immobilized fiber column (PMX cartridge, Toraymyxin; Toray Industries, Tokyo, Japan), which was developed in Japan in 1994, is an extracorporeal hemoperfusion device that uses polymyxin-B fixed to α-chloroacetamide-methyl polystyrene-derived fibers packed in the cartridge. Direct hemoperfusion with PMX (DHP-PMX) therapy can remove circulating endotoxins and reduce various cytokines, even in patients with high levels of plasma cytokines[5]. DHP-PMX has been used for the treatment of endotoxemia[6] and reported to lower inflammatory cytokine and plasminogen activator inhibitor-1 (PAI-1) levels immediately[7]. DHP-PMX therapy has also been attempted for severe sepsis secondary to intra-abdominal infection[8], acute lung injury, and acute respiratory distress syndrome caused by sepsis[9], and its effectiveness has been reported. Recently, we hypothesized that DHP-PMX therapy could reduce I/R injury and demonstrated the usefulness of this therapy on pulmonary warm I/R injury in a canine model[10].

In this study, we evaluated the effectiveness of DHP-PMX on warm I/R injury of the small intestine using a canine model.

MATERIALS AND METHODS

Animals

Eleven adult mongrel dogs of both sexes, weighing 7.5-15.5 kg, were used in this study. The dogs were fasted but had free access to water for 24 h prior to the experiment. All of the animals were cared for in accordance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication 85-23; revised 1985). This study was also approved by the Animal Care and Experimentation Committee, Gunma University, Showa Campus, Japan.

Surgical procedures

After intramuscular administration of ketamine hydrochloride (2 mg/kg), the animals were anesthetized. Endotracheal intubation was performed, and the animals were ventilated with a respirator (Servo Ventilator 900C; Siemens-Elema, Solna, Sweden). The inspired O_2 concentration (FIO_2) was set at 1.0 during the experiment. Mechanical ventilation was performed with a tidal volume of 20 mL/kg and a rate of 10 breaths/min. General anesthesia was maintained with the inhalation of 1% to 2% isoflurane, and muscular relaxation was obtained with additional pancuronium bromide (0.1 mg/kg) every 20 min. The surgery was performed under sterile conditions. A polyethylene catheter was positioned in the abdominal aorta through the right femoral artery and connected to a pressure transducer to record arterial pressure. Another polyethylene catheter was inserted into the right femoral vein to use as a venous infusion line. The infusion rate for the lactated Ringer's solution was set at 30 mL/kg per h for 6 h after reperfusion. Laparotomy was performed *via* a midline incision after the blood pressure and respiration parameters stabilized. The small intestine was isolated with the vascular pedicle, and both the superior mesenteric artery (SMA) and the superior mesenteric vein (SMV) were dissected from the surrounding lymph nodes, plexuses, and tissues. The proximal jejunum and distal ileum were resected to interrupt the intramural blood flow. Warm ischemia was induced by clamping the SMA and SMV for 2 h. Proximal intestinal continuity was restored with an end-to-end anastomosis 1 h after the reperfusion. The resected distal intestine was used as a stoma to measure the tissue blood flow and pHi. During the 6-h experimental period, the abdominal cavity was closed and opened temporarily for each measurement. The experiment was discontinued 6 h after the reperfusion. Catecholamines were not used at any point during the experiment.

Experimental groups

The experimental study was composed of two groups: the DHP-PMX group (n = 6) and the control group (n = 5). The animals were randomly assigned to either the DHP-PMX group or the control group. In the DHP-PMX group, a double-lumen catheter was inserted into the portal vein through the left gastric vein, and DHP with PMX was performed with a flow rate of 80 mL/min for 180 min (from 10 min prior to reperfusion to 170 min after reperfusion) using that catheter. DHP was not performed in the control group.

Arterial blood pressure and heart rate (HR)

The arterial blood pressure and HR were directly monitored through a catheter connected to a transducer (Spectramed TA 1017; San-ei Co., Tokyo, Japan). The rate pressure product (HR × systolic pressure, RPP) was also calculated.

SMA blood flow

The SMA blood flow was measured prior to ischemia and at 1, 3, and 6 h after reperfusion using an electromagnetic blood flow meter (Model MFV-3100; Nihonkohden Co., Ltd., Tokyo, Japan). The SMA blood flow was expressed as the percentage of the level that was determined prior to ischemia.

Tissue blood flow measurements

The tissue blood flow was measured in the small bowel mucosa using a laser Doppler flow meter (ALF 21; Advance Co., Ltd., Tokyo, Japan) prior to ischemia and at 1, 3, and 6 h after reperfusion. Each measurement was made at three points by inserting a probe through the stoma and placing it against the antimesenteric side of the bowel lumen. The laser probe reading reflects tissue blood flow within about 1.0 mm of the surface of the bowel wall. Tissue blood flow was calculated as the mean of the three measurements and expressed as the percentage of the level determined prior to ischemia.

Intramucosal pH (pHi) measurements

pHi was measured prior to ischemia and at 1, 3, and 6 h

after reperfusion. This method has been described previously[11]. In brief, a tonometer (Trip; Tonometrics, Helsinki, Finland) was inserted into the small bowel lumen through the stoma. Within 40 min, the PCO_2 of the saline in the balloon placed at the tip of the tonometer had equilibrated with the intraluminal PCO_2, which reflects the mucosal PCO_2 of the bowel. The HCO_3^- concentration in the bowel wall was assumed to be the same as the HCO_3^- concentration in the arterial blood. The saline PCO_2 and HCO_3^- concentration in the artery were determined with a blood gas analyzer (Stat Profile M; Nova Biomedical Co., Waltham, MA) and were used to calculate the pHi using the Henderson-Hasselbach equation: pHi = 6.1 + log[arterial HCO_3^-/(0.03 × saline PCO_2)].

Measuring serum interleukin-10 (IL-10) levels

Arterial blood samples were collected 170 min after reperfusion (that is just before DHP-PMX removal) for measuring serum IL-10 levels using a commercial sandwich ELISA (Predicta ELISA kit; Genzyme Corp., Cambridge, MA) according to the manufacturer's instructions. In each experiment, a standard curve was obtained with serial dilutions using linear regression analysis of specific samples *versus* expected concentrations. Interleukin measurements were done in duplicate.

Statistical analysis

All results are expressed as the mean ± SEM. The significance of the differences was determined using analysis of variance (ANOVA) or Mann-Whitney *U*-test. $P < 0.05$ was considered to be statistically significant.

RESULTS

All 11 mongrel dogs were successfully observed for 6 h after reperfusion without any complications.

The changes in RPP

The changes in the RPP are shown in Figure 1A. The RPPs gradually decreased with time in the control group. Those in the PMX group also decreased gradually; however, it had improved at 6 h after reperfusion and was significantly different compared to the control group ($P < 0.05$).

The changes in SMA blood flow

The recovery rates of the SMA blood flow expressed as a percentage of the baseline control value obtained before I/R injury are shown in Figure 1B. The SMA blood flow remarkably decreased 1 h after reperfusion in both groups. Additionally, the SMA blood flow gradually decreased until 6 h after reperfusion in the control group. The changes in SMA blood flow in the PMX group, however, were consistently higher after reperfusion than those in the control group, especially with significant ($P < 0.05$) differences at 1 and 6 h after reperfusion (Figure 1B).

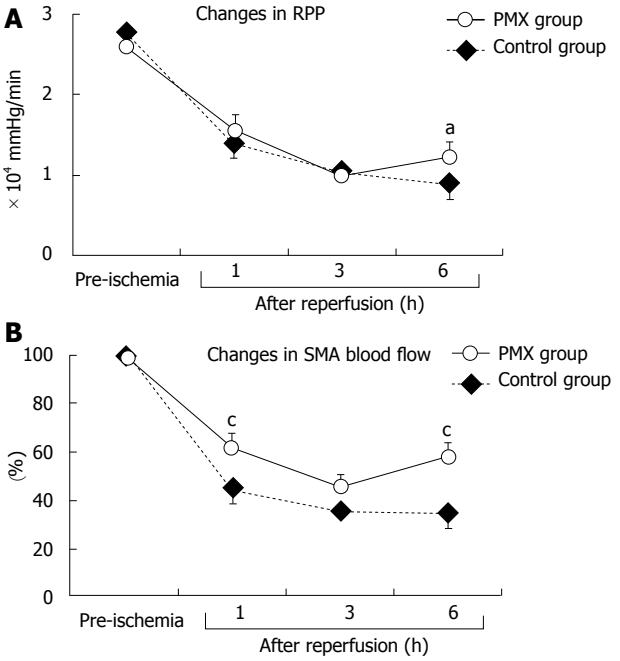

Figure 1 **A**: The changes in the RPP (HR × systolic pressure), $^aP < 0.05$; **B**: The changes in the recovery rates of SMA blood flow, $^cP < 0.05$.

The changes in mucosal tissue blood flow

The recovery rates of the mucosal tissue blood flow expressed as a percentage of the baseline control value obtained before I/R injury are shown in Figure 2A. In both groups, the mucosal tissue blood flow decreased gradually with time except at 6 h after reperfusion in the PMX group. The decreases in the mucosal tissue blood flow after reperfusion were smaller in the PMX group than in the control group. At 6 h after reperfusion, the mucosal tissue blood flow rate was significantly higher in the PMX group than in the control group after reperfusion.

The changes in pHi

As shown in Figure 2B, the changes in pHi also decreased remarkably in both groups. No significant differences in pHi levels were observed at 1 and 3 h after reperfusion in both groups; however, the pHi level in the PMX group was significantly higher ($P < 0.05$) than in the control group at 6 h after reperfusion.

Serum IL-10 levels

The serum IL-10 levels 170 min after reperfusion (that is just before DHP-PMX removal) in both groups are shown in Figure 3. As shown in Figure 3, the serum IL-10 level was significantly ($P < 0.05$) higher in the PMX group than in the control group.

DISCUSSION

Polymyxin B binds to endotoxin, an outer membrane component of gram-negative bacteria that is thought to be an important pathogenic trigger for the production of inflammatory mediators. Several preclinical studies have demonstrated that hemoperfusion or plasmapheresis

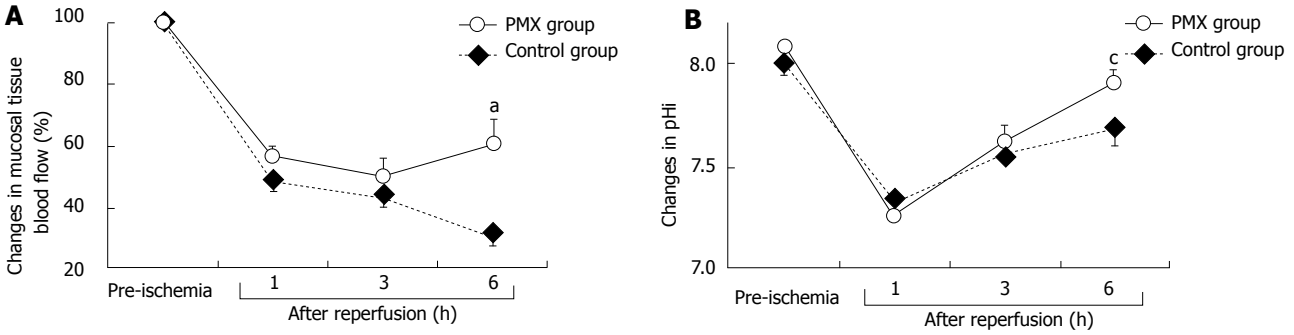

Figure 2 **A**: The changes in the recovery rates of mucosal tissue blood flow, $^{a}P < 0.05$; **B**: The changes in pHi, $^{c}P < 0.05$.

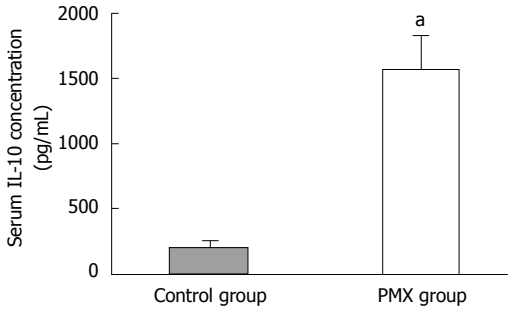

Figure 3 Serum IL-10 levels measured just before DHP-PMX removal, $^{a}P < 0.05$.

over immobilized polymyxin B can remove endotoxin from the blood[12-14]. Recently, some studies reported improved hemodynamic status[15] and improved survival[16] in patients with sepsis treated with PMX, and DHP-PMX therapy is effective for patients with septic shock who are infected not only by gram-negative bacteria but also by gram-positive bacteria without endotoxin release[17]. Therefore, DHP-PMX therapy has been conventionally used in patients with severe sepsis or septic shock, and its clinical effect has been confirmed. In addition, some authors have discussed the mechanism of DHP-PMX action. Kushi *et al* reported that the adsorption of pathogenic bacteria prevented the release of inflammatory cytokines and lessened the stimulation of vascular endothelial cells to lower PAI-1 level rather than directly inhibiting PAI-1 production by DHP-PMX therapy[18]. Tani *et al* speculated that a reduction in plasma endotoxins by endotoxin adsorption contributed to the cessation of cytokine gene expression and the excretion of cytokines[7]. Additionally, improvement in the PaO_2/FiO_2 ratio in patients with acute lung injury or acute respiratory distress syndrome caused by sepsis has been related to DHP-PMX therapy and decreases in blood neutrophil elastase (NE) and IL-8 levels[9]. We hypothesized that DHP-PMX therapy might be valuable in various inflammatory situations. Consequently, we evaluated the usefulness of DHP-PMX therapy on normothermic cardiopulmonary bypass in a pig model[19] and on pulmonary warm I/R injury in a canine model[10], and obtained satisfactory results. In the present study, we investigated the effectiveness of DHP-PMX therapy on small intestinal warm I/R injury in a canine model because intestinal I/R injury produces injury in both the intestines and distant organs including the lungs, kidneys, and liver[3] and is therefore associated with high rates of morbidity and mortality in both surgical and trauma patients[4].

As a result, the RPP was significantly ($P < 0.05$) better in the PMX group than in the control group at 6 h after reperfusion. The SMA and the mucosal tissue blood flow in the control group gradually decreased after reperfusion. Those parameters in the PMX group, however, had improved at 6 h after reperfusion and were significantly ($P < 0.05$) different compared to those parameters in the control group. The pHi level had also improved at 6 h after reperfusion and was significantly ($P < 0.05$) different from that in the control group. Our results showed the effectiveness of DHP-PMX therapy on small intestinal warm I/R injury.

IL-10 is a 35-kDa cytokine that regulates immune and inflammatory responses[20]. Systemic inflammatory response syndrome (SIRS) following major abdominal surgery is characterized by complex alterations in cytokine concentrations, and the balance between tumor necrosis factor (TNF)-α and IL-10 may be related to the occurrence of postoperative complications[21]. Wu *et al* reported that small intestinal ischemia reperfusion increased mucosal inflammatory modulator IL-6 concentration and inhibited anti-inflammatory cytokine IL-10 synthesis[22]. In addition, endogenous IL-10 exerts an anti-inflammatory role during reperfusion injury, possibly by regulating early stress-related genetic response, adhesion molecule expression, neutrophil recruitment, and subsequent cytokine and oxidant generation[23]. Malleo *et al* demonstrated that the absence of endogenous IL-10 enhanced organ dysfunction and mortality associated with multiple organ dysfunction syndrome in mice[24]. In this study, we focused on the serum IL-10 levels after reperfusion, and those in both groups were measured and compared. As a result, the serum IL-10 level measured 170 min (that is just before DHP-PMX removal) in the PMX group was significantly higher than in the control group. In addition, RPP, the recovery rates of SMA blood flow, the mucosal tissue blood flow, and the pHi levels after reperfusion were significantly better in the PMX group than in the control group. Therefore, we suggest that the IL-10 level is associated with the inhibition of small intestinal I/R injury using DHP-PMX therapy.

The authors of a recent study suggested that the absorption of anandamide by PMX might abolish

the diverse negative effects of anandamide such as hypotension, immunosuppression, and cytotoxicity[25]. Taking these results into consideration, the possibility exists that treatment with PMX not only removes endotoxin, but also reduces inflammatory reactions through the inhibition of various inflammatory cascades and has an effective role on I/R injury. Further studies that include the role of endotoxin and alterations of inflammatory factors such as cytokines and chemokines with DHP-PMX therapy are necessary.

In conclusion, DHP-PMX therapy may reduce warm I/R injury in the small intestine, and IL-10 could play an important role in this mechanism.

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COMMENTS

Background

Ischemia-reperfusion (I/R) injury of the small intestine is consequently a critical problem that is important in situations such as the interruption of blood flow to the intestines due to abdominal aortic aneurysm surgery, small intestinal transplantation, surgery involving cardiopulmonary bypass, strangulated hernias, and neonatal necrotizing enterocolitis. Intestinal I/R injury produces injury in both the intestines and distant organs including the lungs, kidneys, and liver, therefore, it is associated with high rates of morbidity and mortality in both surgical and trauma patients.

Research frontiers

A polymyxin B-immobilized fiber column (PMX cartridge, Toraymyxin), which was developed in Japan in 1994, is an extracorporeal hemoperfusion device that uses polymyxin-B fixed to α-chloroacetamide-methyl polystyrene-derived fibers packed in the cartridge. Direct hemoperfusion with PMX (DHP-PMX) therapy can remove circulating endotoxins and reduce various cytokines, even in patients with high levels of plasma cytokines.

Innovations and breakthroughs

DHP-PMX has been used for the treatment of endotoxemia and reported to lower inflammatory cytokine and plasminogen activator inhibitor-1 levels immediately. DHP-PMX therapy has also been attempted for severe sepsis secondary to intra-abdominal infection, acute lung injury, and acute respiratory distress syndrome caused by sepsis, and its effectiveness has been reported. Recently, we hypothesized that DHP-PMX therapy could reduce I/R injury and demonstrated the usefulness of this therapy on pulmonary warm I/R injury in a canine model.

Applications

DHP-PMX therapy may reduce warm I/R injury in the small intestine, and IL-10 could play an important role in this mechanism.

Terminology

The possibility exists that treatment with PMX not only removes endotoxin, but also reduces inflammatory reactions through the inhibition of various inflammatory cascades and has an effective role on I/R injury.

Peer review

The authors demonstrated that DHP with a polymyxin B-immobilized cartridge reduced reperfusion injury in the small intestine. This study was well designed and well investigated.

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RAPID COMMUNICATION

Metabolic syndrome is associated with erosive esophagitis

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Abstract

AIM: To clarify whether insulin resistance and metabolic syndrome are risk factors for erosive esophagitis.

METHODS: A case-control study was performed using the database of the Kangbuk Samsung Hospital Medical Screening Center.

RESULTS: A total of 1679 cases of erosive esophagitis and 3358 randomly selected controls were included. Metabolic syndrome was diagnosed in 21% of the cases and 12% of the controls ($P < 0.001$). Multiple logistic regressions confirmed the association between erosive esophagitis and metabolic syndrome (Odds ratio, 1.25; 95% CI, 1.04-1.49). Among the components of metabolic syndrome, increased waist circumference, elevated serum triglyceride levels and hypertension were significant risk factors for erosive esophagitis (all $P < 0.01$). Furthermore, increased insulin resistance (Odds ratio, 0.91; 95% CI, 0.85-0.98) and fatty liver, as diagnosed by ultrasonography (Odds ratio, 1.39; 95% CI, 1.20-1.60), were also related to erosive esophagitis even after adjustment for a series of confounding factors.

CONCLUSION: Metabolic syndrome and increased insulin resistance are associated with an increased risk of developing erosive esophagitis.

Key words: Metabolic syndrome; Erosive esophagitis; Insulin resistance; Fatty liver

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INTRODUCTION

Metabolic syndrome is a cluster of metabolic abnormalities defined as the presence of an increased waist circumference and two of the following components: high blood pressure, hypertriglyceridemia, low levels of high density lipoprotein (HDL)-cholesterol, or diabetes/hyperglycemia. This syndrome helps to identify individuals at high risk for both cardiovascular disease and diabetes mellitus (DM); therefore, metabolic syndrome has become one of the major health problems worldwide.

Gastroesophageal reflux disease (GERD) and obesity are two of the most common diseases in Korea and the incidences of both have been increasing rapidly. Recently, GERD was shown to affect approximately 3.4%-3.8% of the Korean population[1,2] and in 1995 the prevalence of being overweight [body mass index (BMI) = 25-30 kg/m^2] or obese (BMI > 30 kg/m^2) was, respectively, reported as 11.7% and 2.1% in males, and 18.0% and 2.5% in females; in 2000 the prevalence of being overweight or obese was 33.1% and 3.2% in males, and 32.2% and 4.5% in females, respectively[3].

