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EDITORIAL

Role of endoscopic retrograde cholangiopancreatography in pancreatic diseases

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Abstract

Over the last 15 years, endoscopic retrograde cholangiopancreatography (ERCP) has evolved from a diagnostic tool to one that is primarily used to provide therapy. This development occurred first for biliary disorders and subsequently to a lesser extent for pancreatic diseases. Computed tomography, magnetic resonance imaging, magnetic resonance cholangiopancreatography and endoscopic ultrasonography suggest a diagnosis in the majority of patients with pancreatic diseases today and can help physicians and patients avoid unnecessary ERCP. However, a selected number of patients with pancreatic diseases may benefit from pancreatic endotherapy and avoid complex surgery and chronic use of medications. Pancreatic sphincterotomy, pancreatic stenting and pancreatic cyst drainage are some of the most effective and challenging endoscopic pancreatic interventions and should be performed with caution by expert therapeutic endoscopists. There has been a paucity of randomized studies investigating endoscopic techniques in comparison with surgery and medical therapy for the treatment of most benign and malignant pancreatic disorders due to the limited number of patients and the expertise required to attempt these procedures.

Key words: Endoscopic retrograde cholangiopancreatography; Pancreas; Pancreatic disease; Pancreatic endotherapy; Therapeutic endoscopy

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INTRODUCTION

Pancreatic diseases are among the most challenging disorders of the digestive system. A wide range of benign conditions present both diagnostic and therapeutic challenges to the gastroenterologist and surgeon[1]. These include acute pancreatitis (including recurrent), chronic pancreatitis, pancreatic duct stones, pancreatic leaks, pseudocysts and strictures. Symptoms exhibited by patients with these disorders can be disabling. Endoscopic treatments for these benign disorders have evolved in the last decades and remain a viable, cost-effective alternative to more invasive surgical or radiological methods[2]. In addition, endoscopic therapy can provide palliation for inoperable malignant pancreatic diseases, such as pancreatic cancer with biliary and duodenal obstruction[3].

Endoscopic pancreatic therapy has been developed

much more slowly than the endoscopic treatment of biliary disorders. There are many reasons for this, but the main one appears to be a fear of inducing pancreatitis after even the contrast injection or just sphincteric manipulation. It has become clear however, that techniques initially restricted to biliary endotherapy can also be used in the pancreas in selected individuals[4]. Thus, sphincterotomy and attempts at stone retrieval and stricture treatment were first used in chronic pancreatitis patients in whom the procedure-related risk was much lower than in patients with normal anatomy or sphincter of Oddi dysfunction. More and more use of these techniques has resulted from studies showing that small-caliber stents placed into the pancreatic duct after a sphincterotomy or repeated manipulation of the papilla significantly reduce the incidence and severity of procedure-related pancreatitis. Despite this general observation, however, there have still been very few large series, controlled trials and critical reviews of these techniques.

The introduction of advanced radiological and imaging techniques has limited the diagnostic role of endoscopic retrograde cholangiopancreatography (ERCP), but sometimes the information provided during a therapeutic procedure is also useful for diagnostic purposes. In this editorial, we will focus on the current role of ERCP for the diagnosis and especially the treatment of pancreatic disorders.

ENDOSCOPIC DIAGNOSIS OF CHRONIC PANCREATITIS

ERCP and endoscopic ultrasonography (EUS) are the principal endoscopic methods to assess patients with chronic pancreatitis and complement radiologic methods [computed tomography (CT) scans, magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography]. Both ERCP and EUS can establish the diagnosis of chronic pancreatitis[1,5]. ERCP allows detection of pancreatic duct changes including ductal dilation, strictures, abnormal side branches, communicating pseudocysts, pancreatic duct stones and pancreatic duct leaks. ERCP is highly effective in visualizing these ductal findings (sensitivity for the diagnosis of chronic pancreatitis of 71%-93% and a specificity of 89%-100%). The Cambridge Classification, which assesses the main pancreatic duct and side branches is a widely accepted system for scoring ductal findings seen on ERCP[6]. Unfortunately, pancreatography is imperfect and care should be taken not to overinterpret minor findings seen on ERCP. Conversely, ERCP may not detect changes of less advanced chronic pancreatitis. When the diagnosis of chronic pancreatitis is sought, ERCP should be reserved for patients in whom the diagnosis is still unclear after non-invasive pancreatic function testing or other non-invasive (CT, MRI) or less invasive (EUS) imaging studies have been performed[7,8]. Although ERCP can be used to obtain information about ductal anatomy to define the level and degree of obstruction and the presence of strictures and stones, it does not provide information regarding the surrounding pancreatic parenchyma. EUS can provide high-resolution images of both the ductal structures and the parenchyma[9]. There is good interobserver agreement in the diagnosis of chronic pancreatitis by EUS, and EUS may detect early chronic pancreatitis in a reliable manner compared with ERCP[10].

PANCREATIC DUCT STRICTURES

The finding of a pancreatic duct stricture often poses a diagnostic dilemma regarding the specific cause. The cause of a pancreatic duct stricture is likely to include one or more of the following: chronic pancreatitis, pancreatic neoplasm (benign or malignant), pseudocyst or traumatic injury (blunt or penetrating)[2]. Filling defects such as protein plugs or stones may resemble a stricture. Cancer is the most feared cause of pancreatic duct stricture and should be considered in all patients in whom a pancreatic duct stricture is identified. Patients older than 50 years presenting with single or multiple episodes of acute pancreatitis, who have a pancreatic duct stricture, must have malignancy included in the differential diagnosis, particularly in the absence of alcohol abuse.

Changes in ductal anatomy other than the stricture should be looked for when examining the pancreatogram. This includes irregularity in contour or dilation of the pancreatic duct or of the secondary radicles. The presence of a single stricture with proximal dilation and normal distal ductal anatomy is suggestive of a neoplastic cause. Changes noted throughout the duct, particularly distally to the stricture, in addition to the anticipated proximal dilation, are usually suggestive of chronic pancreatitis. The presence of multiple strictures and dilations in a "chain-of-lakes" appearance is characteristic of chronic pancreatitis. Unfortunately, none of these features suggestive of a diagnosis of chronic pancreatitis is absolute in ruling out pancreatic cancer in individual patients because patients with chronic pancreatitis are at increased risk for pancreatic cancer. Therefore, the pancreatogram alone is not sufficient to rule out pancreatic cancer in patients with chronic pancreatitis, and if there is a clinical suspicion, aggressive attempts to obtain tissue should be made to establish a diagnosis[11]. Physicians should have a low threshold to perform EUS to more closely and thoroughly examine the pancreatic parenchyma, with fine-needle aspiration of any areas felt to be suspicious for possible malignancy. Obtaining serum CA 19-9 levels may be helpful in patients considered to harbor a malignancy, although levels can be elevated in patients with chronic pancreatitis in the absence of cancer.

Benign strictures of the main pancreatic duct are generally due to inflammation or fibrosis around the main pancreatic duct. Because ductal obstruction may lead to pain or acute pancreatitis superimposed on chronic pancreatitis, endoscopic therapy with balloon dilation or pancreatic duct stents for the treatment of dominant pancreatic duct strictures has been evaluated. Stricture dilation may be required to facilitate stent placement or stone removal.

Data regarding the role of endoscopic therapy in treating main pancreatic duct strictures are inconsistent. Some, but not all, authors have reported high success rates (75% to 94%) in treating pain by stenting of pancreatic duct strictures[2,4,12]. In addition, although some authors have correlated clinical improvement to a decrease in the diameter of the main pancreatic duct upstream, others have not. Pancreatic stents are prone to occlusion and patients undergoing endoscopic therapy for pancreatic duct strictures may require frequent stent exchanges. Symptomatic improvement may persist after pancreatic stent removal despite persistence of the stricture. Confounding factors in the literature on pancreatic stent therapy are other therapies performed at the time of stent placement (e.g. pancreatic sphincterotomy, pancreatic stone removal) and the tendency of the chronic pancreatitis pain to wax and wane and decrease with time as deterioration of pancreas function occurs[13]. The optimum duration of stent placement, stent number and diameter and degree of balloon dilation are not known. Complications related to endoscopic therapy of pancreatic duct strictures include pain, pancreatitis, stent occlusion, proximal or distal stent migration, duodenal erosions, pancreatic infection, ductal perforation, and bleeding from pancreatic sphincterotomy.

The role of placing multiple stents in the pancreatic duct has been assessed by Costamagna *et al*[12]. Nineteen patients with severe chronic pancreatitis and with a single pancreatic stent through a refractory dominant stricture in the pancreatic head underwent removal of this stent followed by balloon dilation of the stricture and insertion of the maximum number of stents allowed by the tightness of the stricture and the caliber of the pancreatic duct diameter. Stents were removed after 6-12 mo. The median number of stents placed through the major or minor papilla was three; their diameter ranged from 8.5 to 11.5 Fr and length from 4 to 7 cm. During a mean follow-up of 38 mo after stent removal, 84% of patients were asymptomatic, and 11% had symptomatic stricture recurrence. No major complications were recorded. This study showed that endoscopic multiple stenting of a dominant pancreatic duct stricture is feasible and safe.

PANCREATIC DUCT STONES

Obstructing pancreatic duct stones may contribute to abdominal pain or acute pancreatitis in patients with chronic pancreatitis. ERCP provides direct access to the pancreatic duct for evaluation and treatment of symptomatic pancreatic duct stones. In one randomized trial comparing endoscopic and surgical therapy, surgery was superior for long term pain reduction in patients with painful obstructive chronic pancreatitis[14]. However, because of its lower degree of invasiveness, endotherapy may be preferred, reserving surgery as second-line therapy for patients in whom endoscopic therapy fails or is ineffective. Pancreatic stone removal can be challenging. Frequently the stone configuration and size, coupled with pancreatic duct strictures, occlude the lumen. Adjuvant endoscopic approaches such as stricture dilation, intraductal lithotripsy and pancreatic sphincterotomy may be needed. Even when accessible, pancreatic duct stones (which are often dense and hardened) may be impacted, requiring extracorporeal shock wave lithotripsy (ESWL) to fragment the stones, before endoscopic removal can be achieved. Multiple ESWL sessions may be required and success rate in complete duct clearance and duct decompression exceeds 50%[3,15,16]. Intraductal lithotripsy guided by pancreatoscopy has also been used to fragment pancreatic stones.

Most series have shown improvement in pain with pancreatic endotherapy. Some encouraging short-term results and long-term 5 years follow-up results showing improvements in pain (77%-100% and 54%-86%, respectively) have been reported[17-19]. Although modest, these success rates are acceptable in the context of traditionally difficult-to-manage groups of patients.

ENDOSCOPIC PAIN MANAGEMENT IN CHRONIC PANCREATITIS

The ideal treatment for patients with pancreatic duct stones, dilated pancreatic ducts and pain is not known. The stones can be easily removed coincidently with the performance of a surgical drainage procedure, such as pancreaticojejunostomy. Alternatively, however, they can be fragmented by ESWL and removed endoscopically after sphincterotomy of the pancreatic duct. Stones can be cleared by this approach in roughly 80% of patients, and approximately 50% of these have long-term relief of their symptoms[20]. Dumonceau *et al*[21] conducted a randomized trial comparing pain relief after ESWL alone *vs* in combination with endoscopic drainage of the main pancreatic duct in patients with painful calcified chronic pancreatitis. Two years after trial intervention, 10 (38%) and 13 (45%) patients of the ESWL alone group and of the ESWL combined with endoscopy group, respectively, had presented pain relapse. In both groups, a similar and significant decrease was seen after treatment in the number of pain episodes/year (mean decrease, 3.7 episodes). Thus, there was no difference between the treatment groups, and the treatment costs per patient were three times higher in the ESWL combined with endoscopy group compared with the ESWL alone group.

An alternative involves the use of stents placed in the pancreatic duct endoscopically. Reports indicate that 30%-76% of patients receiving such stents have symptomatic improvement over a period of 14 to 36 mo of observation. Although these results seem encouraging, a criticism is that most of the data reported to date have been from relatively short-term, non-randomized studies. The issue is further complicated by the fact that pancreatic duct stents may not be entirely harmless; for example, they may cause further pancreatic duct changes and potentiation of chronic pancreatitis. Endoprosthesis occlusion and migration also seem to be relatively common.

There have been two randomized controlled trials comparing endoscopic therapy with surgery for the pallia-

tion of pain in chronic pancreatitis[14,22]. After 5 years of follow-up, pain was absent in 14%-16% of patients treated with endoscopy and in 36%-40% of patients treated with surgery. Based on these trials, it appears that surgery provides better pain relief compared to endoscopy, but even surgery fails to provide substantial pain relief in more than half of the patients. Due to its low degree of invasiveness, however, endotherapy can be offered as a first-line treatment, with surgery being performed in cases of failure and/or recurrence.