Several studies have shown the relationship between obesity, erosive esophagitis, and GERD symptoms[4-8]. Also, a recent study demonstrated that metabolic syndrome was associated with reflux esophagitis[9]. However, literature on whether metabolic syndrome and insulin resistance are risk factors for GERD is scant.

In this study, we therefore intended to determine whether metabolic syndrome and insulin resistance are associated with erosive esophagitis in a Korean population.

MATERIALS AND METHODS

Study population and selection of study participants

We conducted a cross-sectional case-control study. The study population consisted of subjects who visited the Medical Screening Center at Kangbuk Samsung Hospital from January to December 2006. Exclusion criteria

consisted of a history of prior gastric surgery, benign gastric or duodenal ulcer, gastric cancer or current proton pump inhibitor medication therapy.

Of the 83032 patients visited the Medical Screening Center, 44718 patients underwent upper gastrointestinal (UGI) endoscopic examination. However, only 28949 patients completed a questionnaire pertaining to their symptoms (heart burn and regurgitation) and provided weights, heights, and waist circumferences. The mean age of the participants was 45 ± 9.6 years and 11375 (39%) were females. After application of the exclusion criteria, 1679 (5.8%) subjects were included as patients with erosive esophagitis. Two controls for each case patient (3358 subjects) were randomly selected from the subjects with normal UGI endoscopic findings and no reflux symptoms.

Questionnaire

All participants completed a self-administered validated questionnaire that identified reflux symptoms, such as irritating heartburn and/or acid regurgitation experienced during the preceding year[6]. In addition, the questionnaire included questions about current smoking and other medical or surgical histories.

Measurement

BMI and metabolic syndrome components, such as waist circumference, lipid profile, blood pressure and fasting glucose level, were measured in all study participants. BMI was calculated as the ratio of weight (kg) to the square of height (kg/m^2) and abdominal obesity was defined as a waist circumference ≥ 80 cm in females and ≥ 90 cm in males[10]. Measurements were made at the World Health Organization (WHO) recommended site (the midpoint between the lower border of the rib cage and the iliac crest) by trained personnel[11]. The mean blood pressure was checked more than twice in the supine position with a sphygmomanometer after 10 min of rest. All blood testing was done after more than 12 h of fasting. Total cholesterol, triglycerides (TG), HDL-C and low-density lipoprotein (LDL)-C were measured with an automatic analyzer (Advia 1650, German). Total C and TG were analyzed by an enzymatic calorimetric test. A selective inhibition method was used in HDL-C measurement and a homogenous enzymatic calorimetric test was used in LDL-C measurement. Fasting glucose was measured by the hexokinase method with an automatic analyzer (Advia 1650) and fasting insulin was assayed *via* an immunoradiometric assay (Biosource, Belgium). The intraassay variation coefficients were 2.1%-4.5% and the interassay variation coefficients for the quality controls were 4.7%-12.2%. The degree of insulin resistance was evaluated by homeostasis model assessment (HOMA-IR) according to the following formula: (fasting insulin in μU/mL × fasting glucose in mmol/L)/22.5[12]. An experienced radiologist who was blind to the laboratory data performed ultrasonographic liver examinations. Fatty liver was defined as a bright liver on ultrasonography (USG). The diagnosis of bright liver was based on abnormally intense and high level echoes arising from the hepatic parenchyma with amplitudes similar to those of echoes arising from the diaphragm.

Participants were diagnosed with metabolic syndrome if they had an increased waist circumference and two of the following components: (1) high blood pressure (≥ 130 mmHg systolic or ≥ 85 mmHg diastolic), (2) hypertriglyceridemia (≥ 150 mg/dL), (3) low levels of HDL-C (≤ 40 mg/dL in males or ≤ 50 mg/dL in females), or (4) DM/hyperglycemia[10].

Upper gastrointestinal endoscopy

Standard endoscopic examination of the esophagus, stomach, and duodenum was performed in all subjects. The severity of erosive esophagitis was graded from A-D according to the LA classification[13]. We considered LA-A to be the cutoff for erosive esophagitis. We also considered a hiatal hernia to be present if diaphragmatic indentation was seen > 2 cm distal to the Z-line and the proximal margins of the gastric mucosal folds, which were observed with considerable air insufflation during inspiration. Distance was measured using the centimeter markings on the endoscope[14].

Statistical analyses

Statistical analysis was done using the χ^2 test for comparison of discrete variables and the *t*-test for comparison of continuous variables. The continuous variables measured in this study were expressed as the mean ± SD. Multivariate analysis was conducted using logistic regression. To examine the risks of potential confounders, including metabolic syndrome for erosive esophagitis, multivariate models included adjustments for age, gender, smoking, alcohol, and metabolic syndrome as categorical factors. For each variable, the odds ratio (OR) and 95% confidence interval (CI) were given. A two-tailed *P* value of < 0.05 was considered statistically significant.

RESULTS

Of the 28949 subjects, 1679 (5.8%) were confirmed to have erosive esophagitis; 1326 (78.9%) cases were classified as LA-A, 328 (19.5%) as LA-B, and 25 (1.6%) as LA-C or LA-D. The mean age was 45.19 ± 9.3 years and 86% of the subjects were men. The study characteristics are mentioned in Table 1. We found a significant increase in the mean BMI, waist circumference, systolic and diastolic blood pressure, fasting blood glucose, HbAlc, TG and HOMA in patients with erosive esophagitis as compared to the controls. Also, patients with erosive esophagitis were more likely to be male, obese, current smokers, regular consumers of alcohol, and more likely to have metabolic syndrome and fatty liver (as diagnosed by abdominal ultrasonography) and less than a college education.

Table 2 shows the results from the multivariate analysis examination of the association between erosive esophagitis and various risk factors. Male gender, current smoking, metabolic syndrome, reflux symptoms, regular

Table 1 Comparisons between participants with and without erosive esophagitis (n = 5037)

	With erosive esophagitis (n = 1679)	Without erosive esophagitis (n = 3358)	P
Age (yr, mean ± SD)	45.2 ± 9.3	45.2 ± 9.7	0.873
Gender (M/F, %)	86/14	59/41	< 0.001
BMI (kg/m^2)	24.8 ± 2.9	23.5 ± 3.0	< 0.001
Waist circumference (cm)	86.8 ± 8.7	81.5 ± 9.8	< 0.001
Current smoking	724 (43%)	786 (23%)	< 0.001
Alcohol use (≥ 3-4/wk)	360 (21%)	387 (12%)	< 0.001
Metabolic syndrome	352 (21%)	433 (13%)	< 0.001
Hiatal hernia	38 (2.2%)	24 (0.7%)	< 0.001
Reflux symptoms[1]	194 (12%)	265 (8.0%)	< 0.001
Systolic BP (mmHg)	118.3 ± 12.7	114.8 ± 13.5	< 0.001
Diastolic BP (mmHg)	77.7 ± 8.8	74.8 ± 9.5	< 0.001
Fatty liver on abdominal USG	809 (48%)	1014 30%)	< 0.001
Fasting plasma glucose	98.4 ± 20.5	95.2 ± 17.2	< 0.001
HbAlc	5.6 ± 0.7	5.5 ± 0.6	0.001
Triglycerides (mg/dL, mean ± SD)	158.9 ± 110.4	123.5 ± 78.6	< 0.001
HDL-C (mg/dL) (mean ± SD)	54.1 ± 12.0	56.4 ± 12.8	< 0.001
HOMA[2]	2.41 ± 1.10	2.18 ± 0.89	< 0.001
H pylori positive	211/555 (38%)	498/922 (54%)	< 0.001
Education (college and higher)	894/1184 (75%)	1639/2391 (69%)	< 0.001

[1]Reflux symptoms: Weekly heartburn and/or acid regurgitation; [2]HOMA: Homeostasis model assessment estimates steady state beta cell function and insulin sensitivity.

Table 2 Multivariate analyses of the risk for erosive esophagitis by gender, smoking, hiatal hernia, reflux symptoms, metabolic syndrome, fatty liver on abdominal USG and HOMA

	Adjusted odds ratio	95% CI	P
Gender	0.29	0.25-0.35	< 0.001
Current smoking	1.60	1.39-1.83	< 0.001
Alcohol use (≥ 3-4/wk)	1.80	1.53-2.14	< 0.001
Hiatal hernia	3.27	1.87-5.70	< 0.001
Reflux symptoms[1]	1.57	1.28-1.94	< 0.001
Metabolic syndrome	1.25	1.04-1.49	0.017
Fatty liver on abdominal USG	1.39	1.20-1.60	< 0.001
HOMA[2]	0.91	0.85-0.98	0.011

[1]Reflux symptoms: Weekly heartburn and/or acid regurgitation; [2]HOMA: Homeostasis model assessment estimates steady state beta cell function and insulin sensitivity.

Table 3 Risk of individual components of metabolic syndrome for erosive esophagitis

	OR (95% CI)[1]	P	OR (95% CI)[2]	P
Increased waist circumference	1.46 (1.28-1.67)	< 0.001	1.33 (1.15-1.54)	< 0.001
Hypertension	1.22 (1.06-1.40)	0.006	1.16 (1.00-1.35)	0.047
DM or elevated FBS	1.09 (0.77-1.54)	0.627	0.95 (0.66-1.38)	0.798
Increased TG	1.98 (1.74-2.26)	< 0.001	1.47 (1.14-1.90)	0.003
Low HDL-C	0.67 (0.56-0.80)	< 0.001	0.90 (0.74-1.09)	0.267

FBS: Fasting blood sugar; TG: Triglycerides; HDL-C: High-density lipoprotein-cholesterol. [1]Unadjusted; [2]Adjusted for gender, current smoking, hiatal hernia, reflux symptoms, alcohol use, fatty liver on abdominal USG, and HOMA.

alcohol use, HOMA and fatty liver (as diagnosed by abdominal ultrasonography) were significant independent risk factors for erosive esophagitis. Among the individual components of metabolic syndrome, increased waist circumference, hypertension, increased levels of TG, and low levels of HDL-C were significantly associated with erosive esophagitis. However, after adjusting for gender, smoking, hiatal hernia, reflux symptoms, regular alcohol use, HOMA and fatty liver (as diagnosed by abdominal ultrasonography), increased waist circumference, increased levels of TG, and hypertension were strongly associated with the development of erosive esophagitis (Table 3).

We also attempted to determine the relationship between the severity of erosive esophagitis, according to the LA classification, and various risk factors. Male gender, current smoking, regular alcohol use, hiatal hernia, metabolic syndrome, reflux symptoms, HOMA and fatty liver (as diagnosed by abdominal ultrasonography) were significantly associated with the severity of erosive esophagitis (Table 4). Among the individual components of metabolic syndrome, increased waist circumference and increased levels of TG were predictive factors for the severity of erosive esophagitis (Table 5).

DISCUSSION

This cross-sectional study in a Korean population showed that metabolic syndrome was strongly associated with the development and severity of erosive esophagitis. Also, insulin resistance, independent of metabolic syndrome, was another significant risk factor for erosive esophagitis.

Recently, the prevalence of metabolic syndrome has rapidly increased in Korea. According to the International Diabetes Federation (IDF) criteria, the age-adjusted prevalence of metabolic syndrome in males was 10.9% in 1997 and 23.3% in 2003. In females, the age-adjusted prevalence of metabolic syndrome was 42.2% in 1997 and 43.4% in 2003[15]. In the current study,

Table 4 Associations of grade of erosive esophagitis, according to LA classification, with risk factors for erosive esophagitis *n* (%)

	Control (*n* = 3358)	A (*n* = 1326)	B (*n* = 328)	C or D (*n* = 25)	*P* for linear trend
Age (yr, mean ± SD)	45.2 ± 9.7	44.8 ± 9.7	46.4 ± 9.3	49.9 ± 10.7	0.094
Males	1991 (59)	1122 (85)	300 (92)	22 (88)	< 0.001
Current smoking	786 (23)	554 (42)	162 (49)	8 (32)	< 0.001
Alcohol use (≥ 3-4/wk)	387 (12)	251 (19)	101 (31)	8 (32)	< 0.001
Hiatal hernia	24 (0.2)	32 (2)	3 (1)	3 (12)	< 0.001
Reflux symptoms[1]	265 (8.0)	141 (11)	50 (15)	3 (12)	< 0.001
Metabolic syndrome	433 (13)	255 (19)	87 (27)	10 (40)	0.001
Fatty liver on Abdominal USG	1014 (30)	625 (47)	166 (51)	18 (72)	< 0.001
HOMA[2] (mean ± SD)	2.18 ± 0.89	2.39 ± 1.10	2.50 ± 1.10	2.70 ± 1.12	0.007

[1]Reflux symptoms: Weekly heartburn and/or acid regurgitation; [2]HOMA: Homeostasis model assessment estimates steady state beta cell function and insulin sensitivity.

Table 5 Associations of grade of erosive esophagitis, according to LA classification, with individual components of the metabolic syndrome *n* (%)

	Control (*n* = 3358)	A (*n* = 1326)	B (*n* = 328)	C or D (*n* = 25)	*P* for linear trend[1]
Increased waist circumference	942 (28)	504 (38)	159 (48)	19 (76)	< 0.001
Hypertension	722 (22)	378 (29)	103 (31)	8 (32)	0.244
DM or elevated FBS	84 (2.5)	49 (4)	11 (3)	0	0.346
Increased TG	850 (25)	561 (42)	138 (42)	11 (44)	0.004
Low HDL-C	497 (15)	164 (12)	37 (11)	5 (20)	0.582

[1]Adjusted for gender, current smoking, hiatal hernia, reflux symptoms, alcohol use, fatty liver on abdominal USG, and HOMA.

the prevalence of metabolic syndrome was lower than previously reported, especially for females. A possible explanation for this difference is that the participants in this study were much younger than those in previous studies. Because the prevalence of metabolic syndrome increases with age[16,17], younger subjects are more likely to have a lower prevalence of metabolic syndrome than older subjects. Additionally, the higher educational level and economic status of the subjects could be another reason for the lower than expected prevalence of metabolic syndrome. Higher income was protective against metabolic syndrome[18] and females in the lower economic group were more likely to be at risk for metabolic syndrome when compared with females in the higher economic group[19,20].

With the increased prevalence of metabolic syndrome, GERD has also become more prevalent in Korea. The overall prevalence of erosive esophagitis was 3.4% in 2001[21] and 6.6% in 2006[6]. In terms of reflux symptoms, 2.5% of adults experienced heartburn and reflux symptoms in 2000[22]. In contrast, 7.1% reported that GERD symptoms were present at least once a week in 2007[1]. This increase may be due to extended life expectancy, greater intake of Westernized food[2], and/or increasing rates of obesity[6]. Moreover, an increase in alcohol consumption[23,24] and a decrease in *Helicobacter pylori (H pylori)* infections[25] could be possible reasons for the increase in erosive esophagitis in Korea.

This study demonstrated that metabolic syndrome and insulin resistance were also risk factors for erosive esophagitis. A recent study verified that elevated triglyceride levels, a component of metabolic syndrome, is an independent predictor for reflux esophagitis and suggested that humoral compounds might alter the lower esophageal sphincter pressure or affect esophageal clearance of refluxate[9]. In fact, human adipose tissue is a major site of IL-6 secretion[26]. IL-6 stimulates hepatic triglyceride secretion in rats[27] and plays an important role in insulin resistance in humans[28]. Moreover, IL-6 reduces esophageal circular muscle contraction[29]. Therefore, cytokines may play important roles in the pathogenesis of reflux esophagitis. However, given that very complex relationships exist among the risk factors for erosive esophagitis[23,30,31], we should be careful when interpreting the clinical significance of these relationships.

An interesting finding of this study was that among the individual components of metabolic syndrome, an elevated level of serum TG was a significant predictive factor not only for the presence of erosive esophagitis, but also for the severity of erosive esophagitis. This result is consistent with a recent study about metabolic syndrome and erosive esophagitis[9] as well as previous studies[32,33]; however, other reports did not find such a relationship between elevated serum TG and erosive esophagitis[34,35]. There are several possible explanations for this association. First, in view of the results of this study, insulin resistance and fatty liver may be responsible for increased serum TG levels because liver fat is a significant correlate of fasting glucose and triglyceride levels[36]. Also, hypertriglyceridemia is associated with increased insulin resistance[37]. Second, considering that *H pylori* infection has been suggested to be a protective factor for erosive esophagitis[38,39] and chronic *H pylori* infections can modify the serum lipid profile, including the increment of total C and TG[40,41], elevated serum TG levels could be just an epiphenomenon accompanying *H pylori* infection. Further studies are needed in order to

verify that point.

There were several limitations to our study. First, *H pylori* infections were not included in the multivariate analysis. *H pylori* infections in this study were diagnosed by histologic analysis of biopsy specimens. However, most of the subjects did not undergo the historical examination for *H pylori* because tissue biopsies were performed only when suspicious lesions were found by UGI endoscopic examination. Therefore, the prevalence of *H pylori* infection was lower in our subjects in comparison with the normal population. Nevertheless, patients undergoing a biopsy were randomly allocated to each comparison group; thus the rate of positive *H pylori* infection in patients with erosive esophagitis was significantly lower than in patients without erosive esophagitis, which is consistent with the result of a previous study[38]. Moreover, the result that metabolic syndrome was a significant risk factor for erosive esophagitis was still persistent after multivariate analysis, including *H pylori* infection ($P < 0.05$). Second, there is a possibility of selection bias because only one-half of the subjects who visited the health care center underwent UGI endoscopy and only a portion of them responded to the questionnaire regarding their symptoms. However, although subjects with reflux symptoms were more likely to participate in this study, the prevalence of patients with reflux symptoms was consistent with that of a recent study[1] and when the limited effects of reflux symptoms on erosive esophagitis are considered, this bias did not seem to affect the primary results of this study.

In conclusion, our study demonstrates that metabolic syndrome is an independent risk factor for erosive esophagitis. In addition, metabolic syndrome is significantly associated with the severity of erosive esophagitis. Therefore, we should take into account not only acid suppression, but also metabolic factors when consulting with patients who have erosive esophagitis.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) and obesity are two of the most common diseases in Korea and the incidences of both have been increasing rapidly.

Research frontiers

Several studies have reported the relationship between obesity, erosive esophagitis, and GERD symptoms. A recent study demonstrated that metabolic syndrome was associated with reflux esophagitis. However, literature on whether metabolic syndrome and insulin resistance, suggested causes of metabolic syndrome, are risk factors for GERD remains scant.

Innovations and breakthroughs

One of the major findings of this study was that metabolic syndrome was strongly associated with the development and severity of erosive esophagitis. Moreover, insulin resistance, independent of metabolic syndrome, was a significant risk factor for erosive esophagitis.

Applications

This study should help to identify patients with particular risk for erosive esophagitis. An early identification of patients at risk for erosive esophagitis would allow more timely treatment and symptom relief.

Peer review

The present study showed a significant correlation between metabolic syndrome and erosive esophagitis and may be helpful for identifying the cause of erosive esophagitis. This study is interesting and valuable.

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RAPID COMMUNICATION

Clinical, virologic and phylogenetic features of hepatitis B infection in Iranian patients

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Abstract

AIM: To characterize the clinical, serologic and virologic features of hepatitis B virus (HBV) infection in Iranian patients with different stages of liver disease.

METHODS: Sixty two patients comprising of 12 inactive carriers, 30 chronic hepatitis patients, 13 patients with liver cirrhosis and 7 patients with hepatocellular carcinoma (HCC) were enrolled in the study. The HBV S, C and basal core promoter (BCP) regions were amplified and sequenced, and the clinical, serologic, phylogenetic and virologic characteristics were investigated.

RESULTS: The study group consisted of 16 HBeAg-positive and 46 HBeAg-negative patients. Anti-HBe-positive patients were older and had higher levels of ALT, ASL and bilirubin compared to HBeAg-positive patients. Phylogenetic analysis revealed that all patients were infected with genotype D (mostly *ayw2*). The G1896A precore (PC) mutant was detected in 58.1% patients. HBeAg-negative patients showed a higher rate of PC mutant compared to HBeAg-positive patients (χ^2 = 9.682, P = 0.003). The majority of patients with HCC were HBeAg-negative and were infected with PC mutant variants. There was no significant difference in the occurrence of BCP mutation between the two groups, while the rate of BCP plus PC mutants was higher in HBeAg-negative patients (χ^2 = 4.308, P = 0.04). In the HBV S region, the genetic variability was low, and the marked substitution was P120T/S, with a rate of 9.7% (n = 6).

CONCLUSION: In conclusion, HBV/D is the predominant genotype in Iran, and the nucleotide variability in the BCP and PC regions may play a role in HBV disease outcome in HBeAg-negative patients.

Key words: Hepatitis B virus; Clinical and virologic features; Genetic variability; Phylogenetic analysis

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INTRODUCTION

Hepatitis B virus (HBV) infection is one of the most important infectious diseases worldwide and is a major global health problem. Approximately one million people die annually because of acute and chronic HBV infection despite the availability of effective vaccines and effective antiviral medications[1]. HBV replicates *via* the reverse transcriptase enzyme system which lacks proofreading ability; therefore, new virions

possess diverse genetic variability[2]. Different selection pressures such as host immunity (endogenous pressure), and vaccine or antiviral agents (exogenous pressure) influence the production of HBV quasispecies in infected individuals. It has been demonstrated that mutations in the HBV genome not only impact the replication fitness of the virus (phonotypical effect) but can also influence the disease outcome, as well as the response to treatment (clinical effect)[3]. Mutations in the HBV surface (*S*), precore (*PC*) and basal core promoter (*BCP*) genes are observed frequently in HBV infected patients, and studies show that these mutations are associated with the clinical outcomes of HBV disease[4-6]. The most clinically relevant mutations in the S region arise in the immunologic "a determinant" domain, and neutralizing antibodies (anti-HBs) are targeted against this epitope[7]. The most frequent and clinically important mutations in the PC and BCP regions are G1896A and A1762T/G1764A, respectively; which are often detected in HBeAg-negative chronic HBV infected patients[5]. Moreover, it has recently been documented that HBV genotypes may also contribute to the clinical features, disease outcome, and response to antiviral therapy[8].

Iran is located in the Middle East, and has an intermediate-to-low prevalence of the HBV infection[9]. The prevalence of HBV infection in Iran is around 2% and it appears that after the implementation of the HBV National Vaccination Programme (started in 1993), the HBV infection rate in young children has diminished significantly[9-11]. There are very few reports on the molecular epidemiology of HBV in Iran[12-14]. Recently, a study on the clinical and serological findings of HBV infection in Iran was published[15], however, there are no reports on the association of the clinical, serologic, virologic (HBV genetic variability) and phylogenetic features of HBV infection. In the present study, we have attempted to determine the HBV genetic variability, and its association with clinical outcome in HBV infected patients at different stages of liver disease.

MATERIALS AND METHODS

Patients

Sixty two HBsAg-positive patients who were referred to the Tehran Hepatitis Centre (2004-2006), were enrolled in a cross-sectional study. The study population consisted of 79% males (n = 49) and 21% (n = 13) females. The mean ± SD age was 37.3 ± 12.3 years (range: 15-64 years, median 36 years). All patients were interviewed and examined by gastroenterologists to evaluate the clinical findings and the results of the investigative workup (liver histology, ultrasonography, and laboratory tests such as serologic, biochemical and virological tests) in order to determine the clinical status of the patient. We followed the American Association for the Study of Liver Disease (AASLD) practice guidelines with regard to the diagnostic criteria. Briefly, inactive carriers had persistent HBV infection without significant necro-inflammatory disease. Chronic hepatitis was defined as HBsAg positivity with or without the presence of HBeAg and a high HBV DNA (> 100 000 copies/mL) level determined by the Amplicor HBV monitor, persistent or intermittent elevation in the serum ALT levels, and compatible liver biopsy. Liver cirrhosis and hepatocellular carcinoma (HCC) were confirmed by liver biopsy. Informed consent was obtained from the patients before collecting blood samples. Sera from the patients was frozen at -20℃ in aliquots, until used for virological examination.

Serologic, virologic and biochemical parameters

All patients were tested for HBV serological markers (HBsAg, anti-HBs, total anti-HBc, HBeAg, and anti-HBe), hepatitis D virus (HDV), hepatitis C virus (anti-HCV) and human immunodeficiency virus (anti-HIV) using commercial kits (DIA PRO Diagnostic Bioprobes, Srl., Italy). Coinfected patients with HIV, HDV and HCV were excluded from the study. Liver function tests such as serum albumin, total bilirubin, ALT, AST and ALP were measured by an auto-analyzer[16]. HBV DNA viral load was determined using the Cobas Amplicor HBV Monitor test (Roche Applied Science, Mannheim, Germany).

Detection of S and BCP/C mutations

HBV DNA was extracted using a nano-particle magnetic beads kit (BILATEC AG, Viernheim, Germany) according to the manufacturer's instructions. The HBV S/pol and BCP/C regions were amplified as previously described[17]. Negative serum samples from subjects with no HBV markers served periodically as a negative control. The PCR amplicons were purified using the AccuPrep™ Gel Purification Kit (Bioneer Inc, Alameda, CA), sequenced bi-directionally with inner primers using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and the data were collected by an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems).

Phylogenetic and sequence analysis

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1[18], as well as BioEdit, version 7.0.4.1 as described previously[14]. Briefly, sequences of BCP/C plus S regions (approximately 1000-bp) were block aligned by the CLUSTAL X program and corrected visually; the Kimura two-parameter algorithm was used for genetic distance calculation. A phylogenetic tree was generated by the neighbour-joining method, and bootstrap re-sampling and reconstruction was carried out 1000 × to confirm the reliability of the phylogenetic tree.

Statistical analysis

The data were statistically analyzed using the SPSS software, version 11.0 (SPSS, Inc., Chicago, IL). $P < 0.05$ was considered significant.

RESULTS

Clinical and demographic data

Based on the clinical and laboratory findings the

Table 1 Clinical, serological, virological and biochemical features of patients infected with HBV (mean ± SD)

Clinica-status	Number (%)	Sex		Age (yr)	HBs Ag+	Anti HBs+	Anti HBc+	HBe Ag+	Anti HBe+	ALT (IU/L)	AST (IU/L)	Alk (U/L)	T-Bil (mg/dL)	Albumin (g/dL)	HBV DNA (log copies /mL)
		M	F												
Inactive HBsAg carriers	12 (19.4)	9	3	30.6 ± 9.5	12	0	12	3	9	38.3 ± 25.8	31.4 ± 12.1	282.2 ± 199.9	0.7 ± 0.241	4.77 ± 1.25	4.3 ± 1.57
Chronic hepatitis	30 (48.4)	25	5	34.5 ± 11.6	30	0	30	8[1]	22[1]	250.9 ± 120.7	104.4 ± 74.7	243.6 ± 125.4	1.17 ± 0.49	4.2 ± 0.7	5.31 ± 1.48
Liver cirrhosis	13 (21.0)	9	4	44.5 ± 9.4	13	0	13	5	8	50.3 ± 18.8	64.6 ± 32.2	274.2 ± 229.3	1.41 ± 0.63	3.6 ± 0.67	5.8 ± 2.03
HCC	7 (11.3)	6	1	47.7 ± 13.7	7	0	7	0	7	123.5 ± 64.1	285.1 ± 196.8	402 ± 114.3	2.92 ± 1.5	3.1 ± 0.48	7.22 ± 2.01
Total	62 (100)	49 (79.00%)	13 (21.00%)	37.3 ± 12.3	62	0	62	16	46	178.7 ± 93.7	120.6 ± 91.1	274.2 ± 165.6	1.36 ± 0.89	3.83 ± 0.91	5.2 ± 1.57

[1]One patient had both positive HBeAg and anti-HBe status.

Table 2 Comparison of demographic and para-clinical features between HBeAg-positive and anti-HBe-positive individuals (mean ± SD)

	HBeAg-positive (n = 16)	Anti-HBe-positive (n = 46)	P
Age (yr)	35.06 ± 12.8	38 ± 12.2	NS
Sex (M/F)	8/8	41/5	0.002
Genotype	D	D	NS
ALT (IU/L)	70.7 ± 63.5	101.7 ± 204.3	NS
AST (IU/L)	69.3 ± 67.6	98.6 ± 134.07	NS
T-Bil (mg/dL)	1.18 ± 0.58	1.43 ± 0.97	NS
HBV DNA (log copies/mL)	6.21 ± 1.7	5.2 ± 1.57	NS

NS: Not significant.

Table 3 The rate and percentage of BCP/C region mutations in HBV isolated among different clinical groups n (%)

Mutation	Inactive HBsAg carriers (n = 12)	Chronic hepatitis (n = 30)	Liver cirrhosis (n = 13)	HCC (n = 7)	Total rate (%)
A1757	12 (100)	25 (83.3)	11 (84.61)	5 (71.42)	85.4
C1753	1 (8.3)	5 (16.6)	5 (38.46)	4 (57.14)	24.2
T1762/A1764	5 (41.6)	11 (36.6)	4 (30.76)	3 (42.85)	37.1
A1899	3 (25)	9 (30)	8 (61.5)	3 (42.85)	37.1
A1896	5 (41.6)	19 (63.3)	6 (46.15)	6 (85.71)	58.1
T1766/A1768	1 (8.3)	4 (13.3)	2 (15.38)	1 (14.28)	12.9
T1764/G1766	1 (8.3)	5 (16.6)	1 (7.69)	2 (28.5)	14.5

patients were divided into four categories: 19.4% patients (n = 12) were inactive HBsAg carriers, 48.4% (n = 30) had chronic hepatitis B infection, 21.0% (n = 13) were diagnosed with cirrhosis, and 11.3% (n = 7) had HCC. The clinical and laboratory findings (serologic, biochemical and virologic) are summarized in Table 1. The clinical features of HBeAg-positive patients and anti-HBe-positive patients are shown in Table 2. Based on the HBeAg serology status, 16 patients were HBeAg-positive and 46 patients were anti-HBe-positive. There was no significant difference in age, ALT, AST, and bilirubin levels (biochemical parameters), and HBV viral load between HBeAg-positive and HBeAg-negative groups; however, a significant difference in the gender distribution was observed (χ^2 = 10.96, P = 0.003) (Table 2). All chronic hepatitis B patients with HCC were HBeAg-negative and were older than the other groups.

HBV genotype and subtype

The phylogenetic tree was constructed using the block alignment of HBV S plus BCP/C gene sequences (62 HBV isolates from this study) along with different HBV genotype (A to H) sequences retrieved from the GenBank[19] as reference genes. The phylogenetic tree revealed that all Iranian isolates were branched with other genotype D of HBV reference isolates with a high bootstrap value, 99%, 1000 × replicates (Figure 1). Thus, all Iranian patients were infected with only genotype D. To assess the HBV subtype, the amino acid mapping on the HBV *S* gene protein was performed. Based on the presence of Arg[122], Thr[125], Pro[127], and Lys[160] residues, 98.4% (n = 61) and 1.6% (n = 1) of isolated HBV were subtyped as *ayw2* and *ayw3*, respectively.

Characteristics of nucleotide substitution in the S and BCP/C regions

Amino acid sequences of a portion of the S region of all 62 isolates of the study patients were compared with the amino acid sequences of the reference genes. Amino acid mapping revealed that the S region was relatively conserved; however, an important substitution of P120T/S was observed. P120T/S substitutions were detected in 9.7% of chronic hepatitis and cirrhotic patients (n = 6). No G145R substitution was identified in the isolates; whereas, some substitutions such as P127T, T131I, Y134H, D144N and I152T were observed in the immunologic domain of the "a determinant" region.

With regard to the mutations in the BCP and C regions (Table 3), a high rate of G1896A PC mutant variants (58.1%, 36/62) was detected in the isolates. The rate of precore mutant isolates was significantly higher (χ^2 = 9.682, P = 0.003) in HBeAg-negative patients (69.5%, n = 32) compared to HBeAg-positive patients (25%, n = 4). In the HBV precore region, mutation of G1899A (Gly-to-Asp, at codon 29) was found in 37.1% isolates (n = 23), and was mostly detected in patients with cirrhosis (61.5%), and HCC (42.8%). All isolates had T1585 which is specific for genotype D.

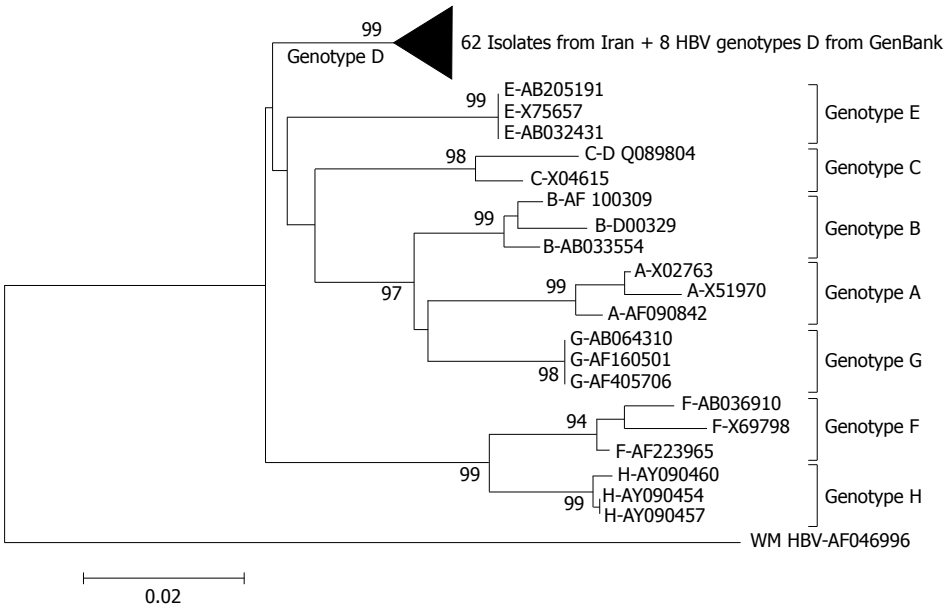

Figure 1 Neighbour joining phylogenetic analysis based on the block-alignment of the S/BCP/C gene regions (approximately 1000-bp) of 62 HBV isolates from Iran and other HBV genotypes form GenBank as reference genes. Bootstrap values indicate 1000-fold replicates. Due to clarity, the 62 isolates from Iran and 8 reference genes of HBV genotype D were collapsed. Woolly monkey HBV was used as an out-group.

BCP double mutation (T1762/A1764) was observed in 37.1% isolates (n = 23); whereas, T1762 and A1764 were also found alone. There was no significant difference in the rate of BCP double mutation between HBeAg-positive and HBeAg-negative patients. The occurrence of precore mutant plus BCP double mutation was detected in 28.2% of HBeAg-negative patients (n = 13); and in 12.5% (n = 2) HBeAg-positive individuals. The G1862T mutation was observed in 4.8% of chronic hepatitis patients (n = 3). This mutation, in which valine (Val) was replaced by phenylalanine (Phe) at position 17 in the PC region, was observed only in HBeAg-negative patients.

DISCUSSION

In the present study, we examined the clinical, serologic, virologic and phylogenetic features in patients with different clinical stages of HBV-related liver disease (inactive HBsAg carriers, chronic hepatitis B, chronic hepatitis B with cirrhosis and HCC). We believe this is the first such study from Iran. Our previous studies on the molecular analysis of HBV, revealed the presence of genotype D in Iran[14,20]. As expected, the present study also showed that genotype D with *ayw2* subtype was present in all 62 patients studied. Genotype D has been reported globally[21]; but has a high prevalence in the Mediterranean area and in the Middle East[22].

The relationship between HBV genotype(s), and the outcome of liver disease, and the response to treatment has been well documented, and has an important impact on public health[8,23]. For example, several studies have shown differences in disease progression between genotype B and C in Asian patients. HBV genotype C is associated with more severe cirrhosis and HCC, and poorer response to interferon therapy[24,25]. Moreover, it has been observed that genotypes can influence HBV replication. For example, HBeAg-negative patients harbouring genotype B had lower viral replication efficiency[25]. Since HBV genotype D was the only predominant genotype in the present study, a comparison between different genotypes was not possible. Non D HBV genotypes are not seen in Iran; whereas, different subtypes of genotype D have been reported[26].

In the present study, amino acid mapping of the *S* gene showed a high rate of homology between the sequences. It has been shown that amino acid substitutions within the "a determinant" domain in the HBV *S* region may lead to conformational changes in the S protein. Some of these changes may create important medical and public health problems including vaccine escape, failure of hepatitis B immune globulin (HBIG) to protect liver transplant patients and babies born to HBV carrier mothers, and failure to detect HBV carriers with certain diagnostic tests[7,27]. In this study, the P120T/S was the most important substitution. This substitution was located at the outside of the "a determinant" immunologic domain. The P120T/S was detected in six isolates. As previously reported, the P120T/S substitution may cause problems with diagnostic assays, and may also cause vaccine escape and poor response to HBIG therapy[27,28].

The G1896A PC mutation truncates the HBeAg protein product by creating a stop codon at position 28 within the precore mRNA. Therefore, patients with HBV variants carrying the A1896 mutant in the genome are usually HBeAg-negative. The G1896A PC mutant may be detected in 20%-95% of HBeAg-negative patients worldwide[3], and is highly predominant in the Mediterranean area where HBV genotype D has a high rate of infection[29]. In a previous study, the rate of HBV precore mutant variants in Iran was reported to be 54%[14]; in the present study, the rate was 58.1%. We observed that patients with a precore mutant variant were older and had a higher rate of AST and ALT elevation (but not statistically significant) compared to patients without this variant, suggesting that this variant occurs in patients with a longer history of HBV infection and worse liver disease. In this study, 85.7%

of patients with HCC (n = 6) carried A1896 variants; whereas, this rate was less in the other study groups. These results are in accordance with previous reports[6,30]. The precore mutant variants have also been reported in HBeAg-positive patients, ranging from 0%-80%[6,31]. In our study, two patients with cirrhosis were infected with the HBV A1896 variants despite HBeAg positivity.

The BCP T1762/A1764 double mutations located at the HBV X gene diminishes HBeAg production, and is associated with more active liver disease[2,32]. In the present study, BCP double mutation T1762/A1764 was detected in 37.1% of patients; but there was no association between these mutations and the status of liver disease (P = 0.7). Moreover, no significant difference (P = 0.9) was observed between the frequency of BCP double mutation in patients with HBeAg-positive and HBeAg-negative phenotypes. By contrast, other studies have shown a relationship between BCP double mutations and the clinical manifestations of HBV infection[6,30]. Moreover, the T1764/G1766 double mutation in the BCP region was detected in 14.5% of isolates (n = 9) (Table 2). However, this study utilized a relatively small study population, and the results suggest that mutations in the PC region were related to more severe liver disease. More studies in larger populations are required to better understand these associations.

In conclusion, the present study has shown that genotype D (predominantly subtype *ayw2*) is the only genotype in Iranian patients. Moreover, a high rate of the precore mutation and BCP double mutation was detected in our study.

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COMMENTS

Background

Heterogeneity of hepatitis B virus (HBV) genome and its mutations may influence the outcome of liver disease as well as the response to antiviral treatment. Considering this fact, we studied the clinical, virologic and phylogenetic features of HBV infection in four groups of patients: inactive carriers, chronic hepatitis, cirrhosis and hepatocellular carcinoma.

Research frontiers

The present study revealed an association between certain HBV mutations and the outcome of liver disease, and the response to treatment.

Innovations and breakthroughs

This is the first report on the association between different clinical presentations of HBV infection and mutations in three regions of the HBV genome: basal core promoter (BCP)/Precore, P and S.

Peer review

The present study is relatively small, but it provides useful information on HBV characteristics in Iranian patients with chronic HBV infection.

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RAPID COMMUNICATION

Polymorphisms of microsomal triglyceride transfer protein in different hepatitis B virus-infected patients

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Abstract

AIM: To identify the two polymorphisms of microsomal triglyceride transfer protein (*MTP*) gene in the Chinese population and to explore their correlation with both hepatitis B virus (HBV) self-limited infection and persistent infection.

METHODS: A total of 316 subjects with self-limited HBV infection and 316 patients with persistent HBV infection (195 subjects without familial history), matched with age and sex, from the Chinese Han population were enrolled in this study. Polymorphisms of MTP at the promoter region -493 and at H297Q were determined by the allele specific polymerase chain reaction (PCR).

RESULTS: The ratio of males to females was 2.13:1 for each group and the average age in the self-limited and chronic infection groups was 38.36 and 38.28 years, respectively. None of the allelic distributions deviated significantly from that predicted by the Hardy-Weinberg equilibrium. There was a linkage disequilibrium between H297Q and -493G/T (D' = 0.77). As the χ^2 test was used, the genotype distribution of *MTP*-493G/T demonstrated a significant difference between the self-limited infection group and the entire chronic group or the chronic patients with no family history (χ^2 = 8.543, P = 0.015 and χ^2 = 7.199, P = 0.019). The allele distribution at the *MTP*-493 position also demonstrated a significant difference between the study groups without family history (χ^2 = 6.212, P = 0.013). The T allele emerged as a possible protective factor which may influence the outcomes of HBV infection (OR: 0.59; 95% CI: 0.389-0.897).

CONCLUSION: The polymorphism of the *MTP* gene, T allele at -493, may be involved in determining the HBV infection outcomes, of which the mechanism needs to be further investigated.

Key words: Hepatitis B virus; Microsomal triglyceride transfer protein; Single nucleotide polymorphism; Self-limited HBV infection; Chronic hepatitis B; Clinical outcomes

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INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of acute and chronic liver disease worldwide, especially in several areas of Asia and Africa. Most infected individuals can clear the virus, while only 5%-10% develop chronic hepatitis and remain in a persistent viral state[1,2]. The reasons for viral persistence are poorly understood, but host genetic factors are likely to influence the disease outcome[3].