In cases of chronic pancreatitis with intractable pain where surgery is clearly indicated, ERCP can give valuable information regarding pancreatic duct configuration and exact ductal changes, according to the Cambridge classification[23,24]. In many cases, efforts such as decreasing smoking and alcohol use, taking oral pancreatic enzyme supplements, and receiving endoscopic therapies such as sphincterotomy and stent placement are usually effective in managing pain and inhibiting disease progression. Surgical options for chronic pancreatitis treatment include drainage procedures such as the Puestow procedure and resections such as pancreaticoduodenectomy, distal pancreatectomy, or total pancreatectomy. ERCP can serve as a preoperative bridge therapy to partial or total pancreatectomy with autologous islet cell transplantation. The latter procedure was developed for both pain management and maintenance of pancreatic endocrine function, especially glycemic control. A few institutes in the world have performed total pancreatectomy with autologous islet transplantation, since it requires special techniques for islet processing. The effectiveness of this procedure has been reported[25,26].

PANCREATIC DUCT LEAKS

Pancreatic duct disruptions or leaks can occur as a result of severe acute pancreatitis or chronic pancreatitis. The causes of the disruption are usually severe inflammation or obstruction of the duct, or severe pancreatic necrosis. Pancreatic leaks can result in pancreatic ascites, pleural effusions, pseudocyst formation and internal and external pancreatic fistulas. Pancreatic duct leaks can often be treated with endoscopic placement of transpapillary stents in a manner similar to the use of biliary stents for closing bile duct leaks[27]. Endoscopic therapy is successful in closing the leaks in approximately 60% of patients. Factors associated with a better outcome in duct disruption include a partial disruption, successfully bridging the disruption with a stent and longer duration of stent placement (approximately 6 wk). There are no comparative studies of surgical, medical and endoscopic therapy for treatment of pancreatic duct leaks.

A novel treatment approach using endoscopic injection of N-butyl-2-cyanoacrylate to achieve closure of the fistula has also been reported[28]. In total, 12 patients underwent ERCP with injection of tissue glue directly into the pancreatic fistulous tract, in addition to endoscopic drainage with stent placement when this was considered to be indicated by the endoscopist. A single session of glue injection was successful in seven patients, and a second session was required in one patient. Inadvertent injection of the cyanoacrylate into the pancreatic duct at the time of glue injection into a pancreatic fistula can be associated with chemical or obstructive pancreatitis. In contrast, the injection of glue to completely fill a disconnected ductal system usually results in glandular atrophy and has been used to avoid surgical resection in high-risk patients by some institutions[29].

PANCREATIC PSEUDOCYSTS

Pancreatic pseudocysts arise as a complication of chronic pancreatitis in 20%-40% of cases[5,18,30]. Endoscopic drainage and management of the pseudocyst is a less invasive alternative to surgical treatment and is safer when the site of the puncture is defined by EUS. Pseudocyst drainage should be considered (1) for symptomatic lesions due to pain, gastric outlet obstruction, early satiety, weight loss or obstructive jaundice; (2) when there are signs of infection of the pseudocyst; and (3) when progressive enlargement of the cyst takes place, even if it is asymptomatic. Special care must be taken to avoid drainage of cystic neoplasms, duplication cysts and other noninflammatory collections[5,31,32].

A retrospective study was conducted to determine the impact of procedure experience on patient outcomes after endoscopic drainage of endoscopic pancreatic fluid collections[33]. In that large review of 175 cases, endoscopic drainage was carried out to treat pancreatic necrosis (33%), acute pseudocysts (23%), or chronic pseudocysts (44%). There was a dramatic improvement in the resolution rates of chronic pseudocysts after the first 20 procedures in comparison with former procedures (45% *vs* 93%) and a reduction in days to resolution of the pseudocyst (50 d *vs* 33 d). In patients with pancreatic necrosis there was a statistically significant decrease in the median hospital stay with greater experience (23 d *vs* 15 d). While these findings require confirmation by other groups, this study for the first time documented the importance of operator experience for patient outcomes after these often technically challenging endoscopic procedures.

Several excellent literature synopses and technical reviews on pancreatic pseudocysts have been published in recent years. These include a technical review by Ballie regarding pseudocysts in general and a subsequent article by the same author on the endoscopic management of pseudocysts[34,35]; a technical review by Hawes[36] that distinguishes between pseudocysts and other types of pancreatic fluid collection; and an excellent article by Giovannini *et al*[37] describing the use of EUS for cystogastrostomy. Finally, Rosso *et al*[38] reviewed 466 cases of endoscopically treated pseudocysts which were reported in 17 publications, comparing the results with previously published surgical series. The authors correctly concluded that pseudocysts are best handled by an integrated multidisciplinary team including pancreatic surgical specialists, gastroenterologists and

interventional radiologists. The conclusions from all these review articles are that treatment of pseudocysts can be complicated but it requires patience, expertise, adequate clinical and endoscopic skills and appropriate endoscopic accessories.

BILIARY OBSTRUCTION IN CHRONIC PANCREATITIS AND PANCREATIC CANCER

Distal common bile duct strictures have been reported to occur in 2.7% to 45.6% of patients with chronic pancreatitis. These strictures can occur from inflammation, fibrosis, or compression from a pseudocyst or a pancreatic stone[17,39]. Because long-standing biliary obstruction can lead to secondary biliary cirrhosis or recurrent cholangitis, biliary decompression is recommended in patients with clinically significant obstruction (e.g. cholestasis or jaundice). Surgical biliary bypass is the standard approach for managing chronic common bile duct strictures. Endoscopic therapy has been used as an alternative to surgery[40]. Plastic biliary stents are a useful short-term treatment for chronic pancreatitis-induced common bile duct strictures in the setting of cholestasis, jaundice or cholangitis and may be used as a long-term treatment approach in poor surgical candidates. Unfortunately, long-term success rates are as low as 7.7%-10% in some studies when single large-bore stents are used[41,42]. The use of multiple stents with frequent stent exchanges and balloon dilations over a long period of time (up to 1-2 years) may be more efficacious than single stents for the treatment of these strictures. Patient selection is critical in this setting because patients need to return frequently for stent changes. Poor compliance to follow-up can lead to biliary sepsis from stent occlusion[43-45].

Self-expanding metal stents (SEMS) have been used for the treatment of benign biliary strictures. Uncovered metal stents have given good 3-year results for poor operative candidates, while reports for covered metal stents have given mixed results. The routine use of metal stents for benign biliary strictures is not recommended at this time[46-49].

Several randomized controlled trials have demonstrated the superiority of SEMS to polyethylene stents for the treatment of malignant distal biliary obstruction, because they have a longer duration of patency (plastic stents occlude at a median of 3 to 6 mo after placement) and consequently have been shown to be more cost-effective[50,51]. The choice of plastic (e.g. polyethylene) stents *vs* SEMS has been debated in the literature and data suggest that SEMS should be preferentially used when life expectancy exceeds 6 mo, whereas polyethylene stents are more cost-effective in patients who are expected to live less than 4 mo[52,53]. However, it is not always easy to predict patient survival at presentation.

There can be significant delays between diagnosis and surgery in patients with resectable pancreatic cancer and obstructive jaundice when neoadjuvant therapy is used or when there is limited access to surgery. In these instances, placement of SEMS at the time of initial ERCP has been advocated for relief of obstructive jaundice. Recently, it was reported that the costs of stenting alone were identical when using either plastic or metal stents for biliary obstruction drained for more than 30 wk before surgery in patients with resectable pancreatic cancer[54]. In the polyethylene group, 16 of 42 patients (38%) required 3 or more ERCPs before surgery and 7 more underwent palliative surgery in the setting of unresectable disease. If actual costs associated with stent-related complications had been included in the calculation, then the balance would have turned in favor of SEMS, because stent-related complications were 15% *vs* 93% after insertion of metal *vs* plastic stents, respectively.

With newly designed stents arriving on the market from different manufacturers, it remains to be established whether covered SEMS are more effective than uncovered in palliating obstructive jaundice and whether complications associated with SEMS (i.e. migration, cholecystitis and occlusion) can be reduced[55,56]. Only comparative multicenter studies can answer these questions.

CONCLUSION

ERCP is useful for the diagnosis of chronic pancreatitis but it should be reserved for patients in whom the diagnosis has not been established by non-invasive or less invasive procedures. ERCP and pancreatic endotherapy can be effective in patients with pancreatic strictures, pancreatic duct leaks, pancreatic duct stones and pancreatic pseudocysts. However, the most important advance with regard to ERCP is the palliative or preoperative treatment of biliary obstruction caused by chronic pancreatitis or malignant pancreatic disease. Metal stents offer better long-term relief compared to plastic stents and should be preferred in patients with a life expectancy of more than 4 to 6 mo. Expertise in ERCP is a prerequisite for effective pancreatic endotherapy.

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TOPIC HIGHLIGHT

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Fat: A matter of disturbance for the immune system

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Abstract

Obesity is increasingly being recognized as a risk factor for a number of benign and malignant gastrointestinal conditions. However, literature on the underlying pathophysiological mechanisms is sparse and ambiguous. There is compelling evidence that both overnutrition and undernutrition negatively interfere with the immune system. Overnutrition has been found to increase susceptibility to the development of inflammatory diseases, autoimmune diseases and cancer. In the regulation of immune and inflammatory processes, white adipose tissue plays a critical role, not only as an energy store but also as an important endocrine organ. The obese state is characterised by a low-grade systemic inflammation, mainly as a result of increased adipocytes as well as fat resident- and recruited-macrophage activity. In the past few years, various products of adipose tissue including adipokines and cytokines have been characterised and a number of pathways linking adipose tissue metabolism with the immune system have been identified. Activation of the innate immune system plays a major role in hepatic steatosis. Non-alcoholic fatty liver disease includes a wide spectrum of diseases, from pure steatosis to non-alcoholic steatohepatitis in the absence of significant alcohol consumption. Although steatosis is considered a non-progressive disease, non-alcoholic steatohepatitis may deteriorate in advanced chronic liver diseases, cirrhosis, and hepatocellular carcinoma. An important parallel between obesity-related pathology of adipose tissue and liver pertains to the emerging role of macrophages, and growing evidence suggests that Kupffer cells critically contribute to progression of non-alcoholic fatty liver disease. Moreover, a close link between specific immune activation and atherosclerosis has been well established, suggesting that fat can directly trigger immune responses. This review discusses the role of fat as "a matter of disturbance for the immune system" with a focus on hepatic steatosis.

Key words: Adipocytokine; Adipose tissue; Fat; Immune system; Kupffer cell; Natural killer; Steatosis

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INTRODUCTION

Obesity predisposes individuals to an increased risk of

developing many diseases, including atherosclerosis, diabetes, non-alcoholic fatty liver disease (NAFLD), cancer and immune-mediated disorders, such as asthma[1-3]. Obesity is typically assessed clinically with the surrogate measure of body mass index (BMI). Individuals with a BMI $\geqslant$ 30 kg/m^2 are considered obese. The incidence of obesity and its associated disorders is increasing markedly worldwide. Data from the most recent NHANES (National Health and Nutrition Examination Survey; 2005-2006) indicate that the prevalence of obesity was 33%-35% among US adults[4]. In another recent NHANES survey based on the combined years of 2003-2006, 16% of children or adolescents aged 2-19 years were obese[5]. In Europe, several surveys conducted since 2000 and using direct anthropometric measurements, showed that the prevalence of obesity ranges from 15% to 30% in men and from 11% to 34% in women, with considerable geographic variation (rates being higher in Central, Eastern, and Southern Europe)[6]. Urbanization and unbalanced diet, associated with genetic susceptibility have allowed the emergence of the obese phenotype.

In mammals, adipose tissue (AT) occurs in two forms: white adipose tissue (WAT) and brown adipose tissue (BAT). Most AT in mammals is WAT and this is thought to be the site of energy storage. In contrast, BAT is found mainly in human neonates and is important for the regulation of body temperature through non-shivering thermogenesis. In addition to adipocytes, which are the most abundant cell type in WAT, adipose tissue also contains pre-adipocytes or stromal vascular cells (which are non-fat cells): endothelial cells, fibroblasts, leukocytes and, most importantly, macrophages. Body fat distribution, rather than adiposity *per se*, is an important risk factor for obesity-related disorders. An excess of intra-abdominal fat rather than subcutaneous fat (central *vs* peripheral obesity) is associated with metabolic syndrome (MS) and cardiovascular disease (CVD). The mechanisms responsible for this association are still unknown, but several hypotheses, which are not mutually exclusive, have been formulated[7]. The first hypothesis proposed a direct effect of visceral AT depots on insulin resistance, lipoprotein metabolism, and blood pressure. Metabolic products of omental and mesenteric AT depots are released into the portal vein, which provides direct delivery to the liver. Lipolysis of omental and mesenteric AT depots releases free fatty acids (FFAs) that can induce hepatic insulin resistance and provide substrate for lipoprotein synthesis and neutral lipid storage in hepatocytes. In addition, specific proteins and hormones produced by omental and mesenteric AT, such as inflammatory molecules, angiotensinogen, and cortisol can also contribute to MS and CVD. Another hypothesis suggests that the limited capacity of subcutaneous fat to store excess energy results in overflow of fatty acids to intra-abdominal fat and "ectopic" sites such as liver, muscle, and islets. In this paradigm, excess intra-abdominal fat is merely a marker of fatty acid overflow from subcutaneous depots.