Molecular genetics methods have increased our

ability to discover variations in the human genome and to correlate them with disease. Single nucleotide polymorphisms (SNPs) are used to characterize gene variations. Genetic associations can provide clues to fundamental questions about the pathogenesis of diseases and lead to new therapeutic avenues[4]. For chronic hepatitis B, this approach may help determine the basis for viral persistence and the development of end-stage complications such as cirrhosis or hepatocellular carcinoma. Initial genetic studies of viral hepatitis focused on human leukocyte antigen (*HLA*) associations[5] and polymorphisms in the promoter or coding region of several genes, such as interleukin-10 (*IL*-10), interferon-γ (*IFN*-γ), vitamin-D receptor (*VDR*), *etc*[6-10], and demonstrate some relationships with the outcome of HBV infection.

The liver is the major organ for the production of plasma lipoproteins, their uptake from plasma and their catabolism[11]. The production of apolipoprotein B (apoB)-containing lipoproteins by the liver is required for the assembly and secretion of very low-density lipoproteins (VLDLs) and low-density lipoproteins (LDLs)[12-16]. The microsomal triglyceride transfer protein (MTP) also plays a key role in apoB secretion by catalyzing the transfer of lipids to the nascent apoB molecule as it is co-translationally translocated across the endoplasmic reticulum membrane[17,18]. Recent studies have shown that the polymorphism at *MTP*-493 is responsible for a change in the *MTP* gene at the transcription level, and that this is prone to influence the intrahepatic triglyceride content[19-21].

Hepatic steatosis frequently occurs during chronic hepatitis B and C. In a transgenic mouse model, hepatitis C virus (HCV) core protein has been shown to inhibit the MTP activity and to modify the hepatic VLDL assembly and secretion[22]. However, no data are available to demonstrate the functional polymorphism of *MTP*-493T/G in HBV-infected patients. The aim of this pilot study was to identify the two polymorphisms of the *MTP* gene in the Chinese population by SNP and to explore their correlation with both HBV self-limited infection and persistent infection.

PATIENTS AND METHODS

Human subjects

In China, 90% of Chinese people are Han and the other 10% derive from 55 minority populations. We enrolled 632 Han Chinese subjects from Ruijin Hospital of Shanghai Jiaotong University Medical School. Among them, 316 had persistent HBV infection (including 195 patients with no family history of chronic hepatitis B) and 316 had previously self-limited HBV infection with no family history. Age and sex were matched between these groups.

The diagnostic criteria for persistent HBV infection were based on the presence of hepatitis B surface antigen (HBsAg) and anti-core IgG-antibody (Anti-HBc), and the absence of anti-hepatitis B surface antibody (Anti-HBs) for more than 6 mo. The mean time from the presumed onset of HBV infection was defined as the first documented seropositivity for HBsAg with or without elevated serum liver enzyme.

Self-limited hepatitis B virus infection was defined as being positive for anti-HBs and anti-HBc, in the absence of previous HBV vaccination, and a negative family history of chronic hepatitis B. Serum HBV-DNA was analyzed to exclude patients with occult HBV infection.

Subjects negative for all HBV markers were not included in the study as these subjects were unlikely to have been exposed to HBV. If they had been exposed to the virus, it would be impossible to predict their outcome.

Patients with concurrent hepatitis A, C, D, E or human immunodeficiency virus (HIV) infection were excluded from the study. Patients with liver disease caused by other factors, such as excess alcohol consumption and autoimmune hepatitis, were also excluded from the study. Our study conforms to the ethical guideline of the 2004 Declaration of Helsinki.

Serological test

Five milliliters of whole blood samples was collected from each subject, the sera were stored at -20℃. Serology for HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc was conducted in accordance with the manufacturer's protocol (Axsym, Abbott).

Genomic DNA extraction

Genomic DNA was isolated using a genomic DNA purification kit (PUREGENE) according to its manufacturer's instructions. DNA samples were quantified with a biophotometer (Eppendorf) and subjected to allele specific real-time polymerase chain reaction (PCR).

Genotyping of gene polymorphisms

Polymorphisms of MTP, including *MTP*-493G/T and H297Q were analyzed. We used the Allele Specific PCR Primer Design Program provided by Roche to design primers (Table 1).

All amplifications were performed on ABI-7000 (real-time PCR) with a 50 μL reaction mixture containing 30 ng of genomic DNA, 0.2 μmol/L per primer, PCR buffer, 0.2 μmol/L of each dNTP (Promega), 4% DMSO (Fisherbrand), 2.4% glycerine, 5 units of Delta Z05 DNA polymerase (Roche), 1 × SYB green (Cambrex). Each genotyping contains 2 amplifications, with one common primer and two specific primers, respectively. To genotype the polymorphisms at the promoter region-493 and at the coding region of MTP at amino acid position 297, primers *MTP*-493-1, *MTP*-493-2, *MTP*-493-cp and MTP H297Q-1, MTP H297Q-2, MTP H297Q-cp were used to analyze the *MTP*-493 G/T and MTP H297Q polymorphisms, respectively (Table 1). Amplification was performed with activation and denaturation at 94℃ and at 95℃ and an annealing temperature of 60℃, respectively.

Genotypes were determined by the difference in cycling numbers (ΔCT) of 2 amplification curbs with the same genomic DNA and the melting curbs, according to the manufacturer's (Roche) instructions.

Statistical analysis

The frequencies of *MTP*-493G/T and *MTP* H297Q alleles were compared between the chronic infection and self-limited infection groups by the χ^2 test. Hardy-Weinberg equilibrium was tested by comparing the expected and observed genotype frequencies using the χ^2 test. To analyze the linkage disequilibrium (LD), pair wise LD was analyzed between two loci on MTP by evaluating the measurement of D'. The difference between the probabilities of observing the alleles independently in the population is: $f(D) = f(A_1B_1) - f(A_1)f(B_1)$, where A and B refer to two genetic markers and *f* is their frequency. D' is obtained from D/Dmax and a value of 0.0 suggests independent assortment, whereas 1.0 means that copies of an allele occur exclusively with one of the possible alleles of the other marker. Analysis of D' was performed using HAPLOVIEW 3.0. The odds ratio with a 95% confidence interval, *P* values and Mantel-Haenszel test were calculated using SAS 8.0 to explore the SNP which may independently influence the outcome of HBV infection.

Table 1 Positions of analyzed SNP and primers used in this study

Genes (Ref.)	SNP	Primers	Sequences (5'-3')
MTP H297Q rs#2306985[23]	C/G	-1	CAGGTCTTCCAGAGCCAC
		-2	CAGGTCTTCCAGAGCCAG
		-cp	ATTTGTCTGCACTTACAGAAGGA
MTP-493[20]	G/T	-1	ATTTAAACTGTTAATTCATACCACA
		-2	TTTAAACTGTTAATTCATACCACC
		-cp	CTTTAACATTATTTTGAAGTGATTGG

Table 2 General characteristics of 632 subjects

	Self-limited HBV infection (*n* = 316)	Chronic hepatitis B (*n* = 316)	
		With family history (*n* = 121)	No family history (*n* = 195)
Male	215	80 (25.3%)	135 (42.7%)
Female	105	41 (13.0%)	60 (19.0%)
Age (yr)	38.28 ± 11.44	36.93 ± 12.28	39.12 ± 10.84
Age range (yr)	9-75	9-69	16-75
ALT level (× ULN)	Normal	3.62 ± 3.48	3.12 ± 2.62
HBV-DNA level (Log)	Undetectable	5.45 ± 2.34	

RESULTS

Demographic characteristics of subjects

In the 2 groups matched for age and sex, the male to female ratio was 2.13:1 (215:101) in each group. The distribution of age in the chronic hepatitis B and self-limited groups, calculated by SAS respectively, was normal ($P = 0.07$ and 0.182). The mean age of subjects in the two groups was 38.28 years and 38.36 years, respectively, with no significant deviation (STDEV was 11.44 and 11.12). In the chronic hepatitis B subgroups, 121 patients (80 males and 41 females) had a family history while 195 (135 males and 60 females) had no family history of liver disease. Serum alanine aminotransferase (ALT) levels in the self-limited group were normal, and 3 times higher than the upper normal limit in the chronic hepatitis B group. Serum HBV-DNA was detectable in each study subject but undetectable (< 3 $\log_{10}$) in the self-limited group, whereas it was positive in the chronic hepatitis B group (5.45 ± 2.34 $\log_{10}$).

The general characteristics of our study subjects are summarized in Table 2.

Allele frequencies and linkage disequilibrium

The polymorphisms of *MTP* H297Q and *MTP*-493G/T were analyzed in 632 subjects of the Chinese Han population in Shanghai. The T minor allele frequency of promoter polymorphisms-493 in the *MTP* gene was 0.123, whereas the G frequency of missense polymorphism H297Q was 0.668. The 2 SNPs of the *MTP* gene showed a statistically significant linkage disequilibrium (D' = 0.77, $P < 0.05$). None of the allelic distributions deviated significantly from that predicted by the Hardy-Weinberg equilibrium (calculated by SAS, Chi-Square $P > 0.05$).

Association of SNP genotypes with outcomes of HBV infection

The genotype distribution of *MTP*, depending on the outcome of HBV infection, is shown in Table 3. The genotype frequencies of *MTP* H297Q, CC, CG and GG were 0.104, 0.468 and 0.428 in the self-limited group while 0.104, 0.443 and 0.453 in the chronic group, respectively. The frequencies of the TT, TG and GG genotypes of *MTP*-493G/T were 0.013, 0.253 and 0.734 in the self-limited group, and 0.025, 0.165 and 0.810 in the chronic group, respectively. The χ^2 test was used to analyze the association of genotype distribution with HBV infection outcomes. The distribution of *MTP*-493G/T was significantly different between the self-limited and chronic hepatitis B groups, both before and after adjustment for family history ($\chi^2 = 8.543$, $P = 0.015$; $\chi^2 = 7.199$, $P = 0.019$). The genotype distributions of *MTP* H297Q demonstrated no significant difference between the two groups and subgroups (i.e. with and without family history).

A significant difference was demonstrated in the allele distribution of *MTP*-493G/T between the self-limited and chronic hepatitis B groups without familial history ($\chi^2 = 6.212$, $P = 0.013$, Table 4). As calculated by Mantel-Haenszel, the T allele emerged as a potential protective factor positively influencing the HBV infection outcomes in the self-limiting group compared with the chronic hepatitis B group without family history ($P = 0.013$, OR = 0.59 < 1).

DISCUSSION

Several studies suggested that HBV-associated chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) are more common in men than in women[24-26], showing that the relative risk for chronic HBV infection is increased in men when compared to that in women. In China, most HBV infections occur during the neonatal or perinatal period, following materno-foetal transmission where the mothers are HBeAg-positive

Table 3 Genotype distributions of *MTP* H297Q and *MTP*-493G/T *n* (%)

SNP	Genotype	Self-limited HBV infection (*n* = 316)	Chronic hepatitis B total (*n* = 316)	Chronic hepatitis B without family history (*n* = 195)	χ^2 test			
					SLHBV *vs* CHB Total		SLHBV *vs* CHB no FH	
					Value	*P*	Value	*P*
MTP H297Q	CC	33 (10.4)	33 (10.4)	20 (10.3)	0.452	0.798	2.588	0.274
	CG	148 (46.8)	140 (44.3)	78 (40.0)				
	GG	135 (42.8)	143 (45.3)	97 (49.7)				
MTP-493G/T	GG	232 (73.4)	256 (81.0)	163 (83.6)	8.543	0.015	7.199	0.019[1]
	GT	80 (25.3)	52 (16.5)	30 (15.4)				
	TT	4 (1.3)	8 (2.5)	2 (1.0)				

[1]Fisher's exact test (2-Tail). SLHBV: Self-limited HBV infection group; CHB total: Chronic hepatitis B total group; CHB no FH: Chronic hepatitis B without chronic hepatitis B family history group.

Table 4 Allele Distributions of *MTP* H297Q and *MTP*-493G/T *n* (%)

SNP	Allele	Self-Limited HBV infection (*n* = 632)	Chronic hepatitis B total (*n* = 632)	Chronic hepatitis B without family history (*n* = 390)	χ^2 test and Mantel-Haenszel logit					
					SLHBV *vs* CHB total			SLHBV *vs* CHB no FH		
					P	OR	95% CI	*P*	OR	95% CI
MTP H297Q	C	214 (33.9)	206 (32.6)	118 (30.3)	0.52	0.903	0.659-1.236	0.232	1.18	0.899-1.549
	G	418 (66.1)	426 (67.4)	272 (69.7)						
MTP-493G/T	G	544 (86.1)	564 (89.2)	356 (91.3)	0.023	0.645	0.442-0.940	0.013	0.59	0.389-0.897
	T	88 (13.9)	68 (10.8)	34 (8.7)						

SLHBV: Self-limited HBV infection group; CHB total: Chronic hepatitis B total group; CHB no FH: Chronic hepatitis B without chronic hepatitis B family history group.

and the infants subsequently become chronic HBV carriers. The predominant mode of HBV spread is intra-familial from mothers to infants or siblings to siblings[25]. This should mean an equal exposure rate of males and females to HBV. The mechanism underlying such a male predominance is unknown. As age and sex were matched when the subjects were enrolled in our study, there was no significant difference in sex and age ($P > 0.05$). We also studied the family history of 632 subjects, in which all of the self-limited subjects and 195 chronic hepatitis B patients had no family history of the disease.

The majority of published studies on HBV persistence correlate to the role of the major histo-compati-bility complex (MHC) in determining the infection outcomes. The most convincing evidence refers to the association between HBV carriage and MHC class Ⅱ and Ⅰ molecules, such as human leukocyte antigen allele *DRB1*1302*[27-29] and allele A*0301[30], which are associated with viral clearance, whilst B*08 is associated with persistent infection[30]. Non-MHC genes have also proved interesting and successful candidates for association studies of hepatitis B viral infection. It was reported that SNPs in the *VDR*[31,32] and *TNF-α* genes, at position-857[33] as TT, are associated with the clearance of HBV in Gambians and the Chinese population, respectively. Two studies showed that the *TNF-α* SNP at position-238 may be associated with persistent infection[33,34]. Several population-based studies also revealed that *IFN-γ* with its +847 and CA repeat allele[35], cytotoxic T-lymphocyte antigen 4 (*CTLA4*) with its -318[36] and *IL-18* with its -607 and -137[37], interferon alpha receptor 1 with its -568C and -408T[38], CC chemokine receptor 5 (*CCR5*) with its 59029G and 59353, heterozygosity of *CCR5* delta 32[31,39], and mannose binding lectin (*MBL*)[40,41] are all associated with chronic HBV infection or HBV clearance.

MTP catalyzes the transport of triglyceride, cholesteryl ester, and phospholipids on phospholipid surfaces[42]. The large subunit of the human *MTP* gene is situated on chromosome 4q22-q24[43]. It has a key function in intracellular apolipoprotein (apo) B lipidation and secretion of VLDL[44]. Abundant MTP has been found on the luminal side of the endoplasmic reticulum and in the liver, intestine, and heart[45]. In the present study, we investigated the two polymorphisms of *MTP*; one is located at -493 of the promoter, the other at the 297th amino acid of the coding region. The polymorphism of *MTP*-493 G-to-T substitution affects the promoter activity of the *MTP* gene[19,20]. It was recently reported that the G allele, which decreases the *MTP* gene transcription, increases intrahepatic triglyceride content[21]. The T allele is associated with an increased expression of the MTP gene[19]. There is linkage disequilibrium between the 2 SNPs[46]. In our study, the genotype of *MTP*-493G/T distribution was significantly different between the two study groups, with different outcomes of HBV infection ($P = 0.015$). This significant difference was observed in allele distribution after adjustment for sex, age and family history ($P = 0.013$), indicating that the T allele may be one of the protective factors against HBV infection, especially against postnatal infection (OR: 0.59 < 1; 95% CI: 0.389-0.897).

A recent study showed that hepatitis C, as a metabolic disease, is associated with liver steatosis involving accumulation of intracytoplasmic lipid droplets[47].

The function of MTP is to lipidate the growing apoB polypeptide chain during translation, allowing apoB to fold correctly and assemble a lipoprotein with a neutral lipid core before secretion[48,49]. It appears to be obligatory for hepatic secretion of apoB[50,51]. It has been shown that the G allele in *MTP*-493 G/T influences the transcriptional activity and is associated with low plasma levels of LDL cholesterol in healthy middle-aged men, and the T allele is associated with an increased expression of the *MTP* gene *in vitro*, and may enhance hepatic secretion of larger VLDL[19]. It was reported that TT and TG in *MTP*-493 increase the *MTP* gene expression and hepatic secretion of VLDL. However, one French study demonstrated that the functional G/T *MTP* polymorphism does not play a role in the development of steatosis in chronic hepatitis C[52].

Brozovic S[53] recently showed that CD1d, a MHC class I-related molecule that functions in glycolipid antigen presentation to distinct subsets of T cells that express natural killer receptors and an invariant T-cell receptor-α chain (invariant NKT cells), is regulated by MTP in hepatocytes. MTP deletion affects the ability of hepatocytes CD1d to activate invariant NKT cells.

In self-limited infections, HBV-DNA falls by more than 90% within 2-3 wk following the viral replication peak and before the peak of antigen-specific CD8 response and liver damage[54]. The role of NK cells in the initial viral containment is confirmed by the observation that the NK cell peak in the circulation of patients infected with HBV precedes the decline of HBV replication[55]. This maximal elevation of the NK cell frequency is then followed by the peak of HBV-specific CD8 cells a few weeks later[55]. Experiments of T cell depletion with anti-CD4 or anti-CD8 antibodies injected into infected chimpanzees showed that NK and NKT cells can contribute substantially to early viral containment[56]. MTP may influence the outcomes of HBV infection by a mechanism mediated by CD1d regulation and NKT cells' activation during the early period of infection.

In summary, the 2 SNPs in MTP have linkage disequilibrium and the T at *MTP*-493G/T may be associated with the clearance of HBV leading to self-limited infection. This might be mediated by CD1d regulation and activation of NKT cells during the early period of infection. The mechanism needs to be further investigated. With the development of SNP detection technology, more SNPs in different genes are likely to be found to be associated with HBV infection. The combination of several SNPs can serve as a predictor for the HBV infection outcomes, leading to new therapeutic methods for HBV infection.

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COMMENTS

Background

Most infected individuals infected with hepatitis B virus (HBV) can clear the virus, while only 5%-10% develop chronic hepatitis and remain in a persistent viral state. The reasons for viral persistence are poorly understood, but host genetic factors are likely to influence the disease outcome.

Research frontiers

This study looked for the potential host genetic factors which may influence the outcomes of HBV infection.

Innovations and breakthroughs

The results show, for the first time, that single nucleotide polymorphisms (SNPs) of microsomal triglyceride transfer protein (*MTP*)-493G/T, which is responsible for MTP transcription level, might be involved in determining the outcomes of HBV infection.

Applications

Based on the results of our study, further investigation should be focused on the mechanism underlying the association between MTP-dependent lipid metabolism and HBV infection, which may lead to new therapeutic methods.

Peer review

The manuscript reports results of an interesting clinical trial. The authors analyzed the polymorphisms of *MTP*-493 at 297 positions in correlation with self-limited and persistent HBV infection in 632 Han Chinese patients. Such an analysis is of importance. The study is well designed and the paper is written in rather good English.

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RAPID COMMUNICATION

Dendroaspis natriuretic peptide relaxes gastric antral circular smooth muscle of guinea-pig through the cGMP/cGMP-dependent protein kinase pathway

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Abstract

AIM: To systematically investigate if cGMP/cGMP-dependent protein kinase G (PKG) signaling pathway may participate in dendroaspis natriuretic peptide (DNP)-induced relaxation of gastric circular smooth muscle.

METHODS: The content of cGMP in guinea pig gastric antral smooth muscle tissue and perfusion solution were measured using radioimmunoassay; spontaneous contraction of gastric antral circular muscles recorded using a 4-channel physiograph; and Ca^{2+}-activated K^+ currents ($I_{K(Ca)}$) and spontaneous transient outward currents (STOCs) in isolated gastric antral myocytes were recorded using the whole-cell patch clamp technique.

RESULTS: DNP markedly enhanced cGMP levels in gastric antral smooth muscle tissue and in the perfusion medium. DNP induced relaxation in gastric antral circular smooth muscle, which was inhibited by KT5823, a cGMP-dependent PKG inhibitor. DNP increased $I_{K(Ca)}$. This effect was almost completely blocked by KT5823, and partially blocked by LY83583, an inhibitor of guanylate cyclase to change the production of cGMP. DNP also increased STOCs. The effect of DNP on STOCs was abolished in the presence of KT5823, but not affected by KT-5720, a PKA-specific inhibitor.

CONCLUSION: DNP activates $I_{K(Ca)}$ and relaxes guinea-pig gastric antral circular smooth muscle *via* the cGMP/PKG-dependent singling axis instead of cAMP/PKA pathway.

Key words: Dendroaspis natriuretic peptide; Cyclic guanosine monophosphate; Protein kinase G; Protein kinase A; Gastric motility

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INTRODUCTION

Natriuretic peptides (NP) include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP), dendroaspis natriuretic peptide (DNP) and urodilatin[1]. DNP is a recently isolated peptide that contains 38 amino residues and shares structural and functional properties with the other members of the natriuretic peptide family[2]. Studies about its physiologic functions mainly focus on cardiovascular[3-5], genital[6], and urinary systems[7]. Evolutionary studies[8] have suggested the presence of DNP-like immunoreactivity

in rat colon, and the DNP-like molecule may control colonic motility as a local regulator. Interestingly, we found that DNP inhibits spontaneous contraction in gastric circular smooth muscle[9]. NPs are similar to nitric oxide (NO), which play important physiological functions by affecting the activity of cGMP and cAMP. Sabbatini *et al* reported[10] that CNP enhances amylase release by reducing cAMP in the exocrine pancreas. Borán *et al*[11] showed that ANP could play a beneficial role in the resolution of neuroinflammation by removing dead cells and decreasing levels of proinflammatory mediators in microglia *via* the cGMP-dependent protein kinase G (PKG) signaling pathway. ANP stimulates lipolysis in human adipocyte through a cGMP signaling pathway[12]. However, Wen *et al*[13] found that CNP activates the pGC-cGMP- phosphodiesterases 3 (PDE3)-cAMP signaling to play a role in hyperthyroid beating of rabbit atria. It is indicated that NP exerts its physiological function by a different pathway. In our previous study, we simply observed that cGMP participates in DNP-induced relaxing of circular smooth muscle, by using a pharmacologic approach. Thus, the aim of this study was to systematically investigate if the cGMP-PKG or cAMP signaling pathway may participate in DNP-induced relaxation using pharmacologic, radioimmunoassay and patch-clamp technique in gastric circular smooth muscle of guinea pigs.

MATERIALS AND METHODS

Preparation of muscle strips

Guinea pigs of either sex, weighing 250-350 g, were purchased from the Experimental Animal Center, Dalian Medical University. The guinea pigs were housed in plastic cages containing corn-chip bedding with free access to food and water for 1 d before they were used for experiments. The care and use of the animals were followed strictly in accordance with the National Institutions of Health Guide for the Care and Use of Laboratory Animals. Guinea pigs were euthanized by a lethal intravenous dose of pentobarbital sodium (50 mg/kg). The abdomen of each guinea pig was opened along the midline, and the stomach was removed and placed in pre-oxygenated Tyrode's solution at room temperature. After the mucous layer was removed, strips (approximately 2.0 mm × 15.0 mm) of gastric antral circular muscles were prepared. The muscle strips were placed in a bath chamber (2 mL volume). One end of the strip was fixed on the lid of the chamber through a glass claw, and the other end was attached to an isometric force transducer (TD-112S, Nihon Kohden-Kogyo Japan). The chamber was constantly perfused with pre-oxygenated Tyrode's solution at 1 mL/min. The temperature was maintained at 37.0 ± 0.5℃ with a water bath thermostat (WC/09-05, Chongqing, China). The muscle strips were allowed to incubate for at least 40 min before the experiments were started.

Isolated cell preparation and electrophysiological recording

The longitudinal layer of muscle was dissected from the other muscle layers using fine scissors and then cut into small segments (1 mm × 4 mm). These segments were kept in modified Kraft-Bruhe (K-B) medium at 4℃ for 15 min. They were then incubated at 36℃ in 4 mL of digestion medium [Ca-free physiologic salt solution (Ca-free PSS)] containing 0.1% collagenase Ⅱ, 0.1% dithioerythritol, 0.15% trypsin inhibitor, and 0.2% BSA for 25-35 min. The digested muscle segments were transferred into the modified K-B medium, and the single cells were dispersed by gentle disruption with a wide-bore, fire-polished glass pipette. The isolated gastric myocytes were kept in modified K-B medium at 4℃ prior to use. Isolated cells were transferred to a 0.1 mL chamber on the stage of an inverted microscope (IX-70 Olympus, Tokyo, Japan) and allowed to settle for 10-15 min. The cells were continuously perfused with an isosmotic PSS at a rate of 0.9-1.0 mL/min. An 8-channel perfusion system (L/M-sps-8, List Electronics, Berlin, Germany) was used to exchange different solutions. The Ca^{2+}-activated K^+ currents ($I_{K(ca)}$) were recorded using the conventional whole-cell patch-clamp technique. Patch-clamp pipettes were manufactured from borosilicate glass capillaries (GC 150T-7.5, Clark Electromedical Instruments, London, UK) using a 2-stage puller (PP-83, Narishige, Tokyo, Japan). The resistance of the patch pipette was 3-5 MΩ when filled with pipette solution. Liquid junction potentials were canceled prior to the seal formation. Whole-cell currents were recorded using an Axopatch 1-D patch-clamp amplifier (Axon Instruments, Foster City California, USA), and data were filtered at 1 KHz. Command pulses, data acquisition, and storage were applied using the IBM-compatible, 486-grade computer and pCLAMP 6.02 software (Axon Foster City, California, USA). Spontaneous transient outward currents (STOCs) were recorded simultaneously by an EPC-10-HEAKA amplifier (HEAKA Instruments, Berlin, Germany). All experiments were performed at room temperature (20-25℃).

Radioimmunoassay

Radioimmunoassay was performed as described elsewhere[14].

Drugs and solutions

Tyrode solution contained (in mmol/L): NaCl 147, KCl 4, $MgCl_2{\cdot}6H_2O$ 1.05, $CaCl_2{\cdot}2H_2O$ 0.42, $Na_2PO_4{\cdot}2H_2O$ 1.81, and 5.5 mmol/L glucose. Ca^{2+}-free PSS was composed of (in mmol/L): NaCl 134.8, KCl 4.5, glucose 5, and N-(2-hydroxyethyl) piperazine-N-(2-ethanesulphonic acid) (HEPES; pH was adjusted to 7.4 with Tris (hydroxymethyl aminomethane). Modified K-B solution contained (in mmol/L): *L*-glutamate 50, KCl 50, taurine 20, KH_2PO_4 20, $MgCl_2{\cdot}6H_2O$ 3, glucose 10, HEPES 10, and egtazic acid 0.5 (pH 7.40 with KOH). PSS contained (in mmol/L): NaCl 134.8, KCl 4.5, $MgCl_2{\cdot}6H_2O$ 1, $CaCl_2{\cdot}2H_2O$ 2, glucose 5, HEPES 10, and sucrose 110 (pH 7.4 with Tris). In order to eliminate delayed rectifier K^+ currents ($I_{K(V)}$), external solution contained 4-aminopyridine (10 mmol/L), a selective inhibitor of $I_{K(V)}$. The pipette solution for recording $I_{K(Ca)}$

contained (in mmol/L): K^+-aspartic acid 110, Mg-ATP 5, HEPES 5, $MgCl_2{\cdot}6H_2O$ 1.0, KCl 20, egtazic acid 0.1, di-tris-creatine phosphate 2.5, and disodium-creatine phosphate 2.5 (pH 7.3 with KOH). Tetraethylammonium (TEA), DNP, LY83583, zaprinast, KT5823 and KT5720 were made up as stock solutions. All chemicals in this experiment were purchased from Sigma (St Louis, MO, USA).

Statistical analysis

All data was expressed as mean ± SD. Statistical significance was evaluated using Student *t*-test. Differences were considered to be significant when $P < 0.05$.

RESULTS

Effect of DNP on cGMP production

Our previous pharmacological study[15] suggests that DNP obviously inhibits spontaneous contraction in gastric antral circular smooth muscle through the cGMP-dependent signaling pathway. To directly confirm the involvement of cGMP on the effect of DNP, in the present study, we measured the content of cGMP in the smooth muscle tissue and in the perfusion solution using radioimmunoassay. The result indicated that cGMP in the smooth muscle tissue and perfusion solution was markedly increased after addition of 10 nmol/L DNP (Figure 1A and B). Pretreatment with 10 nmol/L LY83583 significantly diminished DNP-induced increase in the content of cGMP (Figure 1A and B).

Effect of cGMP-dependent protein kinase on DNP-induced relaxation

Because cGMP activates PKG, we tested the effect of KT-5823 (1 μmol/L), a membrane-permeable PKG-specific inhibitor, on DNP-induced relaxation in the gastric antral circular smooth muscle to determine the potential involvement of PKG. The result indicated that KT-5823 could markedly diminish, although not completely abolish the inhibitory effect of DNP on spontaneous contraction (Figure 1C).

Effect of cGMP on DNP-induced increase of $I_{K(ca)}$

Considering our previous finding that DNP relaxed smooth muscle by increasing $I_{K(ca)}$[15], here we further investigated the relationship between cGMP and DNP-induced increase of $I_{K(ca)}$, and found that the effect of DNP on $I_{K(ca)}$ was observed in the presence of LY83583, an inhibitor of guanylate cyclase to change the production of cGMP. LY83583 (10 nmol/L) significantly blocked DNP-induced increase of $I_{K(ca).}$ The percentage of DNP-induced increase was diminished from 63.24% ± 4.32% to 28.53% ± 3.31% at 60 mV (Figure 2).

Effect of cGMP-dependent protein kinase on DNP-induced increase of $I_{K(ca)}$

To extend our understanding of the role of DNP in the regulation of $I_{K(ca)}$ through the cGMP/PKG pathway, the effect of KT-5823 (a membrane-permeable PKG-specific inhibitor) on channel activity was tested. As the results show in Figure 3, the addition of KT-5823 (1 μmol/L) completely inhibited DNP-induced increase of $I_{K(ca)}$. This data suggests the involvement of PKG-mediated phosphorylation in DNP-mediated regulation of $I_{K(ca)}$.

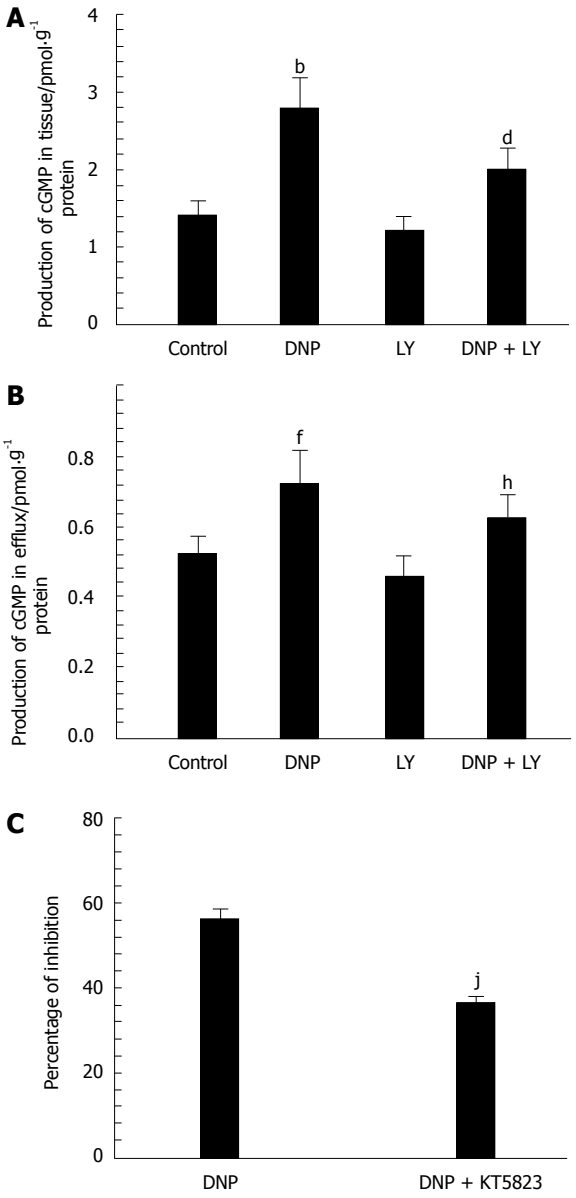

Figure 1 **A**: Effect of 10 nmol/L LY83583 on cGMP production of in tissue ($n = 8$), $^bP < 0.01$ *vs* control group, $^dP < 0.01$ *vs* DNP group; **B**: Effect of 10 nmol/L LY83583 on cGMP production of in efflux ($n = 8$), $^fP < 0.01$ *vs* control group, $^hP < 0.01$ *vs* DNP group; **C**: Effect of a membrane-permeable PKG-specific inhibitor (KT5823) on DNP-induced inhibition of spontaneous contraction in gastric antral circular smooth muscle ($n = 8$), $^jP < 0.01$ *vs* DNP group.

Effect of cGMP-dependent protein kinase on DNP-induced increase in STOCs

STOCs, which can be activated by extracellular Ca^{2+} influx and intracellular Ca^{2+} release, were recorded at -20 mV. The currents were sensitive to TEA (a nonselective K^+ channel blocker) and CHTX (a selective Ca^{2+}-activated K^+ channel blocker). As described in our previous study, DNP increased STOCs in gastric circular myocytes. To

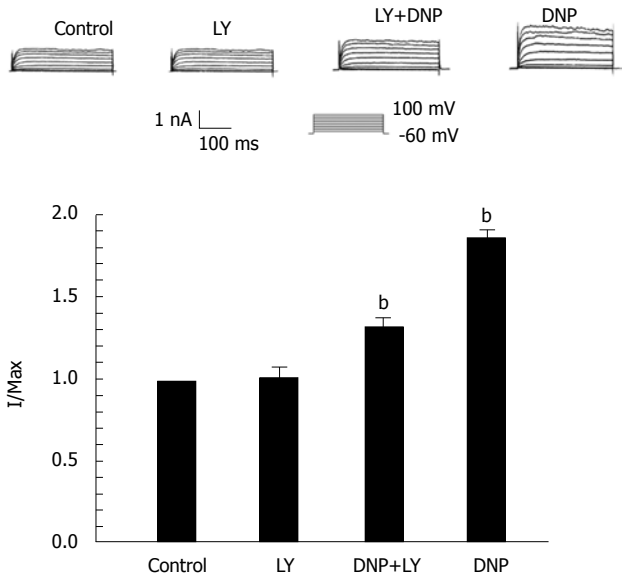

Figure 2 Effect of LY83583 on DNP-induced increase of $I_{K(Ca)}$, $^{b}P < 0.01$ *vs* control group.

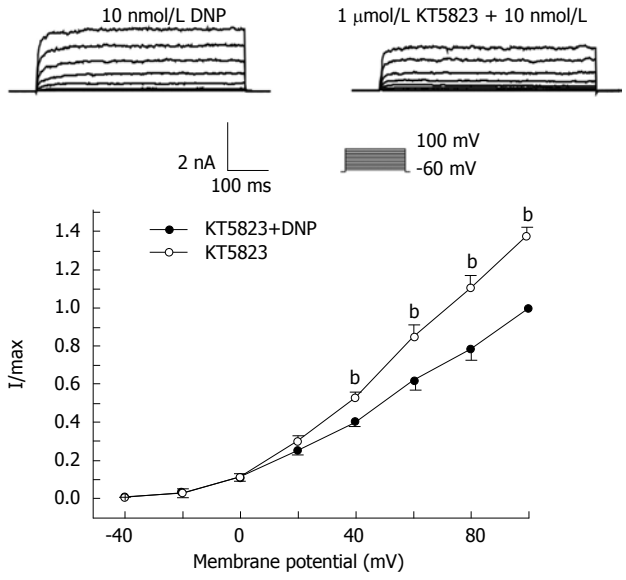

Figure 3 Effect of a membrane-permeable PKG-specific inhibitor, KT-5823, on DNP-induced increase of $I_{K(Ca)}$ (n = 8), $^{b}P < 0.01$ *vs* control group.

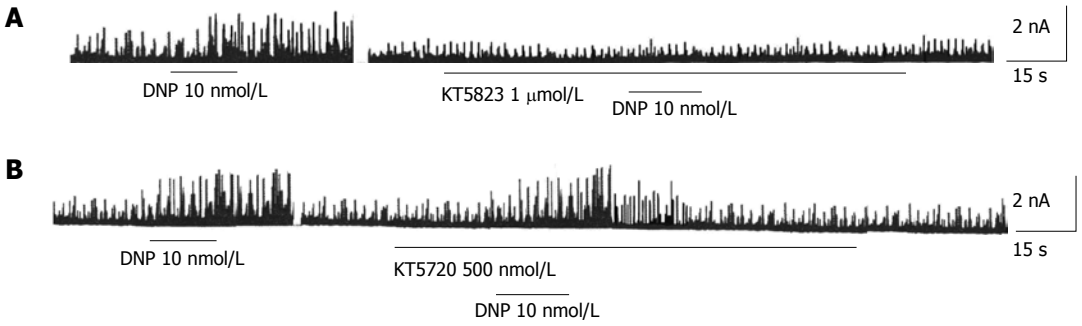

Figure 4 **A**: Effect of a membrane-permeable PKG-specific inhibitor, KT-5823, on STOCs (n = 10); **B**: Effect of KT-5720 (500 nmol/L), a PKA-specific inhibitor, on STOCs (n = 10).

further investigate the relationship between cGMP/PKG pathway and DNP-induced increase in STOCs, we examined the effect of KT5823 on DNP-induced increase in STOCs. The result indicated that KT5823 almost completely abolished DNP-induced increase of STOCs (Figure 4A). However, KT-5720 (500 nmol/L), a PKA-specific inhibitor, could not suppress DNP-induced increase in STOCs (Figure 4B).

DISCUSSION

In the present study, the patch clamp technique, radioimmunoassay and specific pharmacological inhibitors were used to determine involvement of the pGC-cGMP-PKG pathway in DNP-mediated relaxation in guinea-pig gastric antral circular smooth muscle.

In this study, we found that cGMP in the smooth muscle tissue and perfusion solution both were markedly increased after the addition of DNP. The effect of DNP was diminished after treatment with LY83583, an inhibitor of guanylate cyclase to change the production of cGMP. These data indicate that DNP may inhibit spontaneous contraction by increasing cGMP levels. Consistent with this view, KT5823, a PKG inhibitor, markedly diminished the inhibitory effect of DNP on spontaneous contraction. In an attempt to understand how DNP relaxes smooth muscle by affecting the cGMP-dependant pathway, patch clamp experiments were carried out. We observed that KT5823 inhibited DNP-induced increase of $I_{K(ca)}$, and almost completely abolished the DNP-induced increase of STOCs. However, KT-5720 (500 nmol/L), a PKA-specific inhibitor, had no effect on DNP-induced increase in STOCs.

NPs, similar to NO, can increase the generation of cGMP and cAMP, and play important physiological functions in a variety of cell types. In smooth muscle cells, NPs exhibited an inhibitory effect on motility *via* the cGMP pathway. For example, ANP increases intracellular cGMP levels and mediates the role of endothelium- and cardiac-derived NO in regulating sympathetic control functions of the heart and the microvasculature in conscious rats by affecting cGMP-dependent release of catecholamines[16]. Additionally, Kedia *et al*[17] observed that CNP is involved in the cGMP-dependent control of the normal function of human prostatic smooth muscle. Our previous study also found that CNP inhibited spontaneous contraction

by increasing cGMP in gastric antral circular smooth muscle[18]. All these previous reports are consistent with our current findings that DNP-induced relaxation is related to cGMP in gastric circular smooth muscle.

It has been reported that intracellular cGMP may not only result in activation of PKG, but also inhibits activity of PDE3[19]. The latter action would lead to an increase in cAMP, and hence may stimulate another cyclic nucleotide-dependent protein kinase, PKA. NPs exert some physiological functions by affecting PKA. Birukova *et al*[20] have found that Epac/Rap and PKA are novel mechanisms of ANP-induced Rac-mediated pulmonary endothelial barrier protection. However, our present study indicates that DNP-induced increase of $I_{K(ca)}$ and STOCs were significantly blocked by LY83583 and KT-5823. DNP stimulated STOCs even in the presence of a PKA-specific inhibitor (KT-5720), suggesting that the DNP-induced increase of STOCs was due to stimulation of PKG, rather than PKA. The direct effect of cGMP on the activity of ion channels has been reported previously. Yao *et al*[21] showed that a cGMP-gated K^+ channel is expressed in the kidney. Nakamura *et al*[22] revealed that protein kinase G activates inwardly rectifying K^+ channels in cultured human proximal tubule cells. Hirsch *et al*[23] demonstrated the existence of cGMP-regulated K^+ channels that were inhibited by cGMP without PKG-mediated phosphorylation. In our current study, however, DNP-induced relaxation in gastric antral myocytes was inhibited by KT5823. This indicates that PKG-mediated phosphorylation participates in DNP-induced relaxation. Consistent with our data, a previous report has shown that CNP can inhibit L-type Ca^{2+} channel currents, and the inhibitory effect is mediated by pGC-cGMP-PKG-dependent signal pathway in gastric antral myocytes of guinea pigs[24]. However, it should be pointed out that the results of our current study can not determine whether a direct or indirect activation of Ca^{2+}-activated K^+ channels by PKG participates in DNP-induced relaxation of in gastric antral smooth muscle cells. As such, further experiments are necessary to decode this intriguing question.