FAT AND THE IMMUNE SYSTEM

Adipose tissue was once thought to be an inert mass whose sole function was the storage of fat. However, it is now recognized that AT is an active endocrine organ that secretes numerous adipokines, cytokines and chemokines including leptin, adiponectin, resistin, retinol binding protein 4 (RBP4), tumor necrosis factor α (TNF-α), interleukin (IL)-1β, IL-6, and monocyte chemotactic protein 1 (MCP-1)[8,9]. All of these play a central role in the regulation of energy and vascular as well as immune system homeostasis by acting both locally and at distant sites influencing various metabolic and immune processes. Moreover, organs other than AT may contribute to systemic levels of some adipokines.

Obesity is associated with a low-grade inflammation of WAT resulting from chronic activation of the innate immune system, which can subsequently lead to insulin resistance, impaired glucose tolerance and even diabetes[10,11]. In addition to these associations between obesity and disease, research in the past few years has identified important pathways that link metabolism with the immune system and *vice versa*. Many of these interactions between metabolic and immune systems seem to be orchestrated by the complex network of soluble mediators derived from immune cells and adipocytes[1].

The effects of obesity on the immune system are not restricted to local effects within AT. Elevated levels of pro-inflammatory cytokines have been noted in the serum of asymptomatic obese individuals, the cytokine levels being related to the degree of obesity[12]. TNF-α is only present at very low levels in human blood suggesting that TNF-α released by adipose tissue has only autocrine/paracrine actions. IL-6, however, is present at much higher levels. Adipocyte-derived IL-6 has been estimated to comprise 30% of the circulating IL-6 suggesting an endocrine action[13]. Furthermore, these elevated levels of IL-6 are associated with increased circulating levels of C-reactive protein suggesting that although the elevation in levels is modest compared with those seen in sepsis, they could be having real effects on innate immune function.

Obesity is also associated with altered functioning of circulating immune cells[12,14]. Decreased T- and B-cell function, increased monocyte and granulocyte phagocytosis and oxidative burst, and an increase in leukocyte count have been described. More recently, circulating mononuclear cells from obese subjects have been shown to exhibit increased nuclear factor κB (NFκB) nuclear binding with decreased levels of NFκB inhibitor, together with increased mRNA expression of IL-6, TNF-α and migration inhibition factor. Furthermore, there is a good correlation between the markers of macrophage activation and plasma levels of FFAs[15]. It has previously been demonstrated that macronutrient challenges in normal subjects increase NFκB nuclear binding in circulating mononuclear cells, raising the possibility that the activated state of mononuclear cells is due to increased circulating levels of FFAs found in the obese. Indeed, hyperlipidaemia in mice mediates an inflammatory response by the same signalling cascade through which lipopolysaccharide activates the innate immune system (this engages a receptor complex comprising Toll 4 CD14, CD14 and MD-2)[16]. Table 1

Table 1 Adipocytokines, pro-inflammatory cytokines and chemokines, and other factors synthesised by adipocytes and macrophages in white adipose tissue

Adipocytes	Macrophages
Adiponectin	TNF-α
Leptin	IL-1β
Resistin	IL-6
RBP4	MCP-1
TNF-α	Resistin
IL-1β	
IL-6	
MCP-1	
Visfatin	
MIP	

RBP4: Retinol binding protein 4; TNF-α: Tumor necrosis factor α; IL: Interleukin; MCP: Monocyte chemotactic protein; MIP: Macrophage inflammatory protein.

summarises the secretion of adipokines, cytokines and other factors by adipocytes and macrophages in WAT. Finally, recent research has implicated the innate immune system in the pathophysiology of obesity-related liver damage[17,18].

Obesity is a high risk factor for NAFLD. Studies in an animal model of obesity-related liver disease revealed the involvement of dysfunctional hepatic immune cells[19]. In this review we analyse the relationship between hepatic steatosis and the immune system.

HEPATIC STEATOSIS AND THE IMMUNE SYSTEM

Hepatic steatosis is the histological hallmark of alcoholic liver disease (ALD) and NAFLD, which are among the commonest causes of cirrhosis and liver failure in the developed world[20-24]. Steatosis may also alter the natural history of other liver diseases such as chronic viral hepatitis[25]. Excessive consumption of alcohol in humans results in a spectrum of liver abnormalities, ranging from simple fatty liver to steatohepatitis and cirrhosis, which may be present independently or in combination. Infiltration of the liver by lymphocytes and neutrophils is an important feature of alcoholic hepatitis; it initiates a cascade of effector mechanisms that ultimately lead to hepatocyte death, fibrosis, and cirrhosis. Only a minority of consistently heavy drinkers with steatosis ever develop clinically important liver disease[24,26] implying that host or environmental factors determine the evolution of alcohol-related liver damage. Ingestion of alcohol leads to increased production of reactive oxygen species (ROS), which are generated during the metabolism of alcohol by cytochrome P450 2E1 enzyme, and excessive alcohol consumption is associated with increases in lipid, protein, and DNA peroxidation. Consistent with this disease model, risk factors for the development of progressive liver damage in alcohol drinkers include both polymorphisms in alcohol-metabolizing enzymes and polymorphisms in genes associated with a more vigorous inflammatory response in addition to exogenous factors including obesity, exposure to other hepatotoxins, and infection with hepatitis C and/or B virus[27-29].

NAFLD is increasingly recognized as a leading cause of liver dysfunction and cirrhosis in the developed world and is part of a spectrum of metabolic diseases associated with central (intra-abdominal) obesity, hypertension, dyslipidaemia, insulin resistance, and type 2 diabetes mellitus[22,30]. Similar to alcoholic liver disease, NAFLD is a spectrum of disorders, beginning as simple steatosis that is mostly considered an innocent condition. Being both the source and the result of insulin resistance, however, steatosis may be associated with an increased risk for cardiovascular morbidity[31]. Most importantly, in about 15% of all patients with NAFLD, steatosis may evolve into steatohepatitis (NASH), a medley of inflammation, hepatocellular injury, and fibrosis, often resulting in cirrhosis and even hepatocellular carcinoma[32]. Although this full sequence of progression is relatively rare, the overwhelming prevalence of NAFLD predicts a major healthcare burden. Epidemiology, pathogenesis, and approach to treatment of NAFLD follow the same trends as other metabolic disorders, and insulin resistance is the key event linking NAFLD to these diseases[33-35].

ROLE OF ADIPOCYTOKINES IN ALCOHOLIC AND NON-ALCOHOLIC STEATOHEPATITIS

Many of the initial proinflammatory changes seen in NAFLD may be the consequence of altered metabolism rather than the underlying immune pathogenic event, and adipokines provide a link between fat, inflammation, and immunity (for more details see review by Tilg *et al*[9]). More than 50 adipokines have been identified so far. Of these, leptin and adiponectin can influence the immune response, and their serum levels are increased and decreased, respectively, in NASH[9]. While many adipokines are associated with adverse biological functions, adiponectin, the most abundant adipose-derived hormone, seems to have a protective effect in NAFLD. Adiponectin inhibits TNF-α induced endothelial cell adhesion molecule expression, induces production of anti-inflammatory cytokines such as IL-10, and reduces T and B lymphocyte responses. In particular, full-length adiponectin (Acrp30) and its cleavage derivative, globular adiponectin (gAcrp), have been credited with anti-diabetic, anti-inflammatory and anti-atherogenic properties[36]. Adiponectin stimulates hepatic fatty acid oxidation and ketogenesis, while it inhibits cholesterol and triglyceride synthesis[36]. While these metabolic activities primarily occur in hepatocytes, adiponectin has potent anti-inflammatory effects in macrophages. Thus, adiponectin is able to suppress the effects of lipopolysaccharides (LPS) in macrophages, including activation of NFκB and ERK1/2[37-39]. Similarly, adiponectin prevents LPS-mediated inflammatory signalling in Kupffer cells[40]. These anti-inflammatory effects of adiponectin may involve IL-10 signalling pathways[41]. Interestingly, NADPH oxidase is a

major IL-10 target in various cell systems including macrophages[42].

Decreased levels of adiponectin are definitely related to a variety of unfavourable effects, but the precise origin of adiponectin reduction has not been clarified. TNF-α has been demonstrated to suppress the transcription of adiponectin in an adipocyte cell line, which might explain the lower levels of serum adiponectin in obese individuals[9]. Expression of adiponectin is also regulated by other pro-inflammatory mediators such as IL-6, which suppresses adiponectin transcription and translation in an adipocyte cell line[9].

In a recent study, Kolak *et al*[43] evaluated subcutaneous AT biopsies obtained from healthy women both with and without increased liver fat (LFAT) (2.3% ± 0.3% *vs* 14.4% ± 2.9%, respectively), with similar BMIs and percentage body fat. Expression of cytokines and chemokines including CD68 (which correlates with the number of macrophages), MCP-1, macrophage-inflammatory protein (MIP-1α), and PAI-1 were significantly increased, whereas peroxisome proliferator-activated receptors (PPAR)-γ and adiponectin were significantly decreased in women with high levels of LFAT compared with women with normal levels of LFAT, even though subcutaneous fat cell size, BMI, and percentage body fat were similar.

Leptin activates neutrophils, stimulates proliferation in human circulating monocytes, and appears to induce Th1-type cytokine production while inhibiting Th2-type cytokines. In addition, leptin has marked effects on the innate immune response by promoting activation and phagocytosis of macrophages, presumably through JAK/STAT signalling[44]. Expansion of adipocytes in obesity leads to the recruitment of macrophages and the release of TNF-α, IL-6, and MCP-1 from macrophages and lymphocytes. TNF-α and IL-6 suppress the transcription of adiponectin, and TNF-α and IL-1 stimulate the production of leptin[9,44]. Accordingly, hyperleptinaemia associated with obesity may contribute to progression of NAFLD, although this issue remains controversial[45].

Resistin is another pro-inflammatory adipokine secreted by monocytes/macrophages and adipocytes in response to pro-inflammatory signals. Resistin induces NFκB-dependent secretion of TNF-α and IL-6 by monocytes and increases ICAM-1 and VCAM-1 expression in endothelial cells, suggesting that it contributes to endothelial activation and leukocyte recruitment[46]. In particular, in pure steatosis there is no significant increase in adhesion molecule expression but distinctive patterns are associated with both alcoholic hepatitis and cirrhosis, and in murine models of NASH elevated ICAM-1 expression is seen[47]. Alcoholic hepatitis is characterized by increased expression of E-selectin and ICAM-1 on portal and hepatic venous endothelium and of ICAM-1, VCAM-1, and VAP-1 on sinusoidal endothelium as a consequence of local pro-inflammatory cytokines, particularly TNF-α[48-51]. In alcoholic cirrhosis, increased expression of endothelial adhesion molecules including ICAM-1, VCAM-1, and P-selectin is largely restricted to portal and septal vessels. Endothelial ICAM-1 expression is increased in periseptal areas where LFA-1 is also increased in leukocytes, however, in contrast to alcoholic hepatitis, there is little increased ICAM-1 expression on hepatocytes[48].

Visfatin, the characteristic adipokine of mesenteric AT, was previously identified as a protein involved in immune B-cell maturation (pre-B colony enhancing factor)[52]. More recently, visfatin was described to be a highly expressed protein with insulin-like functions that was predominantly found in visceral AT, from which the name visfatin was derived[53]. Thus, visfatin was identified as nicotinamide phosphoribosyltransferase, the rate-limiting enzyme that converts nicotinamide (a form of vitamin B3) to nicotinamide mononucleotide, a NAD precursor[54]. Visfatin also has pro-inflammatory properties by inducing TNF-α and IL-6 in monocytes[55]. Further studies are needed to fully understand the effect of this adipokine in Kupffer cells.

Figure 1 summarises the effects of adipocytokines on the regulation of the immune response.

HEPATIC STEATOSIS AND NATURAL KILLER CELLS

One experimental model which has generated a significant body of evidence regarding potential mechanisms of NAFLD pathogenesis and its relationship with the immune system is the *ob/ob* mouse. *Ob/ob* mice, which are leptin deficient as a result of a spontaneous mutation in the leptin gene, exhibit a number of metabolic and inflammatory features which mimic human NAFLD[56] including insulin resistance, hyperlipidaemia, hepatic steatosis, and TNF-α elevation. One of the principal applications of the *ob/ob* mouse has been the identification of susceptibility of the steatotic liver to inflammatory insult (exemplified by the response to LPS) as a key factor in the development of NASH[57]. A number of immuno-regulatory abnormalities have been identified in *ob/ob* mice which may contribute to their increased susceptibility to inflammatory damage. These include selective depletion in the liver (but not other organs) of Natural Killer (NK) T cells, a key population of immuno-regulatory/effecter lymphocytes which express phenotypic features of both "classical" T cells (CD3) and NK cells [NK1.1 (CD161 in humans)][58,59]. In their most characteristic form, NKT cells show specificity, through a semi-invariant surface T-cell receptor, for highly conserved glycolipid antigens presented by the MHC class I homolog CD1d. NKT cells, which are specifically enriched within the liver, have characteristic cytokine release patterns {Th-1 dominant [interferon (IFN)-γ], mixed, and Th-2 dominant (IL-4) depending on the mechanism of stimulation} which endow, in addition to their effector function, significant immuno-regulatory properties[60]. The observation that liver NKT cells are depleted in steatosis in *ob/ob* mice has led to the suggestion that these cells play a key role in mediating and/or regulating inflammatory effects critical to the development of NAFLD. Although of potential value in the understanding of the pathogenesis of NAFLD, conceptual problems arise with regard to the *ob/ob* mouse

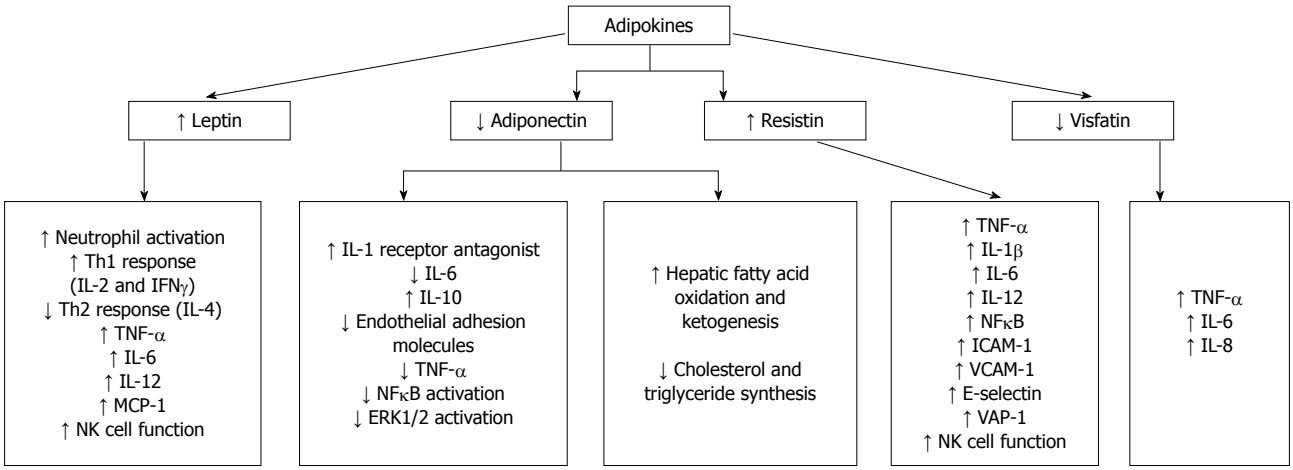

Figure 1 Effects of adipocytokines on regulation of the immune response. IL: Interleukin; IFN: Interferon; MCP: Monocyte chemotactic protein; NK: Natural Killer; TNF-α: Tumor necrosis factor α; NFκB: Nuclear factor κB.

as a model for human disease due to its markedly different leptin phenotype (absent *vs* elevated), and to the fact that leptin is itself a key immunomodulatory cytokine[61]. There are, therefore, potential mechanisms whereby leptin deficiency could modulate the immune response independent of its effects on hepatic fat accumulation. Li *et al*[62] used a natural obese/steatosis model to study the effects of hepatic steatosis on hepatic innate immune system function in leptin complete animals. C57Bl/6 mice fed a high-fat diet showed excess weight gain and the development of hepatic steatosis[63]. Although total hepatic mononuclear cell levels were similar in the high- and low-fat diet groups, the percentage of hepatic (but not splenic) NKT cells was significantly reduced. Within both the hepatic T-cell and NKT fractions, the numbers of cells showing cytoplasmic staining for TNF-α and IFN-γ were, conversely, increased in the high-fat diet group (and serum IFN-γ levels were elevated), suggesting Th1 skewing of the response phenotype resulting from induced NKT cell effects. Finally, the livers of obese mice appeared to be sensitized to LPS injury, presumably reflecting the augmented Th1-type inflammatory cytokine response. These observations suggest that the development of hepatic steatosis *per se* can be associated with significant changes in liver NKT cell function. This finding would be compatible with the NKT cell changes seen in the *ob/ob* mice occurring as a result of hepatic steatosis that occurs in these animals, rather than the specific absence of leptin. The findings do, however, raise a number of issues which will determine whether this model is suitable for the study of human NAFLD. The first issue is the mechanism responsible for liver NKT cell "loss", and Th-1 skewing of the residual cells, in obese C57Bl/6 mice. Theoretically, a reduction in liver NKT cells in obese C57Bl/6 mice could result from a decreased rate of NKT cell recruitment to, or development in, the liver, an increased rate of NKT cell death or migration from the liver, a loss of surface markers identifying the cells as NKT cells or any combination of these effects. The liver recruitment aspect of NKT cell homeostasis was not addressed in the Li's study[60,64,65]. Instead, the authors argue that increased cell loss is the dominant effect, with evidence presented to suggest increased NKT cell apoptosis and increased hepatic expression of IL-12 (postulated to be a promoter of NKT cell apoptosis). There is an emerging consensus, however, that NKT cells are in fact relatively resistant to activation-induced cell death[66]. An alternative (albeit non-mutually exclusive) explanation for the Li's data would be that endogenous IL-12 released by Kupffer cells (KC) at elevated levels in the context of obesity[62,67] acts as a cofactor for the stimulation of IFN-γ release (as opposed to IL-4 release which occurs in the absence of IL-12) by physiologically activated NKT cells, with the resulting "loss" of cells occurring as a consequence of post-activation surface phenotypic shift[68]. If elevation of KC-released IL-12 in response to steatosis were to prove to be a factor in human fatty liver development[69], its well-established ability to promote breakdown of self-tolerance may explain the increasingly recognised tendency towards autoantibody formation reported in NASH patients[70,71]. The possibility that NKT cell activation is responsible, through activation-induced cell death and/or post-activation phenotypic change, for "reduction" in hepatic NKT cells in obese C57Bl/6 mice, and through cytokine release, for liver damage, raises the important question of the mechanism of this activation. Most previous work on NKT cell activation has used non-physiological ligands (anti-CD3 and anti-TCR). Although the recent identification of α-galactosylceramide has highlighted the potential importance of glycolipids as natural ligands for NKT, it is unlikely, given its marine sponge origin, that this agent is a physiological ligand in mice or humans. At present, the identity of the *in vivo* physiological ligand for NKT cells, the extent to which TCR-mediated as opposed to cytokine-driven mechanisms (such as *via* IL-12) are required for activation, and the extent to which different activation pathways result in different cytokine response phenotypes, remain areas of speculation. One potentially highly intriguing link between hepatic steatosis and NKT cell activation has emerged

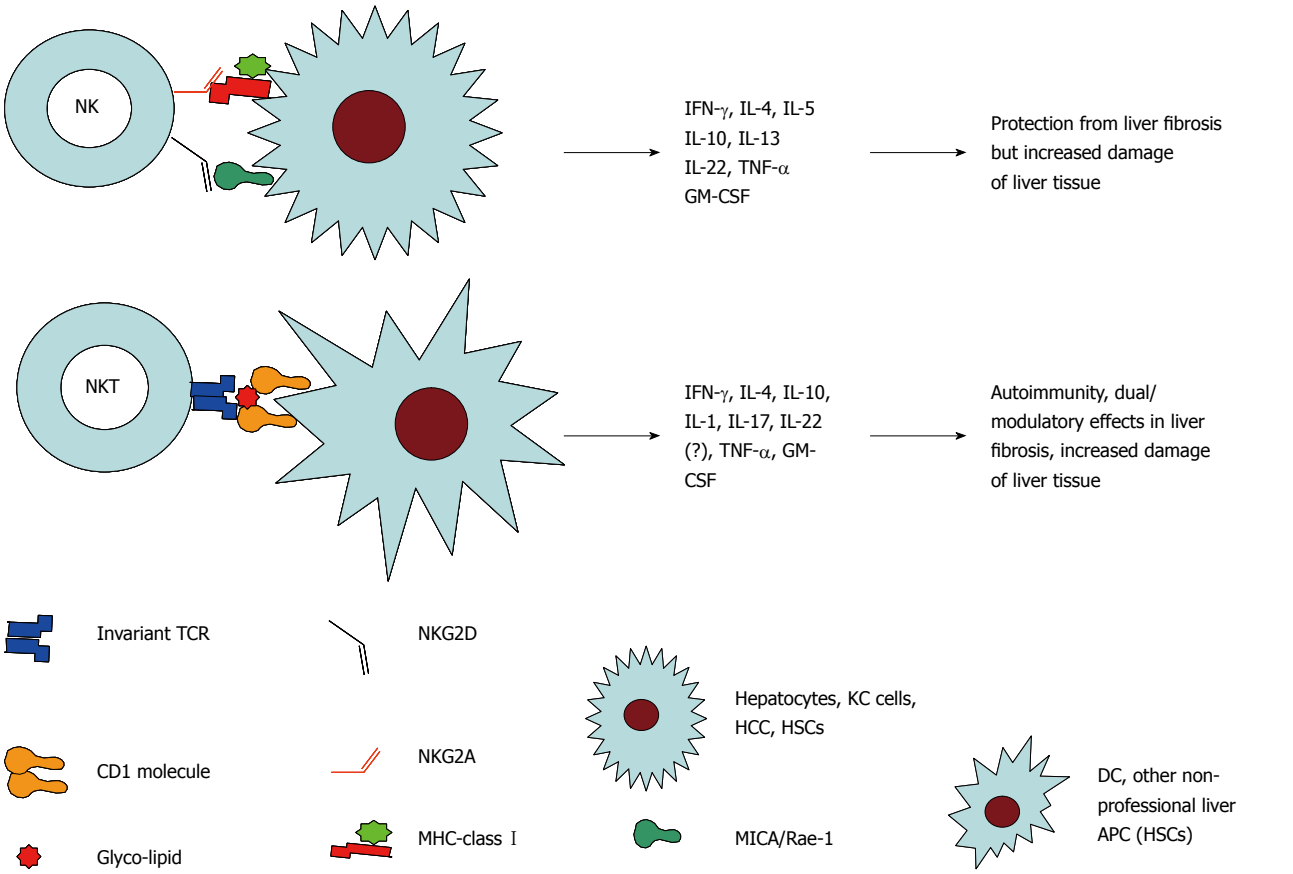

Figure 2 Simplified scheme of Natural Killer/Natural Killer T cell role in liver diseases. Natural Killer (NK) interacts with major and minor histocompatibility antigens expressed on several liver cells and kill and/or produce cytokines having several effects on the tissue. More complex is the role of NKT cells. These cells specifically recognize an antigen expressed in the context of a CD1 molecule and, upon recognition through an invariant TCR, secrete a large amount of cytokines having pleiotropic, sometimes controversial effects, whose overall results are due to the cytokine milieu and to the conditioning of the functions of other immune cells. This scenario is further complicated by the fact that many soluble factors (for instance cytokines) and hedgehog ligands may activate NK or NKT. IL: Interleukin; TNF-α: Tumor necrosis factor α; HCC: Hepatocellular carcinoma; HSCs: Hepatic stellate cells; GM-CSF: Granulocyte-macrophage colony stimulating factor; APC: Antigen presenting cell.

with the observation that microsomal triglyceride transfer protein, plays a key role in the acquisition of glycolipid antigens by CD1d[72]. In partial support of this concept, deficiency of microsomal triglyceride transfer protein in mice is associated with hepatic steatosis, and functional polymorphisms of its encoding gene have shown significant associations with NASH in humans[73]. One approach to dissecting out the mechanisms of NKT cell activation and loss in obese C57Bl/6 mice would be to utilize NKT cell adoptive transfer and tracking methodologies in recombinant NKT cell-deficient mice in combination with NKT cell activation and appropriate cytokine blocking.