Taken together, it can be concluded that DNP relaxes gastric circular smooth muscle by activating Ca^{2+}-activated K^+ channels, mediated by a pGC-cGMP-PKG-dependent signal pathway. cAMP did not participate in the process.

COMMENTS

Background

Dendroaspis natriuretic peptide (DNP) is a recently isolated peptide that contains 38 amino residues and shares structural and functional properties with the other members of the natriuretic peptide (NP) family. Studies about its physiologic functions mainly focus on cardiovascular, nervous, and urinary systems. In a previous study, these authors found that DNP inhibited spontaneous contraction in gastric circular smooth muscle. NP plays important physiological functions by affecting the activity of cGMP and cAMP. However, it is unclear whether cGMP or cAMP participates in regulating DNP-induced inhibition of gastric motility.

Research frontiers

Studies about the physiologic functions of DNP mainly focus on cardiovascular, nervous, and urinary systems. There are few reports about the relationship between DNP and gastrointestinal functions. Studies have demonstrated that the DNP system is present in the rat colon and regulates colonic motility as a local regulator. The relationship between DNP and gastrointestinal function has become a focus of study. A previous study has indicated that DNP inhibits gastric motility, which is the first report about DNP regulating gastric motility. However, the mechanism on how DNP regulates gastric motility is still unclear and is the focus of the author's study.

Innovations and breakthroughs

The author's of this paper have shown that, for the fist time, DNP activates $I_{K(Ca)}$ and relaxes guinea-pig gastric antral circular smooth muscle *via* the cGMP/PKG-dependent signaling axis, instead of the cAMP/PKA pathway. The combined use of pharmacologic, radioimmunoassy and patch-clamp techniques can sufficiently demonstrate the mechanism involved in DNP regulation of gastric motility.

Applications

This work enhanced the understanding of the mechanism on how DNP regulates gastric motility.

Peer review

This is a very interesting study. The authors demonstrated that DNP activates $I_{K(Ca)}$ and relaxes guinea-pig gastric antral circular smooth muscle *via* the cGMP/PKG-dependent signaling axis instead of the cAMP/PKA pathway. This study is well designed, and the analysis is reasonable.

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CASE REPORT

Recovery from respiratory failure after decompression laparotomy for severe acute pancreatitis

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Abstract

We present three cases of patients (at the age of 56 years, 49 years and 74 years respectively) with severe acute pancreatitis (SAP), complicated by intra-abdominal compartment syndrome (ACS) and respiratory insufficiency with limitations of mechanical ventilation. The respiratory situation of the patients was significantly improved after decompression laparotomy (DL) and lung protective ventilation was re-achieved. ACS was discussed followed by a short review of the literature. Our cases show that DL may help patients with SAP to recover from severe respiratory failure.

Key words: Severe acute pancreatitis; Intra-abdominal compartment syndrome; Decompression laparotomy; Intensive care Unit; Respiratory failure

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INTRODUCTION

Severe acute pancreatitis (SAP) is associated with organ failure leading to a mortality rate of 10%-20% that is often related to respiratory failure. Approximately one third of SAP patients develop respiratory complications[1,2]. In the majority of cases, this lung injury is characterized by an increased permeability of pulmonary microvasculature and subsequent leakage of protein-rich exudates into the alveolar spaces[1]. Furthermore, concomitant diseases, such as intra-abdominal hypertension (IAH) defined by intra-abdominal pressure (IAP) greater than 12 mmHg[3], may result in restrictive ventilation disorders and deteriorate the pulmonary situation.

Surgical debridement was the preferred treatment to control necrotizing pancreatitis in the past. However, management of necrotizing pancreatitis has changed since the last decade. The first approach now tends to be non-surgical and relies on conservative strategies including early transfer of patients to intensive care units at specialized centres. Indication for necrosectomy is still given in cases of infected necrosis as well as intestinal infarction, perforation or bleeding, but there is a clear trend towards surgical treatment as late and as rare as possible[2]. In contrast, more and more studies are published promoting decompression laparotomy (DL) for SAP patients developing abdominal compartment syndrome (ACS) defined by IAP greater than 20 mmHg associated with new organ failure[3-7]. This procedure can not only prevent critical decrement of intestinal and renal perfusion, but may lead to improvement in the respiratory situation.

We present three patients with SAP and abdominal compartment syndrome, who developed respiratory insufficiency with limitations of mechanical ventilation associated with high peak pressure levels, low tidal volumes and poor Horowitz-indices (pO_2/FiO_2; HI). The three patients showed a benefit from decompression laparotomy so that ventilation with adequate oxygenation could be re-achieved. Surgery was performed at the Intensive Care Unit of our hospital without transportation of the patients to the operating room.

CASE REPORT

Patient 1

A 56-year-old male electrician was admitted to the

A

	After intubation	Before 1 DL	Before 2 DL
Ventilation mode	Pressure controlled ventilation (PCV)		
FiO_2[4]	0.97	1.0	1.0
Minute volume (L)	10.5	9.0	19.7
Tidal volume (mL)	629	410	310
Ventilation frequency (/min)	16	20	60
Peep (mmHg)	12	20	20
Peak ventilator pressure (mmHg)	14	39	44
Mean ventilator pressure (mmHg)	19	27	32
I:E ratio	1:2	1:1	1:1
pO_2 (mmHg)	67	67	61
pCO_2 (mmHg)	35	53	25
pH	7.44	7.35	7.55

B

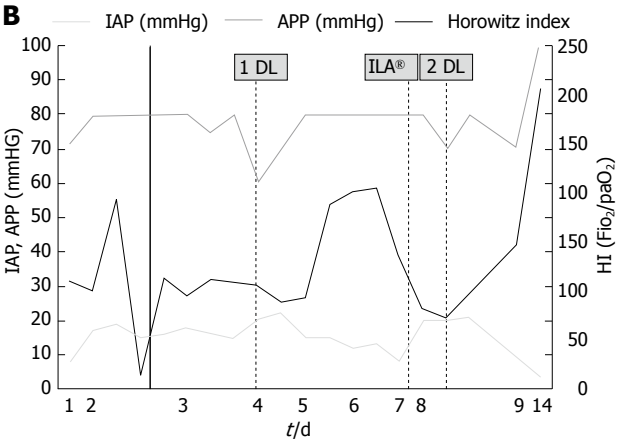

Figure 1 Ventilation adjustment of patient 1 after intubation, before 1 DL and 2 DL (**A**), and development of IAP, APP and HI in this patient over 14 d of ICU hospitalisation (**B**). The spotted lines refer time points of the first and second DL as well as installation of ILA®.

Intensive Care Unit of our hospital because of necrotizing pancreatitis. One week prior to admission, he underwent an ambulant gastroduodenoscopy in the referring hospital and resection of pancreatic papilla minor due to misinterpretation as a polyp. Following endoscopy, progredient upper abdominal pain developed with rising serum lipase and CRP levels. Diagnostic procedures including abdominal sonography, CT-scan and endoscopic-retrograde-cholangio-pancreaticography (ERCP) confirmed pancreatitis. At admission, intubation was performed followed by mechanical ventilation because of respiratory insufficiency (Figure 1A). In the next days, fluid resuscitation was performed, and increasing peak inspiratory and mean airway pressures were needed to achieve sufficient oxygenation. Meanwhile, IAP determined by an indwelling transurethral bladder catheter increased. On day 4, criteria for an ACS were fulfilled (Figure 1B). Simultaneously, Horowitz index decreased (75 mmHg) with 100% oxygen, a positive end expiratory pressure of 20 mmHg and a peak inspiratory airway pressure of 39 mmHg. Sonography revealed an elevation of the diaphragm without any movement. On day five, we decided to perform decompression laparotomy to improve pulmonary gas exchange. A median laparotomy of approximately 30 cm was performed. The abdominal cavity was closed with absorbable Vicryl®-Mesh. Postoperative oxygenation improved with decreasing ventilation pressures and chest wall compliance. Nevertheless the respiratory situation deteriorated again with low oxygenation values and hypercapnia, while IAP equally rose. A pumpless extracorporeal lung assist system (ILA®) was installed on day eight, resulting in a marked decrease in pCO_2, but the Horowitz index did not improve. Since the IAP values increased again (> 20 mmHg), the laparotomy was extended from xiphoid process to symphysis, thereby leading to a significant improvement in the respiratory situation. Again, the abdominal cavity was closed with Vicryl®-Mesh. During the next days, CT scan-guided retroperitoneal drainage was performed for debridement of pancreatic necrosis. As a result, IAP further decreased (Figure 1B). The patient was successfully weaned, therapy with ILA® system could be quickly terminated and the abdomen healed by secondary wound healing.

Patient 2

A 49-year-old man with morbid obesity (172 cm, 150 kg, BMI = 50.7 kg/m^2) presented in the Emergency Department of our hospital for colicky abdominal pain. His past medical history included a limited cardiac function due to ischemic heart disease and recurrent ventricular tachycardia. Laboratory and radiological findings as well as endoscopy revealed biliary pancreatitis. Three days after admission, his respiratory situation worsened and he was transferred to the Intensive Care Unit. Mechanical ventilation had to be initiated. On the second day of intensive care treatment, intra-abdominal inflammation, massive fluid resuscitation (fluid intake about 10 L/d, urine output 1 L/d the first day, up to 7 L/d the next days) and pre-existing obesity led to ACS with a measured IAP above 25 mmHg. Limitations of ventilation therapy with decreased lung and chest compliance prompted us to perform decompression laparotomy. IAP, APP, Horowitz index as well as compliance values significantly improved after surgical therapy and lung protective ventilation could be re-achieved. Secondary wound healing of the open abdomen was improved after vacuum-assisted closure (VAC) therapy (Figure 2B). Unfortunately, the patient developed cardiovascular complications and died of fulminant lung bleeding two weeks later.

Patient 3

A 74-year-old woman was admitted with post-ERCP pancreatitis after an ERCP was performed for symptomatic cholecystolithiasis. She was transferred to our hospital from another hospital due to aggravation of her condition with rising inflammation parameters. On the day of admission, she had to be intubated, mechanical ventilation was started and fluid resuscitation was performed. Within 3 d the HI dramatically dropped with simultaneously increasing peak pressure levels (Figure 3). Under 100% oxygen ventilation, inverse ventilation ratio (2:1), peak pressure of 45 mmHg and positive end-expiratory pressure (PEEP) of 22 mmHg, and PO_2 of 70 mmHg could be achieved. Clinically, she presented with massive abdominal tenderness. IAP-values above 20 mmHg were measured

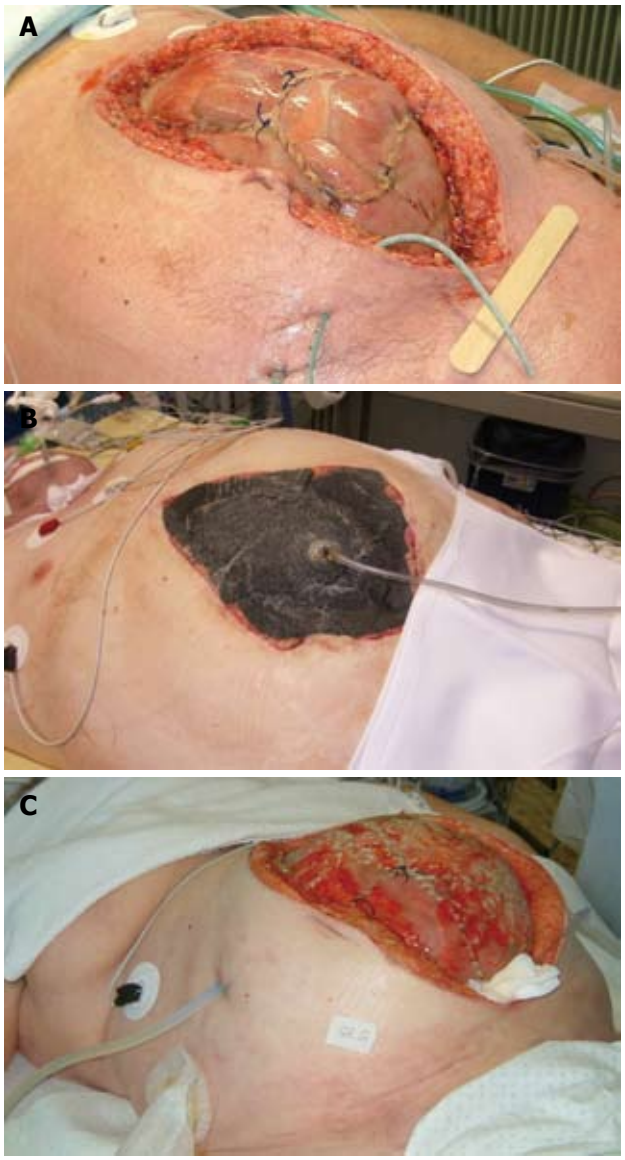

Figure 2 Open abdomen after surgical decompression. **A** and **C**: Intra-abdominal drains further reduce intra-abdominal tension; **B**: Vacuum bondage improves wound healing. **A**: Patient No. 1; **B**: Patient No. 2; **C**: Patient No. 3.

and decompression laparotomy was performed due to respiratory failure. Immediately after the intervention, her respiratory situation improved significantly. During the next days, interventional CT-guided drainage therapy was performed and led to a further decrease in intra-abdominal pressure. Weaning was successful and spontaneouse breathing was re-achieved after dilatation tracheotomy and prolonged respiratory therapy due to septic complications. Three months after recovery, the abdominal laparotomy wound was closed along with elective cholecystectomy.

DISCUSSION

We present three cases of patients with SAP developing ACS leading to severe limitations of respiratory therapy. In each of these patients, decompression laparotomy caused immediate improvement in pulmonary function and led to definite survival in two cases.

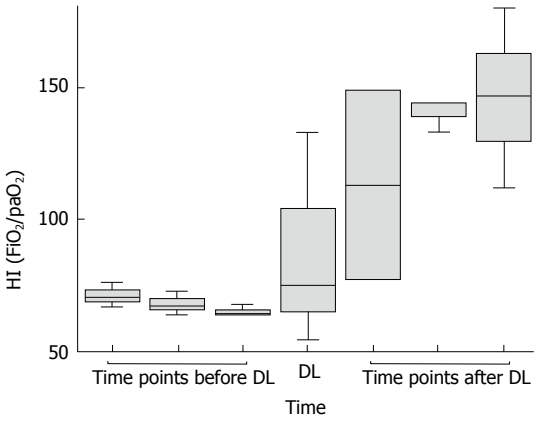

Figure 3 Development of Horowitz-indices (HI). In the Box blots, the HI 3, 2 and 1 d before, at and 1, 2 and 3 d after DL of all 3 patients is shown. The line refers to the median, whereas the boxes refer to the interquartile ranges. The whiskers represent the 5th and 95th percentiles, respectively.

Approximately 30%-40% of SAP patients develop ACS because of pancreatic-retroperitoneal inflammation, edema of peripancreatic tissue, fluid formation or abdominal distension, subsequently leading to intestinal ischemia with ileus and renal failure[5]. Besides, additional fluid resuscitation is known to further increase IAP. It was reported that ACS generally affects cardiac, pulmonary and renal function, and contributes to multi-organ dysfunction with a mortality rate ranging 10%-50% within two weeks[6,8-11]. The degree of IAP in patients with SAP seems to correlate with the degree of organ dysfunction, the severity of disease, the length of intensive care unit stay and mortality[6,8-11].

Pulmonary side effects mediated by IAH, such as atelectasis, edema, decreased oxygen transport and increased intrapulmonary shunt fraction, are caused by compression of pulmonary parenchyma. IAP is transmitted to the thorax through the elevated diaphragm and causes pulmonary parenchyma compression. The abdominal pressure on lung parenchyma is aggravated in mechanically ventilated patients due to high positive airway pressure, thereby leading to an elevated risk of alveolar barotraumas[12]. Furthermore, a study in ACS trauma patients demonstrated an increased rate of pulmonary infections[13].

Animal and human studies showed that abdominal decompression laparotomy can reverse the cardiopulmonary and abdominal effects of ACS[9,10]. In our patients, decompression laparotomy led to decreasd IAP levels, improved Horowitz indices and increased lung compliance (Figure 3). Ventilation pressures could be reduced in order to re-achieve a lung protective ventilation regime. In addition, all three patients benefited from improving renal perfusion (rising urine volume and decreasing creatinine and urea levels), mesenterial perfusion (declining lactate levels) and cardiac output (less need for catecholamines). Moreover, patients with SAP may specifically profit from decompression laparotomy since elevated IAP influences pancreatic and intestinal perfusion and might therefore contribute to pancreatic necrosis[14].

Although decompression laparotomy can lead to

recovery of patients with SAP from respiratory failure, its complications, such as massive intra-abdominal bleeding, need to be taken into account. Moreover, not only surgical complications but also persisting open abdomen is associated with risks and subsequent extensive abdominal wall reconstruction. New surgical concepts like subcutaneous anterior abdominal fasciotomy[15] may minimize complications and reach comparable clinical improvement in patients with SAP and ACS. Randomized studies are few so far, and the use of decompression laparotomy for ACS has been criticized by several authors since the mortality remains high in these patients[5,16].

Abdominal decompression laparotomy may help to overcome respiratory failure in patients with abdominal compartment syndrome and severe acute pancreatitis. Therefore, serial indirect measurement of IAP through an indwelling transurethral bladder catheter should be used routinely in critically ill patients with SAP in order to detect ACS. We hold that decompression laparotomy should be performed in patients with SAP and IAH/ACS with severe limitation of mechanical ventilation. However, prospective randomized studies are needed to further define the role of surgical decompression in SAP.

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CASE REPORT

Atypical presentation of pioderma gangrenosum complicating ulcerative colitis: Rapid disappearance with methylprednisolone

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Abstract

Piodermal gangrenosum (PG) is an uncommon ulcerative cutaneous dermatosis associated with a variety of systemic diseases, including inflammatory bowel disease (IBD), arthritis, leukaemia, hepatitis, and primary billiary cirrhosis. Other cutaneous ulceration resembling PG had been described in literature. There has been neither laboratory finding nor histological feature diagnostic of PG, and diagnosis of PG is mainly made based on the exclusion criteria. We present here a patient, with ulcerative colitis (UC) who was referred to the emergency section with a large and rapidly evolving cutaneous ulceration. Laboratory and microbiological investigation associated with histological findings of the ulcer specimen allowed us to exclude autoimmune and systemic diseases as well as immuno-proliferative disorders. An atypical presentation of PG with UC was diagnosed. Pulse boluses of i.v. methyl-prednisolone were started, and after tapering steroids, complete resolution of the skin lesion was achieved in 3 wk. The unusual rapid healing of the skin ulceration with steroid mono-therapy and the atypical cutaneous presentation in this patient as well as the risk of misdiagnosis of PG in the clinical practice were discussed.

Key words: Ulcerative colitis; Pioderma gangrenosum; Steroids; Cutaneous lesion; Immunosuppression

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INTRODUCTION

Piodermal gangrenosum (PG) is an uncommon ulcerative cutaneous dermatosis associated with a variety of systemic conditions including inflammatory bowel disease (IBD), arthritis, haematological malignancies, paraproteinemia and hepatitis[1-5]. Many other cutaneous ulcerations resembling PG have been described in literature[6-10]. There has been neither laboratory finding nor histological feature diagnostic of PG, and diagnosis of PG is mainly established by exclusion criteria. We described here a patient with ulcerative colitis (UC) who manifested atypical presentation of PG. After diagnosis, a rapid healing of the large and painful skin ulceration was obtained by high doses of i.v. steroid therapy.

CASE REPORT

An 82-year-old man was referred to the emergency section with a round painful cutaneous ulcer of 15 cm in diameter in the left mammary region. The edges were undermined and presented with granulated tissues, crusts, and purulent exudates (Figure 1A). One month before a lesion appeared in the same skin area presenting as a small red plaque with surrounding erythema. This was supposed to be a consequence of a mosquito bite according to his family doctor. The lesion rapidly progressed to a wider and painful cutaneous ulceration over the past month. Antimicrobial treatment with amoxicillin and ciprofloxacin was totally ineffective and the patient required paracetamol and codeine every 6 h for pain relief.