In a recent paper, Hua *et al*[74] examined the mechanism of dietary fatty acid induced hepatic NKT cell deficiency and its causal relationship to insulin resistance and NAFLD, and found that dietary saturated fatty acids (SFA) or monounsaturated fatty acids (MUFA), but not polyunsaturated fatty acids (PUFA), caused hepatic NKT cell depletion with increased apoptosis. Dietary SFA or MUFA also impair hepatocyte presentation of endogenous, but not exogenous, antigen to NKT cells, indicating alterations of the endogenous antigen processing or presenting pathway. *In vitro* treatment of normal hepatocytes with fatty acids also demonstrates impaired ability of CD1d to present endogenous antigen by dietary fatty acids. Furthermore, dietary SFA and MUFA activate the NFκB signaling pathway and lead to insulin resistance and hepatic steatosis.

Recently, a new subset of T helper cells, named Th17 due to the ability to produce IL-17 and other cytokines, has been correlated to processes underlying hepatic steatosis. In particular, Th17 largely express a NKT marker, CD161, and they have been described to be closely involved in the immune responses in several anatomical sites including skin, liver and gut[75,76]. Th17 produce cytokines besides IL-17 such as IL-22 which is indicated to play a pivotal role in hepatic steatosis as recently shown[77].

Figure 2 shows a simplified Scheme of NK/NKT cell role in liver diseases.

KUPFFER CELLS AND STEATOSIS

Hepatocellular accumulation of lipids is a key morphologic feature of NAFLD. Lipidomic analysis of human liver tissue is a promising novel approach to associate abnormal fat composition with various stages of NAFLD. Thus, total and damaged phospholipids are more abundant in simple steatosis at the expense of triglycerides[78], while the increased ratio of stearic to arachidonic acid in NASH may

correlate with fibrosis[79]. Altered abundance and composition of liver tissue lipids may modulate the biological activity of KC in NAFLD through a number of mechanisms. First, the space-occupying effect of fat-laden hepatocytes may lead to impaired sinusoidal perfusion[80]. Leukocytes trapped in narrowed sinusoids may increasingly engage KC in the microvascular inflammatory response[80]. Second, excessive exposure of KC to fatty acids may modulate pathways of inflammation and insulin resistance through interaction with cell surface receptors and intracellular mediators[81]. Third, anomalous deposition of lipids in the plasma membrane may alter the structure of lipid raft domains and interfere with clustering and function of cell surface receptors[82]. Altered lipid composition may also affect proper functioning of intracellular membranes as seen with free cholesterol loading of mitochondria[83]. Finally, abundant or abnormal lipids may confound recognition of fatty hepatocytes as dangerous and promote adverse interactions with KC[17]. Nevertheless, the existence of a lipid-derived quintessential alarm expressed or released by steatotic hepatocytes remains speculative.

Recent findings indicate that TLR-mediated recognition of fatty acid moieties is an important mechanism by which lipids regulate pathways of inflammation and innate immunity[82]. Depending on fatty acid composition, the outcome of this effect may be highly variable. Saturated fatty acids, implicated in the development of chronic conditions such as atherosclerosis, have been shown to activate TLR4 signalling in adipocytes and macrophages through both Myd88-dependent and TRIF-dependent pathways[84,85]. In contrast, polyunsaturated fatty acids inhibit these events in several cell types including macrophages[85]. Consequently, TLR4 is a sensor of endogenous fatty acid levels and composition, and KC most likely benefit from this ability.

Emerging evidence indicates that altered cholesterol metabolism may directly affect the function of KC. Thus, high-fat diet fed to LDL receptor deficient mice rapidly results in significant hepatic inflammation, but only if the diet contains cholesterol[86]. The presence of "foamy" KC suggests that scavenging of modified lipoproteins may induce this early inflammatory response[86]. While these findings need to be extrapolated to human NAFLD with caution, they point to the importance of altered cholesterol metabolism. In addition, some of these observations challenge the "second-hit" concept since steatosis is not necessarily a forerunner of hepatic inflammation as these events may develop simultaneously[86,87].

There is evidence that steatosis promotes Th1 polarization of the cytokine balance favouring innate or classic activation of macrophages in NAFLD[88]. PPAR-α, PPAR-γ, and PPAR-σ and liver X receptors LXR-α and LXR-β are members of the nuclear hormone receptor superfamily of transcription factors that coordinate complex genetic programs of metabolism[89,90]. Therapeutic use of synthetic ligands to target these receptors and exploit their biological functions is increasing. The beneficial effects of PPAR-γ in hepatocellular lipid homeostasis have prompted large clinical trials to assess impact on NAFLD and these efforts have been recently reviewed elsewhere[91]. However, the recognition that nuclear hormone receptors link lipid metabolism to alternative activation of macrophages adds a new dimension to their potential use in the treatment of NAFLD[88,92]. While PPAR-γ promotes alternative activation of macrophages that contribute to valuable metabolic changes such as improved insulin sensitivity[93,94], recent research indicates that PPAR-σ is specifically required for a similar program in KC[95,96]. Thus, signature gene expression of PPARσ-deficient KC is greatly reduced in the livers of obese mice and in response to IL-4 stimulation[95,96]. Moreover, PPARσ ablation results in severe steatosis and insulin resistance[95,96]. Notably, the effect of PPARσ in KC is modulated by fatty acids[95] and may fail due to altered lipid homeostasis and hepatic microenvironment in NAFLD. Thus, hepatocytes as a previously unsuspected source of Th2 cytokines stimulate M2 gene expression in KC and this important regulatory circuit may be altered in steatosis[96]. These findings raise the intriguing possibility that specific targeting of PPAR-σ in KC to induce alternative activation may improve both inflammation and steatosis in NAFLD. One important caveat is that the M2 phenotype includes stimulation of the extracellular matrix that may contribute to hepatic fibrosis[97]. Figure 3 shows the effects of activation of Kupffer cells in NAFLD.

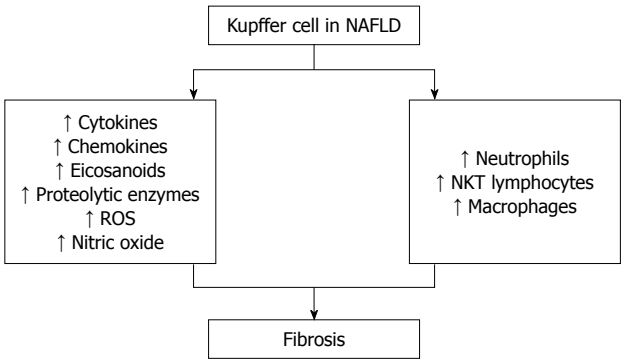

Figure 3 Effects of the activation of Kupffer cells in non-alcoholic fatty liver disease. NAFLD: Non-alcoholic fatty liver disease; NKT: Natural Killer T cells; ROS: Reactive oxygen species.

In the last few years, there is increasing evidence that ligands of Hedgehog (Hh) may have a critical role in processes leading to liver fibrosis. The Hh mediated activity is quite low in healthy liver but increases during the course of several liver diseases, as recently reviewed[98]. In particular, it has recently been shown that damaged/dying hepatocytes may produce Hh ligands that mediate proliferation of myofibroblasts in the liver, thus promoting fibrosis[99]. Moreover, Hh seems to be critical due to its properties in regulating NKT growth and functions in liver fibrosis[100,101].

IMMATURE MYELOID CELLS AND STEATOSIS

Immature myeloid cells ($CD11b^{+}Gr\text{-}1^{+}$) play a role in the

induction of inflammatory cytokines[102] through activation of innate immune pathways. The role that immature myeloid cell populations play in obesity-related liver disease is unknown. In a recent study, Deng *et al*[103] hypothesize that accumulation of immature myeloid cells in the liver may be an important component in the development of inflammatory responses in liver tissue that are triggered by obesity, which in turn contributes to metabolic consequences, such as steatohepatitis. In this study, the liver of obese mice was demonstrated as the major organ where $CD11b^{+}Ly6C^{+}$-$Ly6G^{-}$ immature myeloid cells accumulate. It is not clear why these cells are preferentially recruited into the liver. Chemotactic cytokines and chemokines could direct the migration of immune cells including myeloid cells and may be responsible for the cell accumulation. Several hepatic cell populations, including hepatocytes, KC, sinusoidal endothelial cells, and hepatic stellate cells, can secrete chemokines upon activation. High-fat diet-derived products could activate one of these cells in the liver, resulting in the recruitment of these circulating activated immature myeloid cells into the liver. IL-6 is overexpressed in the NAFLD patient[104], and IL-6 has been shown to block immature myeloid cell differentiation[105]. As a result, these activated cells are accumulated in the liver. The specific role of chemokines or other factors in the recruitment of these cells to the liver warrants further investigation.

CONCLUSION

It is now recognized that adipose tissue is an active endocrine organ that secretes numerous molecules that play a central role in the regulation of energy and vascular as well as immune system homeostasis by acting both locally and at distant sites influencing various metabolic and immune processes. Many of these interactions between metabolic and immune systems seem to be orchestrated by this complex network of soluble mediators derived from immune cells and adipocytes that are briefly summarized in Figure 4.

NAFLD is becoming an increasingly relevant clinical issue, especially in the developed world. One of the unmet challenges of NAFLD is to satisfactorily predict its progression from simple steatosis into steatohepatitis. This transition represents a milestone in the natural history with a considerable probability for developing end-stage liver disease. Elucidation of molecular and cellular events that may lead to this outcome is therefore critically important. Fortunately, the past few years have brought remarkable advances in our understanding of NAFLD pathogenesis, often by extension of research in adipose tissue biology, obesity, and insulin resistance. These efforts point to the intricate relationship of the innate immune system and lipid homeostasis in NAFLD with a prominent role for Kupffer, myeloid and NKT cells and a number of biochemical and cellular mechanisms involved.

However, a number of questions regarding the role of macrophage infiltration in human obesity remain to be answered. For example, what is the cause/s of macrophage infiltration? Does moderate fat gain alter macrophage number and/or macrophage phenotype in humans? Are some individuals predisposed to this? Is macrophage infiltration causal in the development of insulin resistance? The activation of NKT cells exacerbates macrophage infiltration in adipose tissue and glucose intolerance with obesity. Therefore, NKT cells enhance chronic inflammation in visceral adipose tissue and contribute to the development of metabolic disorders in obesity. The NKT cells may be the novel therapeutic targets in atherosclerosis, metabolic syndrome, and type 2 diabetes.

Further studies are needed to fully understand the interaction between fat, the immune system and steatosis.

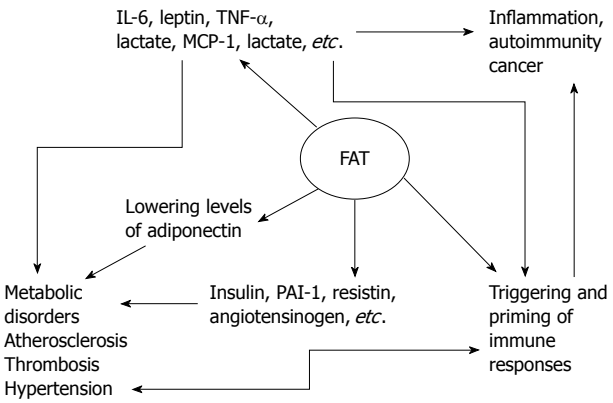

Figure 4 Complex network of soluble mediators derived from immune cells and adipocytes. MCP: Monocyte chemotactic protein; TNF-α: Tumor necrosis factor α; IL: Interleukin.

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TOPIC HIGHLIGHT

Giovanni Tarantino, MD, Professor, *Series Editor*

Hepatic steatosis, low-grade chronic inflammation and hormone/growth factor/adipokine imbalance

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Abstract

Non-alcoholic fatty liver disease (NAFLD), a further expression of metabolic syndrome, strictly linked to obesity and diabetes mellitus, is characterized by insulin resistance (IR), elevated serum levels of free fatty acids and fatty infiltration of the liver, which is known as hepatic steatosis. Hepatocyte apoptosis is a key feature of this disease and correlates with its severity. Free-fatty-acid-induced toxicity represents one of mechanisms for the pathogenesis of NAFLD and hormones, growth factors and adipokines influence also play a key role. This review highlights the various pathways that contribute to the development of hepatic steatosis. Circulating concentrations of inflammatory cytokines are reckoned to be the most important factor in causing and maintaining IR. Low-grade chronic inflammation is fundamental in the progression of NAFLD toward higher risk cirrhotic states.