The patient was admitted to our hospital 3 years before due to rectal bleeding and anaemia. UC was

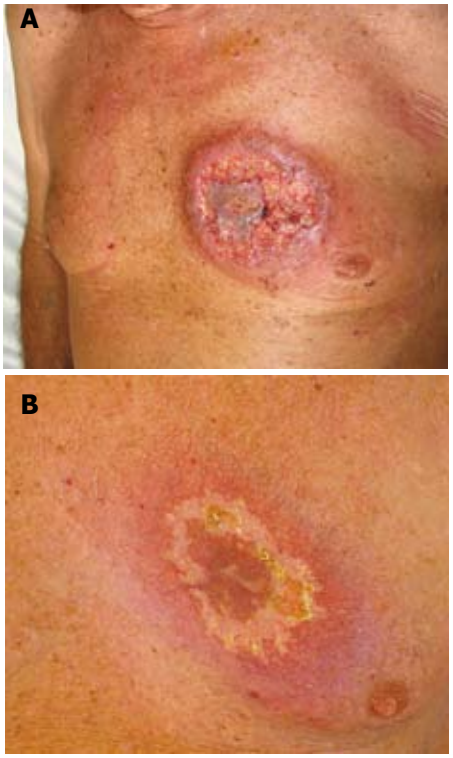

Figure 1 **A**: Patient with chronic ulcerative colitis presenting a round and painful cutaneous ulcer of 15 cm in diameter in the left mammary region. The edges were undermined. Granulated tissue, crusts, and purulent exudates are evident; **B**: Resolution of the skin lesion after 20 d of metilprednisolone therapy.

diagnosed. Therefore, the patient received prednisone and mesalazine therapy (10 mg/d and 800 mg thrice/d, respectively). Prednisone was tapered and stopped after 3 mo, whereas mesalazine administration was continued.

On examination, the patient presented with mild hyperthermia (37.5-38℃). He complained of 3-5 daily episodes of diarrhoea but without rectal bleeding. No lymphoadenopathy was observed. The lesion was very painful. A swab and microbiological examination of the specimen from the ulcer was negative for bacteria and fungi. Routine laboratory investigations revealed white cell count of 13.8×10^9/L with neutrophilia. The erythrocyte sedimentation rate was 32 mm/h. Liver and kidney function tests, immunoglobulin, protein electrophoresis, anticoagulation panel were normal. Venereal Disease Research Laboratory (VDRL) test, HIV test, anti-neutrophilic cytoplasmic, antinuclear and anti-DNA antibodies, rheumatoid factor, LE test, were all negative, and cryoglobulins were absent.

Chest X-ray, venous and arterial functional studies were normal. A skin biopsy of the lesion was performed under local anaesthesia. Histological analysis showed focal necrotizing flogosis associated with ulceration and peripheral lymphocytic and neutrophilic infiltration extending through the dermis and subcutaneous tissue; extravascular red blood cell infiltration was also present.

Necrotizing vasculitis was not observed and the histological changes were consistent with pioderma gangrenosum. Methylprednisolone pulse boluses (500 mg/d for 3 d) were given i.v. Steroid was reduced to 80 mg/d and then tapered to 20 mg/d for 3 wk. The patient healed from skin lesion 20 d after beginning of steroid therapy (Figure 1B).

DISCUSSION

Brunsting *et al*[11] in 1930 first described five patients with rapidly progressive and painful suppurative skin ulceration with necrotic and undermined borders that were called PG. This lesion is a neutrophilic dermatosis associated with a variety of systemic diseases, such as paraproteinemia, arthritis, and myeloproliferative diseases, and IBD. In about 50% of the cases, UC is the underlying condition and PG may parallel the severity of the disease[1,9,12]. The pathogenesis of PG is poorly understood and over-expression of interleukin (IL)-8 and IL-16 has been reported, suggesting an over-reactive inflammatory response to a traumatic process. Although the lesion can occur in any surface it is more common on the legs in perineal, vulvar, penile and neck region. Atypical presentations are considered on the arms or in the chest. Weenig *et al*[6] reported two cases of livedoid vasculopathy, a rare thrombo-occlusive disease of post-capillary venules, which may occur with cutaneous ulcers of the legs characterized by a very similar macroscopic and histological pattern. These lesions may be confused with PG. However, livedoid vasculopathy is not responsive to steroid therapy. Therefore, PG is an excluded diagnosis on the basis of laboratory findings and histology, associated with a high rate of clinical suspicion. The good and rapid clinical responses to steroids associated with other immunosuppressive therapy such as cyclosporine, azathioprine and ciclophosphamide are also important "*ex-adiuvantibus*" criteria.

Patients with vasculitis associated with or not associated with cryoglobulinemia or those with antiphospholipid-antibody syndrome, and those with Wegener granulomatosis and poliartrite nodosa, may present lesions resembling PG[5,6,8,9,13]. These lesions may be misdiagnosed with PG due to initial response to steroid therapy, but without evidence of complete healing. The clinical pattern of a patient with very painful skin lesion, suffering from IBD should raise the suspicion of PG; however laboratory findings and functional and radiologic analysis to rule out other systemic disease are mandatory for a correct diagnosis.

Other rare malignant lesions, such as lymphoma, leukaemia cutis and Langherans cell hystiocytosis can be ruled out according to the histological studies of the specimen.

PG is a diagnosis of exclusion and its misdiagnosis can result in serious clinical consequences.

The chronic UC in our patient based on the exclusion criteria, convinced us to start therapy with a high dose of corticosteroid. The rapid healing of such a large skin lesion is unusual. Some patients refractory to steroid treatment can benefit from the combination of steroid with cyclosporine[14,15]. At the moment, our patient is disease free at 12 mo after diagnosis without

clinical symptoms related to UC under a maintenance therapy of 7.5 mg/d prednisone.

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CASE REPORT

Development of autoimmune hepatitis type 1 after pulsed methylprednisolone therapy for multiple sclerosis: A case report

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Abstract

A 43-year-old woman with multiple sclerosis (MS) was treated with pulsed methylprednisolone and interferon β at a hospital. Four weeks after initiating treatment, liver dysfunction occurred and she was referred and admitted to our hospital. Clinical and laboratory findings were consistent with and fulfilled the criteria for drug-induced hepatitis, but not for autoimmune hepatitis (AIH). She was successfully treated with corticosteroids. As ataxia developed after 1 year, she was treated with pulsed methylprednisolone for 3 d, then readmitted to our hospital when liver dysfunction occurred. Clinical and laboratory findings led to the diagnosis of AIH. To the best of our knowledge, this is the second case of AIH developed after pulsed methylprednisolone for MS.

Key words: Multiple sclerosis; Autoimmune hepatitis; Pulsed methylprednisolone

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Takahashi A, Kanno Y, Takahashi Y, Sakamoto N, Monoe K, Saito H, Abe K, Yokokawa J, Irisawa A, Ohira H. Development of autoimmune hepatitis type 1 after pulsed methylprednisolone therapy for multiple sclerosis: A case report. *World J Gastroenterol* 2008; 14(35): 5474-5477 Available from: URL: http://www.wjgnet.com/1007-9327/14/5474.asp DOI: http://dx.doi.org/10.3748/wjg.14.5474

INTRODUCTION

Intravenous methylprednisolone pulse therapy is the standard treatment for relapsing multiple sclerosis (MS). Interferon (IFN)-β is the most commonly used drug in the treatment of MS, and has been proven to reduce the disease activity, progression and relapse rate[1,2]. IFN-β is associated with hepatotoxicity, although it rarely induces severe liver injury. It was reported that autoimmune hepatitis (AIH) occurs during IFN-β therapy for MS[3,4], but only one report has described the occurrence of AIH after intravenous methylprednisolone pulse therapy for MS[5]. We describe herein a case of a MS patient who developed AIH after treatment with IFN-β and pulsed methylprednisolone.

CASE REPORT

A 43-year-old woman with abdominal discomfort and nausea was referred to our hospital on August 7, 2006. She was diagnosed with MS on the basis of clinical and laboratory findings 7 years ago. Three years ago, she was treated with pulsed methylprednisolone (1000 mg/day for 3 d) followed by 50 mg/day of oral prednisolone because of ataxia. Although oral prednisolone was tapered and stopped for 1 month, she remained healthy until June 2006, when ataxia developed again. On June 28, 2006, she was treated with pulsed methylprednisolone (1000 mg/day for 3 d) followed by 50 mg/day of oral prednisolone. Despite pulsed methylprednisolone therapy, symptoms did not improve. She was therefore retreated with pulsed methylprednisolone (1000 mg/day) for 3 d from July 5, 2006. Moreover, she was treated with IFN-β 8 at MU every other day from July 11 to 26, 2006. After pulsed methylprednisolone, oral prednisolone was not administered. On August 3, 2006, the patient

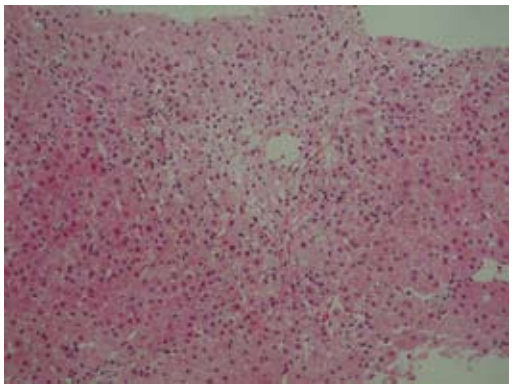

Figure 1 Histological examination of a liver biopsy specimen showing bridging perivenular necrosis and infiltration of inflammatory cells including eosinophils (hematoxylin-eosin staining × 100).

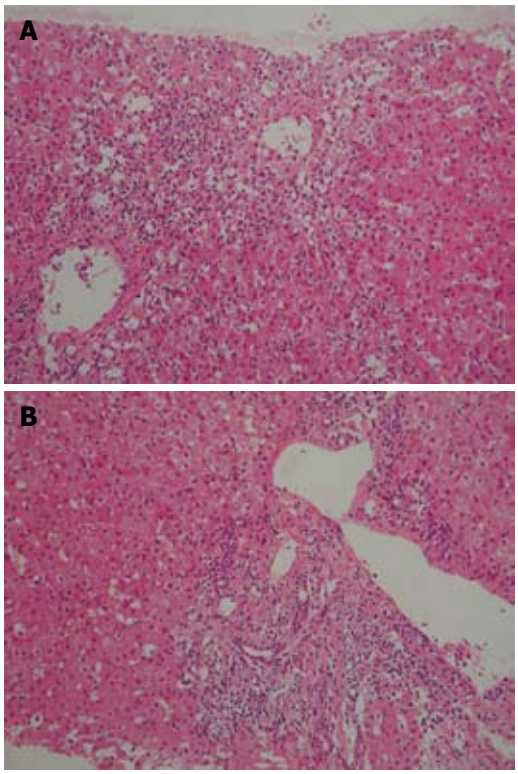

Figure 2 Histological examination of a liver biopsy specimen showing bridging perivenular necrosis (**A**) and interface hepatitis (**B**) (HE staining × 100).

became nauseous and vomited, and these symptoms did not improve. On August 7, 2006, she was referred to our hospital and admitted after blood testing revealed severe liver dysfunction. Three years ago, she developed acute hepatitis due to Epstein-Barr (EB) virus after treatment with pulsed methylprednisolone. Since then, she had been free of liver dysfunction.

On admission, her blood pressure was 156/89 mmHg and heart rate was 102 beats/min, body temperature was 37.3℃, and the areas of skin at sites of IFN-β injection became welts. Her conjunctivae were not jaundiced, heart and respiratory sounds were normal. No abnormalities were noted in the chest or abdomen. The liver and spleen were not palpable. Neurological examination showed no abnormalities suggestive of MS. Laboratory findings were as follows: 1102 IU/L aspartate aminotransferase (AST) (normal, 10-35 IU/L), 1067 IU/L alanine aminotransferase (ALT) (normal, 12-33 IU/L), 377 IU/L alkaline phosphatase (ALP) (normal, 300-500 IU/L), 3.4 mg/dL total bilirubin (TB) (normal, < 1.1 mg/dL), 2.2 mg/dL direct bilirubin (DB) (normal, 0.2-0.4 mg/dL), 26 IU/L γ-glutamyl transpeptidase (γGTP) (normal, 10-47 IU/L), 6.4 g/dL total protein (TP) (normal, 6.0-8.5 g/dL), 3.7 g/dL albumin (normal, 4.0-5.3 g/dL), 1370 mg/dL serum immunoglobulin (Ig)G, 147 mg/dL IgA, 272 mg/dL IgM, and 71.4% prothrombin time (PT). Anti-nuclear antibody (ANA), anti-smooth muscle antibody and anti-LKM-1 antibody were all negative. HBs antigens, IgM-HA and HCV antibodies were negative. Other viral infections including EB virus and cytomegalovirus infection were excluded by serological testing. Abdominal computed tomography showed no abnormalities. Biopsy specimen of the liver showed bridging perivenular necrosis with infiltration of inflammatory cells including eosinophils (Figure 1). A lymphocyte-stimulation test for IFN-β yielded negative results, but the patient displayed a score of 9 according to the criteria for drug-induced liver injuries[5], indicating a high probability of drug-induced liver injury. All these findings led to the diagnosis of drug-induced liver injury caused by IFN-β. Despite intravenous administration of stronger neo-minophagen C (60 mL/day) and prostaglandin, jaundice developed with a serum TB level of 19.1 mg/dL. Methylprednisolone (125 mg/day for 3 d) and ursodeoxycholic acid (UDCA, 600 mg/day) were therefore administered. Symptoms subsequently improved and serum TB level normalized. Prednisolone was decreased gradually and stopped on April 10, 2007. UDCA was stopped on May 10, 2007. Liver function remained normal even after withdrawal of prednisolone and UDCA.

However, ataxia developed and the patient was again treated with pulsed methylprednisolone (1000 mg/day) for 3 d from October 1, 2007. After pulsed methylprednisolone, oral prednisolone was not administered. Two weeks later, she was readmitted to our hospital due to fatigue and liver dysfunction. Laboratory findings on admission were as follows: 566 IU/L AST, 875 IU/L ALT, 214 IU/L ALP, 1.7 mg/dL TB, 12 IU/L γGTP, 1785 mg/dL IgG, and 71.4% PT. Anti-nuclear antibody (ANA) titer was × 80 with a homogeneous pattern, positive results were obtained for anti-smooth muscle antibody, and HLA DR was 4. Viral infections were excluded by serological testing. Biopsy specimen from the liver revealed bridging perivenular necrosis and interface hepatitis (Figure 2A and B). In this case, IgG was not elevated, which is atypical for AIH. However, according to the criteria for AIH[6], the patient had a score of 16 on the second admission, indicating definite AIH, compared to a score of 9 on the first admission. Conversely, according to the criteria for drug-induced liver injury[7], our patient displayed a score of 2, indicating a low possibility that this case represented drug-induced liver injury. Moreover,

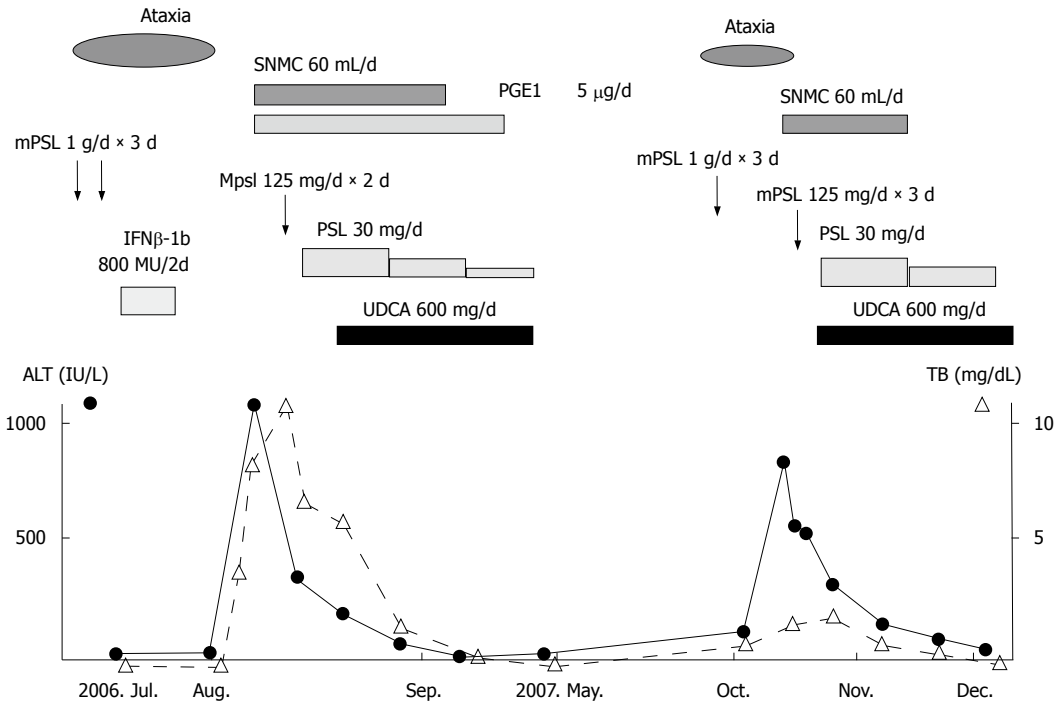

Figure 3 Clinical course of the disease. SNMC: Stronger neo-minophagen C, PGE1: Prostaglandin, mPSL: Methylprednisolone, PSL: Prednisolone, IFN-β: Interferon β.

lymphocyte-stimulation testing for methylprednisolone yielded negative results.

These clinical and laboratory findings supported the diagnosis of AIH. After administration of prednisolone and UDCA, symptoms and liver function improved. The charts for the overall clinical course are shown in Figure 3. Her condition is now under control with prednisolone, 10 mg/day.

DISCUSSION

MS is an inflammatory demyelinating disease of the central nervous system. Liver dysfunction is not always caused by MS itself, but can result from many factors, such as drug toxicity, fatty infiltration and viral infection. Liver dysfunction in patients with MS is most commonly caused by drugs. IFN-β, which raises serum ALT level as a side effect, is one of the drugs well known to cause liver injury in patients with MS.

Tremlett *et al*[8] reported that 36.9% of patients with MS develop new elevations of ALT, although only 1.4% reach grade 3 hepatotoxicity (> 5-20 upper limit of normal). In patients with MS receiving IFN-β, if *de novo* elevation of aminotransferases is mild, IFN-β treatment is often continued, and elevated aminotransferases return to almost normal[4]. However, severe liver dysfunction does not resolve simply after stopping IFN-β, and prompt treatment is needed. A case of fulminant liver failure occurring during IFN-β treatment has been reported[9]. Our patient satisfied the criteria for drug-induced hepatitis, but not for AIH on the first admission. Byrnes *et al*[10] have also reported drug-induced liver injury secondary to IFN-β in patients with MS. However, the precise mechanisms underlying IFN-β-induced hepatotoxicity remain unclear.

IFN-β may cause autoimmune complications including thyroiditis, lupus erythematosus and rheumatoid arthritis[11]. Duchini *et al*[3] have reported a case of AIH occurring during treatment with IFN-β. Conversely, Reuβ *et al*[5] have reported a case of AIH that developed after high-dose intravenous methylprednisolone pulse in MS and speculated that AIH may occur in patients with multiple autoimmunity as an immune rebound phenomenon after immunosuppressive regimens.

The typical histological pattern of AIH is chronic active hepatitis that shows portal inflammation with fibrosis, interface hepatitis and rosette formation of hepatocytes. However, few cases of AIH with centrilobular necrosis (CN) as the dominant finding have been reported[12]. Recently, some cases of CN with autoimmune features have been confirmed as early-stage AIH[13,14]. Acute-onset AIH sometimes does not satisfy AIH criteria serologically and shows CN histologically[14-16]. Although our patient showed a typical pattern of AIH at the second admission, liver dysfunction at the first admission may have been due to early-stage AIH.

The cause of AIH in this patient was an immune rebound phenomenon after pulsed methylprednisolone, because the second episode of liver dysfunction occurred after pulsed methylprednisolone therapy rather than after IFN-β therapy. In fact, some reports have described AIH occurring in patients with multiple autoimmunity after pulsed methylprednisolone therapy[5,17-18]. In particular, withdrawal of glucocorticoids after pulsed methylprednisolone therapy might have induced immune rebound phenomenon in the present

case. However, we cannot deny the possibility that AIH was induced by IFN-β in this patient. She received IFN-β treatment before the first admission. Moreover, Misdraji *et al*[12] reported that AIH with CN occurs after IFN-β therapy in patients with MS.

In conclusion, the prevalence of AIH seems to be about 10-fold higher in patients with MS than in the general population[19]. Attention should be paid to the development of AIH after pulsed methylprednisolone or IFN-β treatment in patients with MS, and if AIH develops, immediate treatment with corticosteroids or azathioprine should be initiated. Moreover, administration of corticosteroids or azathioprine after pulsed methylprednisolone might be effective for preventing the development of AIH.