Key words: Hepatic steatosis; Low-grade chronic inflammation; Adipokines; Hormones; Growth factors

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INTRODUCTION

There are many genetic, evolutionary, environmental, behavioral and physiological factors that induce and exacerbate obesity. Teleologically, our early ancestors had a survival advantage if they were able to store energy to be used in famine or during very stressful situations. Those individuals with 'thrifty genes' survived and reproduced more metabolically efficient offspring. In modern society, thrifty genes are coupled with increased food availability and sedentary lifestyle. This often results in a net positive energy balance. The current view is that obesity leads to hyperinsulinemia/insulin resistance (IR) and IR exacerbates obesity, which frustrates most attempts at weight loss. Adipocytes, while utilizing glucose and fat excess during nutritional affluence and storing them as triglycerides, release energy as free fatty acids (FFAs) and glycerol by lipolysis. These FFAs do not produce significant metabolic disturbances as long as they are oxidized in target tissues by the leptins of adipocytes. Long-chain fatty acids (LCFAs) in non-adipocytes, which cannot undergo mitochondrial β-oxidation, cause signal transduction defects[1] or result in apoptosis *via* accumulation as cytosolic triglycerides. The consequences of increased lipid delivery to peripheral tissues are multiple and the integrated response

to central adiposity is complex and involves several organs and tissues.

Concerning the cardiovascular risk, some observations suggest that different lipid accumulation processes begin in the subendothelial or deep intimal regions, which contributes to complicated atheroma core formation in peripheral arteries. Furthermore, it has been demonstrated that the presence of deep intimal lipid accumulation is associated with reduced endothelium-dependent relaxation in large arteries. The functional and morphological abnormalities might contribute to human coronary atherogenesis that progresses slowly with age[2]. Besides, adipocytes can also prevent atherosclerotic vascular damage by its product adiponectin[3].

LIPOTOXICITY: THE CLASSICAL VIEW

Acute elevations in FFAs can provoke peripheral IR in humans[4]. In addition, acute lowering of FFAs with the antilipolytic drugs can enhance peripheral insulin-mediated glucose uptake[5]. A defect at the level of reduced glucose transport *per se* results from an effect of FFAs to inhibit proximal insulin signaling steps, including tyrosyl phosphorylation of insulin receptor substrates[6]. Obesity results in the accumulation of muscle (intramyocellular) triglycerides and activated lipids in the form of long-chain fatty acyl-CoA esters[7]. This accumulation is also implicated in the impairment of insulin signaling, possibly *via* activation of selected protein kinase C isoforms[8]. Similar effects occur in the liver in association with hepatic lipid accumulation, a ubiquitous finding in obese patients. Lipids can also accumulate in pancreatic islets, which impairs insulin secretion, such as in the development of diabetes in ZDF rats[7]. In addition, decreased catabolism contributes to tissue lipid accumulation, because hepatic and intramyocellular lipid content is associated with reduced mitochondrial oxidative and phosphorylation activity in muscle of elderly humans[9]. Obesity is clearly associated with increased levels of circulating FFAs. Patients with various grades of obesity and IR are generally resistant to the antilipolytic effects of insulin[10]. Furthermore, adipocyte constituents of visceral fat are more metabolically active and have a higher rate of lipolysis[11]. Increases in FFAs can provoke peripheral IR in animals and humans[4]. In addition to the role of FFAs in producing IR in muscle, the impaired ability of insulin to suppress FFA release boosts hepatic glucose production, because insulin-mediated anti-lipolysis contributes to insulin regulation of hepatic glucose output[12]. The glucose-fatty acid cycle that has the potential to increase fatty acid utilization to inhibit glucose oxidation in muscle was first proposed by Randle *et al*[13] in 1963.

LOW-GRADE CHRONIC INFLAMMATION: THE MAIN ROLE OF INTERLEUKIN 6

Growing evidence links a low-grade, chronic inflammatory state to obesity and its coexisting conditions such as IR, type 2 diabetes, metabolic syndrome and non-alcoholic fatty liver disease (NAFLD)[14,15] that includes a large spectrum that ranges from fatty liver (FL), non-alcoholic steatohepatitis (NASH) and cryptogenetic cirrhosis. The spleen also plays a key role in this chronic process[16]. Anti-inflammatory drugs can reverse IR[17], which suggests that inflammation is directly involved in its pathogenesis. Inflammatory mediators that are biosynthesized in the liver and increased in NAFLD patients include C-reactive protein (CRP)[15], interleukin (IL)-6[16], fibrinogen and plasminogen activator inhibitor-1 (PAI-1)[18]. Fat in the liver represents a site beyond adipose tissue that independently contributes to synthesis of inflammatory mediators. In support of a sequence of cellular and molecular events that mediate hepatic IR in NAFLD, recent data lend credence to the fact that hepatic steatosis activates IκB kinase (IKK)-β and nuclear factor (NF)-κB[19]. Among the inducible transcription factors that control inflammatory gene expression, NF-κB plays a central and evolutionarily conserved role in coordinating the expression of various soluble pro-inflammatory mediators (cytokines and chemokines) and leukocyte adhesion molecules. In non-stimulated cells, NF-κB is sequestered in the cytosol by the inhibitor of NF-κB (IκB) that masks the nuclear localization signal present along the NF-κB protein sequence. Treatment of cells with pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1, or with bacterial products such as lipopolysaccharide, leads to the activation of a specific-IKK complex that phosphorylates IκB and thereby tags it for ubiquitination and degradation by the proteasome[20]. The degradation of IκB thus allows NF-κB to translocate into the nucleus where it can act as a transcription factor that upregulates IL-6 production and secretion. IL-6 works locally through paracrine and/or endocrine mechanisms to activate IL-6 signaling in the liver. IL-6 is known to induce IR in hepatocytes[21]. Hepatic production of IL-6 also provides a further pathogenic link to extrahepatic organs such as muscle. NF-κB target genes are not upregulated in transgenic mouse muscle, but IL-6 target genes are, including suppressor of cytokine signaling (SOCS) and signal transducer and activator of transcription (STAT) proteins. These genes are reversed during IL-6 neutralization, which is consistent with the pathogenic involvement of IL-6. Activation of NF-κB leads to a severe syndrome of muscle wasting, without IR[22].

VISCERAL ADIPOSITY AND ADIPOCYTE DIFFERENTIATION

Although many studies have reported strict correlations between insulin sensitivity and visceral fat deposition[23], associations between the amount of subcutaneous fat on the trunk and IR have also been reported in obese non-diabetic men[24] and those with type 2 diabetes[25]. Thus, subcutaneous fat, not draining into the portal vein, determines IR by a mechanism that is not linked to the liver. At

the same time, IR in obese women is strictly related to increased overall fat mass, or to an elevation in truncal subcutaneous fat mass as measured by skinfold thickness[26] or magnetic resonance imaging[27]. Moreover, IR is predicted independently by an enlargement of truncal subcutaneous fat mass and an increased amount of visceral fat[26]. Total and subcutaneous fat mass is important to the IR syndrome; in fact, adipocytes, while responding sensitively to systemic influences, affect important target tissues with their secretions. They specifically detect the changes in energy equilibrium of the organism and adequately respond by drawing excess glucose and lipids from the bloodstream[28], storing them as triglycerides[29], releasing depot fat to non-adipocytes as FFAs and glycerol[30], and increasing fatty acid oxidation in adipose and non-adipose tissues to maintain fat homeostasis and cellular integrity[31]. The main transcription factor designating the characteristics of adipocytes is most likely the adipocyte determination and differentiation factor 1 (ADD1)/sterol regulatory element binding protein-1c (SREBP-1c)[32]. Its regulatory functions consist in sensing the glucose and fat excess and drawing them into the adipocytes to preserve energy and maintain constant blood levels. Otherwise, fat accumulation in non-adipocytes could be deleterious to their functions[33]. An inverse correlation has been found between cytosolic cholesterol concentration and ADD1/SREBP-1c, meanwhile, plasma insulin and glucose levels have a positive impact on ADD1/SREBP-1c[34].

Insulin crucially controls almost all aspects of adipocyte pathobiology. Almost all anabolic effects of insulin in the adipocytes are regulated by the transcription factor ADD1/SREBP-1c that also controls other mature adipocyte markers *via* transactivation of peroxisome proliferator activated receptor (PPAR)-γ and leptin[35]. PPAR-γ has major influences on various aspects of adipogenesis, such as adipocyte differentiation from preadipocytes and differentiation of fibroblasts into mature adipocytes[36]. ADD1/SREBP-1c and PPAR-γ also regulate the genetic expression of the enzymes for *de novo* lipogenesis and glucose transporter GLUT4[37].

Adipocytes do not have unlimited capacity for expansion by storing fat as triglycerides. When adipocytes reach a critical fat cell size, adipogenesis is triggered that increases the number of fat cells[38]. ADD1/SREBP-1c, and in particular PPAR-γ, efficiently cause the differentiation of pre-adipocytes and even fibroblasts or myoblasts into mature adipocytes[32]. Old adipocytes are protected by diverting the fuel excess to more competent (in terms of lipogenesis) younger adipocytes. When adipogenesis cannot occur, fat cells produce factors that strongly inhibit the anabolic actions of insulin. Two of these are TNF-α[39] and resistin[40]. These adipocyte products might result in the development of metabolic syndrome by creating insensitivity to insulin action, mainly in the fat tissue, and partly in the liver and muscle[33]. Resistin, a novel signaling molecule isolated in mice has been suggested as the putative hormone that links obesity with type 2 diabetes. Research confirms the expression of resistin in human adipose tissue and increased expression in abdominal fat[41]. Glucose firstly determines the fate of nutritional energy, whether it is oxidized or stored as triglycerides[34]. It then inhibits oxidation of LCFAs, which causes accumulation of LCFAs and their metabolites in the cytosol, which results in impaired signal transduction[42], and finally stimulates apoptosis, when unoxidized LCFA metabolites continue to accumulate in the long term (so-called glucolipotoxicity)[43].

ADD1/SREBP-1c, which is mostly dependent on insulin as outlined above, has central importance in fat tissue for the regulation of energy metabolism[30]. The amount and type of fat and associated cholesterol content of food generally inhibit ADD1/SREBP-1c expression *via* an effect on cytosolic cholesterol level[44]. Although activation of this transcription factor changes mainly according to the same intracellular cholesterol concentrations[45], the glucose component of the nutritional excess and insulin are the predominant stimulators for the genetic expression of ADD1/SREBP-1c in fat tissue[34]. It has been proposed that the amount and quality of nutritional carbohydrate, which determines the glycemic index and the responding insulin level, control the activation of ADD1/SREBP-1c. Extracellular glucose level is not only the operating lipogenic machinery of fat cells, but also controls the adipocyte secretions that are effective in non-adipocytes and their energy regulation, which determines fuel partitioning, and oxidation or storage in cells, including fat tissue. Ultimately, chronic over-nutrition, particularly rich in carbohydrates, causes impairment in transcriptional regulatory effects of ADD1/SREBP-1c, which leads to the development of a variety of metabolic disorders. In this way, IR has the potential to provide information on healthy and non-healthy obesity[46].

GROWTH FACTORS AND ADIPOKINES

Differentiation of precursor cells into mature fat cells is stimulated by multiple hormones, including glucocorticoids, growth hormone (GH), insulin-like growth factor (IGF)- I , and insulin.

As reported in a recent published review[47], a relevant role for GH in metabolism has been known since the late 1940s, when its effects on lipid and protein metabolism were demonstrated in experimental animal models[48]. After the binding of GH to specific monomeric or dimeric receptors, members of the cytokine receptor superfamily, the GH intracellular signal that is involved in lipid metabolism results in activation by trans-phosphorylation of adjacent Janus-kinase 2, with subsequent recruitment of the STAT pathway[49]. The GH signal cascade induces ultimately the transcription of specific genes, such as those for IGF- I , IGF-binding proteins (IGFBPs), acid-labile subunit (ALS), or SOCS proteins[47].

IGF- I , the main anabolic mediator of GH effects, is primarily GH-dependent and influences GH secretion through a negative feedback system[50]. IGF- I exhibits a 45% amino acid homology with insulin, acts as an insulin

sensitizer, and is a member of the IGF family, along with IGF-Ⅱ. Consequently, IGF-Ⅰ and insulin, apart from high affinity for their specific receptors, share lower affinity for their cognate insulin and IGF-Ⅰ receptors, respectively. The IGFBPs, present in serum, other biological fluids, and tissue extracts, bind IGF-Ⅰ and IGF-Ⅱ with affinities comparable to those of IGF-Ⅰ receptors. IGF-Ⅰ, in particular, circulates in plasma as a ternary complex along with IGFBP-3 or -5 and ALS, which prolongs the half-life of IGF-Ⅰ and modulates its bioavailability to peripheral tissues[51]. IGF-Ⅰ intracellular signal triggers metabolic mechanisms different from GH, by inducing tyrosine phosphorylation of the insulin receptors substrate proteins, with subsequent activation of phosphatidylinositol-triphosphate kinase and mitogen-activated protein kinase pathways[50,52]. However, a close interplay between the signaling pathways activated by GH, IGF-Ⅰ, and insulin has been demonstrated *in vitro*[47].