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CASE REPORT

Acute pancreatitis successfully diagnosed by diffusion-weighted imaging: A case report

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Abstract

Diffusion-weighted imaging (DWI) is an established diagnostic method of acute stroke. The latest advances in magnetic resonance imaging (MRI) technology have greatly expanded the utility of DWI in the examination of various organs. Recent studies have revealed the usefulness of DWI for imaging of the liver, kidney, ovary, and breast. We report a patient with acute pancreatitis detected by DWI and discussed the efficacy of DWI in diagnosing acute pancreatitis. A 50-year old man presented with a primary complaint of abdominal pain. We performed both DWI and computed tomography (CT) for this patient. The signal intensity in a series of DWI was measured and the apparent diffusion coefficient (ADC) values were calculated to differentiate inflammation from normal tissue. Two experienced radiologists evaluated the grade of acute pancreatitis by comparing the CT findings. Initially, the pancreas and multiple ascites around the pancreas produced a bright signal and ADC values were reduced on DWI. As the inflammation decreased, the bright signal faded to an iso-signal and the ADC values returned to their normal level. There was no difference in the abilities of DWI and CT images to detect acute pancreatitis. However, our case indicates that DWI can evaluate the manifestations of acute pancreatitis using no enhancement material and has the potential to replace CT as a primary diagnostic strategy for acute pancreatitis.

Key words: Diffusion-weighted imaging; Apparent diffusion coefficients; Magnetic resonance imaging; Acute pancreatitis

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INTRODUCTION

Acute pancreatitis is a potentially fatal disease with an overall mortality rate of 7%-11%[1-3]. Patients suffering from acute pancreatitis (AP) often have additional complications such as sepsis, systemic inflammatory syndrome (SIRS) and multiple organ failure (MOF), resulting in a life-threatening condition[4-6]. Therefore, it is important to accurately evaluate the grade of inflammation and absence of necrotizing pancreatitis to improve its prognosis. Diffusion-weighted imaging (DWI) is an established diagnostic tool of acute stroke and brain tumors[7-11]. Due to the latest technical advances in magnetic resonance imaging (MRI), DWI has also been applied in detecting various disorders of abdominal organs[12-16]. This report describes the efficacy of DWI in evaluation of acute pancreatitis.

CASE REPORT

A 50-year-old man presented with a primary complaint of epigastric pain after drinking alcohol. Laboratory tests upon admission revealed slightly higher levels of white blood cells (108 × 10^2/mL), C-reactive protein (53.3 mg/L),

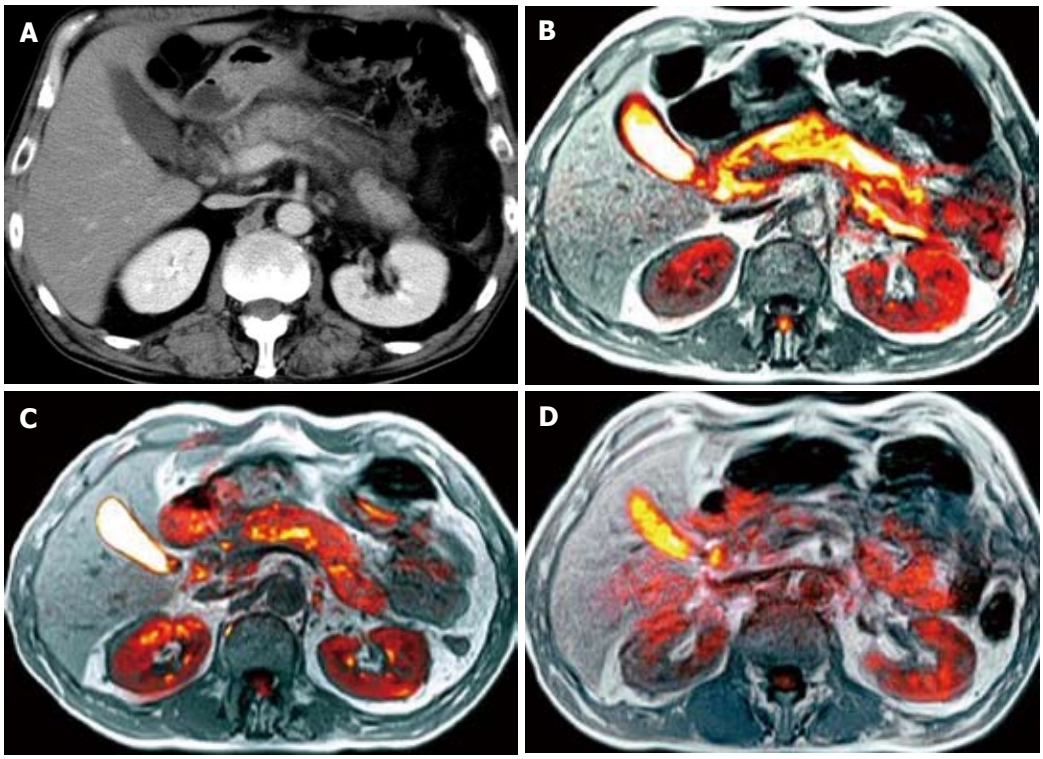

Figure 1 A CT scan at admission revealing enlarged pancreas complicated by acute multiple ascites (**A**), a fusion image at admission (**B**), on days 10 (**C**) and 50 (**D**) showing bright signals in the whole pancreas and ascites around it, diminished pancreatic enlargement and slightly decreased signal-intensity, as well as disappearance of all signs of acute pancreatitis, respectively.

serum amylase (276 IU/L). His APACHI Ⅱ score and Ranson score were 2 and 0, respectively. An enhanced abdominal computed tomography (CT) scan revealed an enlarged pancreas complicated by multiple acute ascites (Figure 1A). An abdominal DWI at 1.5 T (Toshiba; Excelart vantage AGV, screw ratio 130 mT/m per ms) showed bright signals in the whole pancreas and multiple ascites around it (Figure 1B). Furthermore, the apparent diffusion coefficient (ADC) map in that area revealed a reduced ADC value. Following admission, the patient received drip infusion of 300 mg gabexate per day. Seven days after admission, laboratory tests revealed almost normal levels of WBC (54×10^2/mL), CRP (4.1 mg/L) and serum amylase (93 IU/L). Ten days after admission, DWI revealed diminished pancreatic enlargement, slightly decreased signal-intensity (Figure 1C), slightly increased ADC values and disappearance of ascites. His symptoms improved significantly at that time. After 50 d, DWI showed complete disappearance of the manifestations of acute pancreatitis (Figure 1D).

DISCUSSION

Severe acute pancreatitis is often associated with pancreatic necrosis and has a rather high mortality rate. It was reported that necrotizing pancreatitis and inflammatory changes are related to its various complications and prognosis[4-6]. To improve its mortality, it is essential to accurately evaluate the grade of inflammation and the absence of necrotizing pancreatitis. Plane CT can show the changes in inflammation around the pancreas, but cannot detect necrotizing pancreatitis without the use of enhancement material[17]. However, enhancement material has been reported to aggravate acute pancreatitis[18] and it is hard to employ enhanced CT in patients with renal failure due to severe acute pancreatitis. At present, CT is the only available diagnostic imaging method of acute pancreatitis. Clearly, it is urgent to develop new diagnostic strategies for this condition. DWI is a MR imaging technique that provides information about the diffusion of water protons, such as brownian motion in living tissues. DWI has been applied in the diagnosis of brain ischemia and brain tumors[7-11]. Recent technical development in MRI has expanded the utility of DWI in examinations of the liver, kidney, breast, *etc*[12-16]. The apparent diffusion coefficient (ADC) is a quantitative parameter, which reflects the microenvironment of diffusing water molecules. It was reported that reduced ADC is observed in most malignant tumors[8,12,13,15,16]. This present study demonstrated that DWI could detect acute pancreatitis with reduced ADC values at the time of diagnosis. As serum WBC, CRP, and amylase became normal, the signal-intensity and ADC values returned to their normal levels. The decreased ADC value is thought to result from the increased number and size of cells. Therefore, intercellular spaces become smaller, restricting the movement of water molecules[15,16]. If a malignancy is found in abnorm, the ADC value would remain low. These results suggest that inflammation may be closely related to the bright signal. In addition, changed ADC values are useful in differentiating malignant from benign tumors. DWI has a potential to evaluate the manifestations

of acute pancreatitis. Furthermore, the greatest advantage of DWI in diagnosing this condition is that no enhancing material is needed.

In conclusion, DWI is a powerful tool for evaluating acute pancreatitis and has a potential to replace CT as a primary diagnostic strategy for acute pancreatitis.

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CASE REPORT

Asymptomatic colonic metastases from primary squamous cell carcinoma of the lung with a positive fecal occult blood test

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Abstract

We describe a 74-year-old man with a colonic metastatic squamous cell carcinoma (SCC) from the lung. His chest X-ray revealed an abnormal shadow in the right upper lobe. Computed tomography (CT) of the chest demonstrated a large lung tumor in the right upper lobe obstructing the right upper bronchus. Bronchoscopy revealed an easy-bleeding tumor in the right upper bronchus that was diagnosed as poorly differentiated squamous cell lung carcinoma. He underwent colonoscopy because he had a positive fecal occult blood test. Colonoscopy revealed a large protruding lesion with central ulceration in the descending colon. Histological examination of the biopsy specimen obtained from the colonic lesion revealed SCC. The lesion was diagnosed as metastatic colonic SCC. He had no abdominal symptoms. He underwent chemotherapy with an infusion of cisplatin 130 mg i.v. day 1, and docetaxel hydrate 100 mg i.v. day 1, repeated every 4 wk, followed by 4 courses of chemotherapy. The primary lesion shrank by less than 10% and was judged to be "Partial Response" (PR) after 3 courses of treatment. The patient still lived 23 wk after the diagnosis of metastatic colonic SCC. Colonic metastasis of primary SCC of the lung is rare.

Key words: Gastrointestinal metastatic tumor; Colonic metastasis; Large intestine; Colonoscopy; Chemotherapy

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INTRODUCTION

Primary lung cancer is a common neoplasm, and frequently metastasizes to internal organs such as the lung, liver and adrenal gland; however, it is relatively rare for lung cancer to metastasize to the gastrointestinal (GI) tract. Colonic metastasis from primary carcinoma of the lung has rarely been described. Clinically, patients may present with symptoms of colonic obstruction, lower GI bleeding, bowel perforation, or GI fistula[1-4]. Herein, we describe a rare case of metastatic colonic squamous cell carcinoma (SCC) from the lung.

CASE REPORT

A 74-year-old man presented with the symptom of shoulder pain. His chest X-ray revealed an abnormal shadow in the right upper lobe (Figure 1A). He was in good health with no specific family or past medical history. His body temperature was 36.7℃, blood pressure was 148/82 mmHg, radial pulse rate was 72 beats/min and regular. He had anemia, but no jaundice. Neurological examination revealed no abnormal findings or lymphadenopathy. Abdominal palpation revealed tenderness in the left lower quadrant. Laboratory tests showed a red blood cell count of $318 \times 10^4/\mu L$ [normal range (NR), $430\text{-}570 \times 10^4/\mu L$], a white blood cell count of 7600 /μL, and a platelet count of $30.8 \times 10^4/\mu L$, and a hemoglobin concentration of 8.9 g/dL (NR, 14-18 g/dL). Liver function tests were normal except for lactate dehydrogenase (LDH) of 340 IU/L (NR, 106-211 IU/L). A test for C reactive protein revealed a level of 0.8 mg/dL (NR, < 0.5 mg/dL). Renal function tests showed that blood urea nitrogen

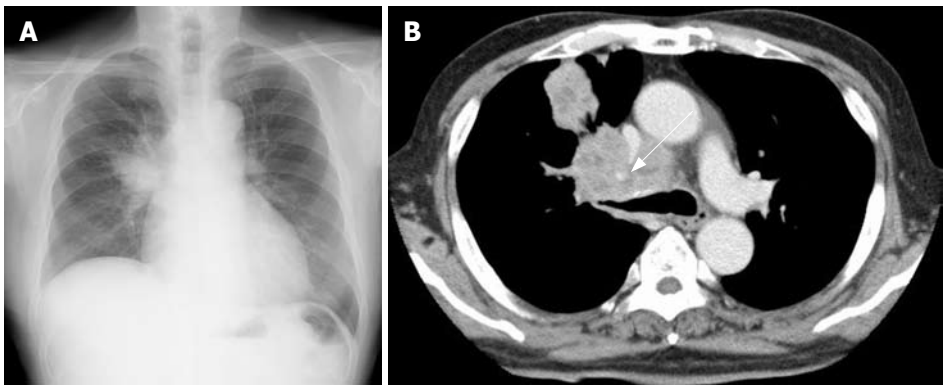

Figure 1 **A**: Chest X-ray revealed a large abnormal shadow in the right upper lobe; **B**: A computed tomography (CT) of the chest demonstrated a large lung tumor in the right upper lobe obstructing the right upper bronchus.

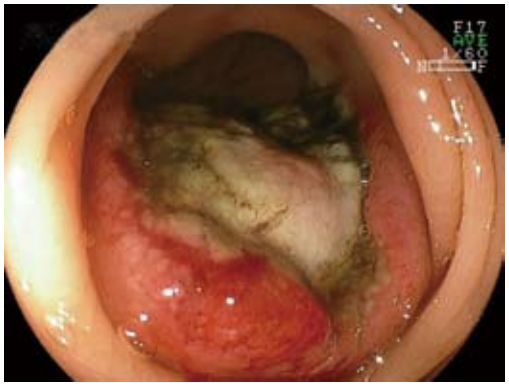

Figure 2 Endoscopic appearance of the descending colon. A large protruding lesion with central ulceration, about 40 mm in diameter, was seen.

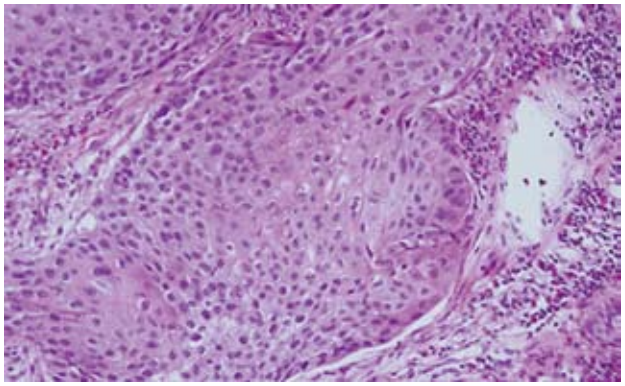

Figure 3 Histological examination of the biopsy specimen obtained from the colonic protruding lesion revealed tumor cells diagnosed as SCC (× 50).

and creatinine were normal. Computed tomography (CT) of the chest demonstrated a large lung tumor in the right upper lobe obstructing the right upper bronchus (Figure 1B). Bronchoscopy revealed an easy-bleeding tumor in the right upper bronchus. Poorly differentiated SCC was diagnosed by punch biopsy.

Colonoscopy revealed a large protruding lesion with central ulceration in the descending colon (Figure 2). This finding suggested a clinical diagnosis of metastatic colonic tumor. Biopsy specimens obtained from the lesion showed SCC (Figure 3). Based on these findings, the patient was diagnosed with metastatic colonic SCC from primary lung cancer. Subsequent positron emission tomography (PET) with radiolabeled-18 fluorine fluorodeoxyglucose (FDG) imaging revealed an abnormal lesion in the descending colon. The patient underwent chemotherapy with an infusion of cisplatin 130 mg i.v. day 1, and docetaxel hydrate 100 mg i.v. day 1, repeated every 4 wk, followed by 4 courses of chemotherapy. The primary lesion shrank by less than 10% and was judged to be "Partial Response" (PR) after 3 courses of treatment. The patient still lived 23 wk after the diagnosis of metastatic colonic SCC.

DISCUSSION

GI metastasis from lung cancer is considered to be rare, although there is about 4.7%-14% prevalence at autopsy[5-7]. A recent report described by Kim *et al*[8] revealed that GI metastases were detected in 10 (0.19%) of 5239 lung cancer patients. In an autopsy study from Japan, the rate of GI tract metastasis, excluding the esophagus, was 1.8% and the colonic metastasis rate was only 0.5%[6]. The histological type of lung cancer that causes colonic metastasis varies according to different studies. The most common types were large cell or SCC[5,9,10]. We believe that it is hard to pinpoint the type of primary lung cancer causing colon metastasis because of the small number of cases. Reviewing the English literature, there are several reports on colonic metastasis[11-14]. Patients with GI metastasis of lung cancer are often asymptomatic, as in the present case. Yang *et al*[14] reported that the clinical prevalence of symptomatic GI metastasis of lung cancer is 1.77% (6/339). Habesoglu *et al* described that about 1/3 of colonic metastases from lung cancer are asymptomatic and the diagnosis is made at autopsy[4]. The most common symptoms are abdominal pain, nausea, vomiting, anemia, and weight loss[1,5]. Other symptoms of colonic obstruction, lower GI bleeding, bowel perforation, or GI fistula may occur[1-4]. These findings generally present after the diagnosis of primary disease but can occur synchronously or before diagnosis of the primary lesion[1,4,10].

Lung cancer with intestinal metastasis has been reported to have a poor prognosis of less than 16 wk in several studies[4,6,12,14]; however, because of advanced improvement in chemotherapy, supportive care for lung cancer, and extending life expectancy, we may come across an increasing number of GI metastasis in the future.

Thus, we should pay attention to GI metastatic signs such as GI bleeding, abdominal pain, nausea, vomiting, or less commonly, ileus. Development of GI symptoms after chemotherapy should be carefully managed in patients with GI metastasis because of the possibility of chemotherapy-induced perforation. The present patient underwent colonoscopy for further evaluation of a positive fecal occult blood test, and a metastatic colonic lesion was detected before chemotherapy. He has been receiving chemotherapy without chemotherapy-induced perforation or abdominal symptoms. If we aggressively examine lung cancer patients with a positive fecal occult blood test by colonoscopy, more latent patients with metastatic colonic tumor from the lung might be discovered in the future.

Recently, PET-FDG imaging has become useful to diagnose the extent of GI metastasis[13]. Cases of small intestinal[15] and large intestinal metastasis[13] from lung cancer have been reported. In the present case, we could also detect colonic metastatic lesions with PET-FDG; however, the definite role of PET-FDG in the diagnosis of GI metastasis from primary lung cancer is still controversial because of the few cases and lack of clinical data. To clarify the usefulness of PET-FDG for the diagnosis of GI metastasis from lung cancer, we should accumulate and analyze more cases of GI metastasis from primary lung cancer.

In conclusion, we reported a rare case of metastatic colonic SCC from the lung with a positive fecal occult blood test. It is necessary to be aware that primary SCC of the lung may cause colonic metastasis, although lung SCC rarely causes colonic metastasis.

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Falk Workshop: Perspectives in Liver
Transplantation

International Gastroenterological Congresses 2008
February 14-16, Paris, France
EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies
www.easl.ch/hepatitis-conference

February 14-17, Berlin, Germany
8th International Conference on New Trends in Immunosuppression and Immunotherapy
www.kenes.com/immuno

February 28, Lyon, France
3rd Congress of ECCO - the European Crohn's and Colitis Organisation
Inflammatory Bowel Diseases 2008
www.ecco-ibd.eu

February 29, Québec, Canada
Canadian Association of Gastroenterology
E-mail: general@cag-acg.org

March 10-13, Birmingham, UK
British Society of Gastroenterology Annual Meeting
E-mail: BSG@mailbox.ulcc.ac.uk

March 14-15, HangZhou, China
Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea
Asian Pacific Association for the Study of the Liver
18th Conference of APASL: New Horizons in Hepatology
www.apaslseoul2008.org

March 29-April 1, Shanghai, China
Shanghai-Hong Kong International Liver Congress
www.livercongress.org

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco
OESO 9th World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas
E-mail: robert.giuli@oeso.org

April 9-12, Los Angeles, USA
SAGES 2008 Annual Meeting - part of Surgical Spring Week
www.sages.org/08program/html/

April 18-22, Buenos Aires, Argentina
9th World Congress of the International Hepato-Pancreato Biliary Association
Association for the Study of the Liver
www.ca-ihpba.com.ar

April 23-27, Milan, Italy
43rd Annual Meeting of the European Association for the Study of the Liver
www.easl.ch

May 2-3, Budapest, Hungary
Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA
Digestive Disease Week 2008

May 21-22, California, USA
ASGE Annual Postgraduate Course
Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion
E-mail: education@asge.org

June 4-7, Helsinki, Finland
The 39th Nordic Meeting of Gastroenterology
www.congrex.com/ngc2008

June 5-8, Sitges (Barcelona), Spain
Semana de las Enfermedades Digestivas
E-mail: sepd@sepd.es

June 6-8, Prague, Czech Republic
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June 10-13, Istanbul, Turkey
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June 11-13, Stockholm, Sweden
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June 13-14, Amsterdam, Netherlands
Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic
Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management
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June 25-28, Barcelona, Spain
10th World Congress on Gastrointestinal Cancer
Imedex and ESMO
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15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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