Human adipocytes express GH receptors. Adult patients with GH deficiency (GHD) characteristically develop an increase in abdominal obesity, total cholesterol, triglycerides and fibrinogen levels, and a decrease in high-density lipoprotein (HDL)-cholesterol levels, which indicates the metabolic syndrome[53]. GHD is correlated with the severity of alterations in lipid metabolism[54] and GH treatment in GHD patients is associated with improved lipid profiles and cardiovascular risk[55]. GH exerts insulin-like and insulin-antagonistic metabolic effects[56], which include increased gluconeogenesis, enhanced lipolysis, and inhibition of insulin action. In particular, GH displays its lipolytic effect mainly in the visceral adipose tissue, by increasing adipose tissue hormone-sensitive lipase activity *via* enhanced stimulation of the β-adrenergic receptors[49]. This effect results in increased FFA flux from the adipose to peripheral tissues[48]. No definite effects have been reported on lipoprotein lipase (LPL), thus suggesting that GH might not affect triglyceride uptake in adipose tissue. GH might also directly induce adipogenesis *via* activation of STAT-5/PPAR-γ pathway[47], although its role has been demonstrated only during the early phase of the process[47]. Finally, GH inhibits serum leptin and increases circulating resistin levels, while the effect on adiponectin remains controversial[47]. In this context, it is still not yet clear whether GH metabolic actions are exerted directly, or indirectly *via* IGF-1, or are part of GH antagonism of insulin signaling.

The effects of IGF-Ⅰ on lipolysis, gluconeogenesis, and SOCS protein are the opposite of those of GH. In particular, metabolic IGF-Ⅰ effects are similarly to those of insulin, and mainly consist of increased tissue glucose uptake, inhibition of gluconeogenesis, and enhanced adipogenesis[56]. IGF-Ⅰ has been suggested to be a major regulator of cell proliferation, differentiation and metabolism, thus regulating, among other biological processes, adipose tissue growth and differentiation of pre-adipocytes into adipocytes. The role of IGF-Ⅰ in the accumulation of adipose tissue has been investigated using transgenic mice that overexpress the *IGFBP-1* gene. In response to a sucrose-enriched diet, the transgenic mice gain significantly less body weight, and adipocyte size and epididymal fat mass are significantly reduced compared with wild-type mice[57]. Moreover, fewer colonies are generated from adipose tissue of transgenic mice, and the mitogenic response of these cells to IGF-Ⅰ is significantly lessened compared with those from wild-type mice[57]. Finally, the induction of glycerol-3-phosphate dehydrogenase, a measure of adipocyte differentiation, is reduced in pre-adipocytes from transgenic mice by IGF-Ⅰ, but not insulin. In line with the lipogenic properties of IGF-Ⅰ, long-term IGF-Ⅰ treatment of patients with GH insensitivity syndrome results in increased adipose tissue[58]. Although it has been shown that *in vitro* GH treatment of 3T3-L1 pre-adipocyte cultures is associated with a concomitant increase in IGF-Ⅰ expression[57], the effects of GH on lipolysis are not mediated by IGF-Ⅰ, because there are no functional IGF-Ⅰ receptors in adipocytes[59]. Nevertheless, a direct and independent effect of GH-induced IGFBP-3 on adipocytes has also been reported[60]. These data indicate that IGF-Ⅰ has a crucial role in the proliferation of adipocyte precursors, the differentiation of pre-adipocytes, and the development of obesity in response to caloric excess[61]. However, in healthy individuals, IGF-Ⅰ levels are inversely related to the percentage of body fat[62], and epidemiological studies have demonstrated the relationship, in subjects without pituitary or cardiovascular diseases, between low IGF-Ⅰ and hypertension and type 2 diabetes[63], and cardiovascular risk[64].

Hepatic GH signaling is also essential to regulate intrahepatic lipid metabolism. In contrast with its effects on adipose tissue, GH induces triglyceride uptake in the liver by increasing LPL and hepatic lipase expression in a STAT-5 independent manner; however, the net effects of GH in intrahepatic lipid metabolism might be affected by GH antagonism of insulin signaling in the liver, or by GH-mediated secretion of IGF-Ⅰ[47]. Intrahepatic lipid accumulation and other histological liver markers characterize patients with NAFLD. NAFLD represents a spectrum of disease that ranges from simple steatosis to NASH, NAFLD-associated cirrhosis and end-stage liver disease. Ninety percent of circulating IGF-Ⅰ originates in the liver, and hepatocytes are the largest source of IGFBP-1 and IGFBP-3. Thus, both NASH and liver cirrhosis result in a progressive decline of hepatic IGF-Ⅰ output[65]. In this context, a possible link between hepatic steatosis, GH/IGF-Ⅰ axis, and inflammatory cytokines, probably *via* SOCS signaling, might be suggested as one of the mechanisms involved in the development and/or progression of metabolic syndrome and its cardiovascular and hepatic consequences[66-68].

Transforming growth factor (TGF)-β1, in addition to playing a certain role as a pro-fibrogenetic cytokine mainly in NAFLD[69], is an anti-proliferative and pro-apoptotic factor for mammary epithelial cells, in which it acts in an auto/paracrine manner and is thus considered an important local regulator of mammary tissue involution. A recent study has supported additional evidence that stimu-

lation of IGF- I is associated with complete abrogation of TGF-β1-induced activation of pro-apoptotic Bad and Bax and in the consequent protection against apoptosis. In conclusion, apoptotic effects of TGF-β1 are mediated by IGFBPs and occurs through IGF- I sequestration, which results in inhibition of the protein kinase B/Akt-dependent survival pathway[70].

Leptin, which has autocrine, paracrine and endocrine effects, is one of the most important substances secreted by fat cells[71]. Leptin controls peripheral fatty acid oxidation *via* PPAR-α stimulation, and plays a key metabolic regulatory role in fat tissue more than in muscle, liver and pancreatic β cells. Leptin levels correlate directly with the severity of hepatic steatosis but not with inflammation or fibrosis in NAFLD patients[72].

Adipocytes that increase lipogenic activity by sensing the fuel excess also secrete leptin to prevent cytosolic fat accumulation that would compromise functions of non-adipocytes[31]. Leptin limits the lipid accumulation by its autocrine effect in adipocytes, thus maintaining cellular fat balance[30]. Its fatty acid oxidative effects are also enhanced by upregulation of mRNA of uncoupling protein-2 (UCP-2) in adipocytes and in some non-adipocytes[73], such as muscle and β cells[74]. UCP leads to energy loss by converting the energy from the Kreb's cycle to thermogenic heat dissipation in the mitochondrial electron transport chain[75]. Recent studies have indicated that leptin is an independent risk factor for coronary artery disease[73]. Although leptin is highly correlated to the overall adipose tissue, leptin is associated with IR independently of fat mass, which suggests that hyperleptinemia is an independent component of the metabolic syndrome[76,77].

Although the exact interactions between insulin and leptin are still confusing, a putative leptin resistance, like IR, has been postulated in obesity[78], and a few data also suggest peripheral leptin resistance[79]. A soluble form of the soluble leptin receptor (sOb-R) has been demonstrated. sOb-R represents the main leptin-binding compound in plasma, which results in a fraction of bound and free leptin in plasma[80]. The exact function of the sOb-R is not clear. In obesity, levels of the sOb-R are decreased compared with lean controls, which resulted in an increased fraction of free leptin[81]. A reduction in body weight through diet or bariatric surgery significantly increases the concentration of circulating sOb-R, and therefore, increases the fraction of bound leptin[82].

Thus, sOb-R might act as a modulating factor of leptin action and plays an important role in leptin resistance. The high concentrations of free leptin are indicative of leptin resistance[83]. Recent findings suggest that the insulin-degrading capacity of morbidly obese patients is linked to venous leptin levels. Insulin controls leptin synthesis but leptin can, in turn, influence insulin cleavage and thus insulinemia. If insulin cleavage is to be interpreted as a way to decrease hyperinsulinemia, leptin could be a signal that limits the extent of insulin physiological actions. The fact that leptin is produced in the same insulin-degrading tissue (i.e. visceral adipose fat pad) supports this association[84]. IR and clustering of components of the metabolic syndrome decrease the concentration of the sOb-R and increase leptin levels in obese or overweight middle-aged men, which results in a decreased fraction of bound leptin, which further emphasizes the close relationship between the insulin and leptin axes. It is likely that low levels of sOb-R and a high concentration of free leptin are independent components of the metabolic syndrome.

Adiponectin is a novel adipose-specific molecule that possesses possible anti-atherogenic and anti-inflammatory properties. The plasma levels of adiponectin are lower in obese subjects and in patients with type 2 diabetes, which contributes to the development of atherosclerotic complications[85]. It has been demonstrated that secretion of adiponectin from adipocytes is stimulated by insulin[86]. Furthermore, ADD1/SREBP-1c has been recognized as being responsible for the control of adiponectin at the transcriptional level[87].

In NAFLD patients, adiponectin and adiponectin receptor Ⅱ (AdipoRⅡ) staining is less evident in biopsies from those suffering from NASH than FL. Hepatic expression of adiponectin and AdipoRⅡ is reduced in NAFLD[88]. Patients with NASH have significantly lower levels of serum adiponectin than do controls. Although no significant correlation exists between serum adiponectin and anthropometric data, it is independently associated with age, HDL, and triglycerides. Type of meal has no effect on serum adiponectin either in patients with NASH or in controls. There is no expression of adiponectin mRNA in liver samples. However, AdipoRⅡ mRNA expression is higher in NASH than in FL and normal liver tissue[89]. Obesity, particularly visceral adiposity, might also contribute to IR by altering the levels of key adipocyte-derived circulating proteins, referred to as adipokines, including adiponectin and resistin. Adiponectin is produced by adipocytes, and its levels are generally lower in patients with IR and the metabolic syndrome[90]. A role in the regulation of insulin sensitivity and glucose homeostasis was demonstrated in studies that have shown that recombinant adiponectin lowers glucose in diabetic rodents and enhances insulin action in hepatocytes[91]. Exogenous adiponectin, or transgenic adiponectin overexpression, also can reduce lipid accumulation in muscle and liver and enhance insulin sensitivity in mice. Importantly, adiponectin-deficient mice display delayed FFA clearance and are sensitive to diet-induced IR[72]. The mechanisms that mediate the beneficial effects of this protein might involve the activation of AMP-activated protein kinase in muscle or liver; adiponectin signaling *via* this pathway might involve two recently identified, distinct receptors[91].

Administration of exogenous resistin to rodents increases plasma glucose and hepatic glucose production, whereas resistin-null mice have reduced fasting glucose levels[92]. Resistin is reckoned as an adipocyte-specific secretory factor that can cause IR and decrease adipocyte differentiation. Conversely, based on various studies, IGFs can improve IR and stimulate adipocyte adipogenesis. Whether IGFs exert their effects by controlling resistin

production or modulating resistin action is not known. These data demonstrate that IGF- I downregulates resistin gene expression *via* IGF-1R-dependent and MEK1-, p38 MAPK-, and phosphoinositide 3-kinase-independent pathways, and probably modifies the distribution of resistin protein between the intracellular and extracellular compartments *via* a p38 MAPK-dependent pathway. Decreases in resistin production and secretion induced by IGF- I might be related to the mechanism by which IGF- I modulates body weight and diabetes in animals[93].

Adipose tissue was once thought of as a reservoir for surplus energy, but more recently, it has been recognized as an active endocrine organ that contributes to metabolic homeostasis by secreting several adipokines such as leptin, adiponectin, TNF-α, IL-6, PAI-1 and resistin. Initially, resistin was reported as an adipose-tissue-specific protein[40] but ensuing studies *in vitro* and *in vivo* have shown conflicting data regarding the expression of resistin in relation to IR or obesity[94]. Moreover, a longitudinal analysis has shown that serum resistin is higher in obese than in lean subjects, and that changes in serum resistin are positively correlated with changes in body mass index (BMI), fat mass, plasma glucose and insulin levels after a weight reduction program entailing dieting and exercise[95]. In normal control rats, *in vivo* insulin infusion and *ex vivo* administration of TNF-α to cultured fat pads increases resistin gene expression significantly. These results imply that hyperinsulinemia and increased TNF-α levels might upregulate the adipose resistin gene in bile-duct-ligation-induced liver cirrhosis[96].

CARDIOVASCULAR RISK

Obesity is considered to be a major contributor to overall and cardiovascular morbidity and mortality[97]. Epidemiological studies have demonstrated that the incidence and prevalence of obesity are increasing. Metabolic syndrome, which comprises IR, visceral obesity, hypertension, dyslipidemia, and microalbuminuria, is considered a major risk factor for atherosclerosis in obesity[98,99]. Fat accumulation in the visceral depot and liver are strongly correlated, and both are highly correlated with the development and severity of IR[100].

Given that CRP level is a strong predictor of cardiovascular events in men[101], the mechanisms that underlie elevated CRP levels among unhealthy subjects are important. CRP is the main acute phase protein and is a marker of systemic inflammation. Adipose tissue secretes pro-inflammatory cytokines, such as IL-6 and TNF-α. The synthesis of CRP, mostly under the control of IL-6[102] and TNF-α, can stimulate the production of CRP[103]. About 30% of total circulating levels of IL-6 originate from adipose tissue in healthy Caucasian subjects[104]. Adipose tissue is an important factor in the increased CRP levels, *via* IL-6.

VISCERAL ADIPOSITY

Given that omental adipose tissue is a pure depot of visceral adipose tissue, it is of interest to investigate the regulation of lipid metabolism in human omental tissue *in vivo*. It has been proposed that IR of the liver derives from a relative increase in the delivery of FFA from the omental fat depot to the liver (*via* the portal vein). Increased delivery results from: (1) more stored lipid in the omental depot; (2) severe IR of the central fat depot; and (3) possible regulation of visceral lipolysis by the central nervous system. The significance of portal FFA delivery results from the importance of FFAs in the control of liver glucose production. Insulin regulates liver glucose output primarily *via* control of adipocyte lipolysis. Thus, because FFAs regulate the liver, it is expected that visceral adiposity will enhance delivery of FFAs to the liver and make the liver relatively insulin resistant. It is of interest how the intact organism compensates for IR secondary to visceral fat deposition. Although part of the compensation is enhanced B-cell sensitivity to glucose, an equally important component is reduced liver insulin clearance, which allows for a greater fraction of β-cell insulin secretion to bypass liver degradation, to enter the systemic circulation, and to result in hyperinsulinemic compensation. The signals that result in β-cell upregulation and reduced liver insulin clearance with visceral adiposity are unknown, but it appears that the glucagon-like peptide hormone plays an important role[105].

In patients who have undergone abdominal surgery, two specific adipokine concentrations have been measured in venous blood from the omentum to obtain information on some processes of synthesis in the presence of abdominal obesity. Although vascular endothelial growth factor (VEGF) and IL-6 concentrations are increased in the systemic circulation, the contribution of visceral adipose tissue to circulating levels of VEGF and IL-6 is modest[106]. In contrast, in a recent study on rat tissues, the omentum has been found to have the greatest VEGF concentrations of those examined and the highest VEGF secretion rate. Fractionation studies of the omentum furthermore have demonstrated that omental adipocytes, rather than the stromal-vascular cells, are the primary source of VEGF. An endothelial cell mitogenic assay has showed that a major portion of the mitogenic activity of heparin-binding proteins and conditioned media derived from omentum is abolished by VEGF antibody. Additional studies with the transcription inhibitor actinomycin D have demonstrated that the VEGF gene is continuously transcribed in the rat omental adipocytes. Incubation of the omental adipocytes under hypoxic conditions has induced approximately a 1.7-fold increase in VEGF protein expression, which is abolished by actinomycin D[107]. However, what is the importance of VEGF? Liver regeneration is dependent upon coordinated proliferation of hepatocytes and endothelial cells. VEGF promotes angiogenesis. Hepatic steatosis increases liver resection morbidity and delays regeneration. As a counter-reacting mechanism, serum VEGF concentration increases in more severe forms of NAFLD. Some researchers have hypothesized that VEGF overexpression stimulates hepatic regeneration[108].

Another debate has arisen over the so-called "portal hypothesis" that implicates increased lipolytic activity in visceral fat, and therefore, increased delivery of FFA to the liver, which ultimately leads to hepatic IR. The mechanism by which increased central adiposity causes hepatic IR has been clarified by research at the transcriptional level, by studying the expression of several genes that are involved in glucose and lipid metabolism in the fat-fed canine model. Northern blot analysis has revealed an increase in the ratio of visceral to subcutaneous mRNA expression of LPL and PPAR-γ. In addition, the ratio for SREBP-1 tends to be higher in fat-fed dogs, which suggests enhanced lipid accumulation in the visceral fat depot. The visceral to subcutaneous ratio of HSL increases significantly, which implies a higher rate of lipolysis in visceral adipose tissue despite hyperinsulinemia in obese dogs. Liver SREBP-1 expression is increased significantly, with a tendency for increased fatty-acid-binding protein expression. In addition, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase increases significantly, consistent with enhanced gluconeogenesis[109].

MITHOCONDRIAL INVOLVEMENT

ATP is crucial for maintaining cellular integrity, therefore, abnormal production might predispose to hepatocellular injury, and mitochondrial dysfunction could be the key mechanism. Estimates of energy metabolism are mainly based on basal metabolic rate (BMR). BMR involves measurement of subjects at rest, under thermo-neutral temperatures (i.e. no thermogenic stress), in a post-absorptive (not digesting food) and inactive state. The underlying machinery that fuels BMR is identical to that which fuels all the other sources of energy utilization, namely, oxidative phosphorylation. ATP is generated in mitochondria, and is subsequently hydrolyzed to ADP and phosphate to release energy for useful work. This process of electron transport during oxidative phosphorylation is the primary source of oxygen radical species. Total energy expenditure (TEE) is commonly predicted on the basis of patient weight, activity level, and degree of metabolic stress (metabolic demands). BMR accounts for about 70% of TEE; the remainder is provided by energy dissipated by metabolism of food (10% of TEE), and energy expended during physical activity (20% of TEE). Conditions that increase metabolic stress, such as infection, critical illness, or trauma, having inflammation in common, can increase BMR. BMR in obese patients is generally augmented, in contrast to common belief, and it is a strong body response to overfeeding, probably cytokine-mediated. BMR is generally measured by indirect calorimetry using a canopy system and single-frequency bio-impedance analysis. Increased energy expenditure, observed in morbidly obese patients with NAFLD as a consequence of a systemic, low-grade, inflammatory process, might explain progression from obesity to metabolic syndrome, independent of the presence of NAFLD. In this context, increased BMR might be indicative of metabolic syndrome, strictly linked to IL-6 levels[110]. Indeed, energy expenditure in obese patients is increased not only because the increased fat-free mass results in a rise in BMR, but also because of the higher energy cost of weight-bearing activities. NAFLD, which is characterized by mitochondrial dysfunction, can predispose to drug-induced hepatotoxicity that probably shares the same pathophysiological mechanism[111].

An up-to-date study has documented that hepatic mitochondrial dysfunction precedes the development of NAFLD and IR in Otsuka Long-Evans Tokushima fatty rats. This evidence suggests that progressive mitochondrial dysfunction contributes to the natural history of obesity-associated NAFLD[112].

INTRINSIC FACTORS LEADING TO HEPATIC STEATOSIS

Although we have previously focused on adipocyte biology and development of obesity, with an emphasis on IR, steatosis of the liver could be independently influenced by some aforementioned transcription factors. It is now clear that several members of the nuclear receptor superfamily are co-expressed by macrophages, lymphocytes and other cell types that are involved in the regulation of inflammatory and immune responses. Beyond PPAR-γ and SREBP-1c, nuclear liver X receptors[113] are members of this family that are known to be activated by lipid-derived endogenous (such as fatty acids, eicosanoids and cholesterol) and pharmacological ligands. Such transcription factors, as well as PPAR-γ co-activator 1α (PGC-1α)[114], farnesoid X receptor[115] and AMP-activated protein kinase[116], a key regulator of fatty acid oxidation in the liver, represent fundamental issues in the development of NAFLD and hepatic IR. In addition to peripheral IR and pancreatic β-cell dysfunction, it should be emphasized that type 2 diabetes mellitus is also characterized by aberrant hepatic gluconeogenesis. cAMP response element-binding protein (CREB), a key regulator of hepatic gluconeogenesis, mediates its actions through transcriptional induction of the nuclear hormone receptor PGC-1α. Recently, CREB-induced activation of the NR4A orphan nuclear receptor family, including the three highly homologous isotypes, NR4A1, NR4A2, and NR4A3, has been identified as a novel PGC-1α-independent mechanism for regulating hepatic gluconeogenesis.

CONCLUSION

It remains to be established whether IR is also a phenotypic expression and to what extend it has a genetic determinant. Although it is generally thought that organ fat deposition begins when visceral and subcutaneous abdominal adipose tissue stores are full, a recent study has not been able to confirm this. Given that IR is not related to fat deposition, it has been hypothesized that the chain of events does not presuppose that obesity is the cause of IR. This is supported by the clear association between inflammatory status (CRP level and spleen volume) and the hepatic score

at ultrasound[15]. Could the high fat liver content be the breaking point between benign and progressive obesity? This is the first intriguing question that could be answered by successive follow-up of the obese population. A possible confirmation of these findings is found in a study that suggests that the contribution of visceral fat to inflammation might not be completely accounted for by clinical measures of obesity (BMI and waist circumference)[117]. A second point to stress is whether weight control can slow down the progression of IR and the worsening of fat deposition in organs in obese patients and overweight subjects as soon as possible, such as in adolescence. Could a possible anti-inflammatory approach be used to cure metabolic syndrome and NAFLD? In fact, inflammatory mechanisms are fundamental to the progression of NAFLD towards higher-risk cirrhotic states. Finally, does hepatic IR follow peripheral IR[118]? In other words, are hepatocytes the last adipocytes? It is likely that they are[119].

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TOPIC HIGHLIGHT

Giovanni Tarantino, MD, Professor, *Series Editor*

Noninvasive investigations for non alcoholic fatty liver disease and liver fibrosis

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Abstract

Non-alcoholic fatty liver disease (NAFLD) includes a spectrum of diseases that have insulin resistance in common and are associated with metabolic conditions such as obesity, type 2 diabetes mellitus, and dyslipidemia. NAFLD ranges from simple liver steatosis, which follows a benign course, to nonalcoholic steatohepatitis (NASH), a more severe entity, with necroinflammation and fibrosis, which can progress to cryptogenic cirrhosis and end-stage liver disease. Liver biopsy remains the gold standard for evaluating the degree of hepatic necroinflammation and fibrosis; however, several noninvasive investigations, such as serum biomarkers, have been developed to establish the diagnosis and also to evaluate treatment response. These markers are currently neither available in all centers nor validated in extensive studies. Examples include high-sensitivity C reactive protein and plasma pentraxin 3, which are associated with extensive liver fibrosis in NASH. Interleukin-6 correlates with inflammation, and cytokeratin-18 represents a marker of hepatocyte apoptosis (prominent in NASH and absent in simple steatosis). Tissue polypeptide specific antigen seems to have a clinical utility in the follow-up of obese patients with NASH.

Key words: Non-alcoholic fatty liver disease; Biomarkers; Necroinflammation; Liver fibrosis

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) represents a group of conditions ranging from simple liver steatosis, usually asymptomatic, to nonalcoholic steatohepatitis (NASH), which is characterized by the presence of apoptosis/inflammation and fibrosis, and also by a progressive course, evolving to cryptogenic cirrhosis.

Non-alcoholic steatohepatitis (NASH) was first described in 1980 by Ludwig *et al*[1] in patients with abnormal liver tests and fatty infiltration accompanied by inflamma-

tory changes at histological examination. Prior to 1980, hepatic steatosis was documented in patients with associated metabolic conditions, especially in obese patients who underwent liver biopsy before and after bariatric surgery[2].

The prevalence of NAFLD has increased over the last two decades and it affects approximately 30% of adults in the United States[3] and almost a third of the general population[4]. The most common form of NAFLD encountered in clinical practice is liver steatosis, also known as non-alcoholic fatty liver (NAFL), if it occurs in the absence of significant alcohol consumption (more than 10 g/d in females and 20 g/d in men). Data analysis from two recent prospective cohort studies concluded that raised body mass index (BMI) and alcohol consumption are both related to liver disease, with evidence of a supra-additive interaction between the two[5]. The study by Liu *et al*[6] confirmed that excess body weight increases the incidence of liver cirrhosis. In middle aged women in the UK, an estimated 17% of incident or fatal liver cirrhosis is attributable to excess body weight, as compared with an estimated 42% attributable to alcohol[6].

Fatty liver disease has a benign clinical course, as long as inflammatory injury of the liver does not develop. It is essential to differentiate between this form of simple steatosis, which is associated with a favorable long-term prognosis, and NASH, with a different natural history; approximately 20% of patients with NASH will develop cryptogenic cirrhosis and even end-stage liver disease[7]. Cirrhosis associated with NASH might even progress to hepatocellular carcinoma[8] and death related to NASH was reported to be approximately 12%-25% over a 7-10 years period[9]. NAFLD is an important cause of cryptogenic cirrhosis, as Powell *et al*[7] suggested, although other disorders could progress to this type of cirrhosis[10]. Another important issue concerning NAFLD is related to the inner mechanisms of the disease. Even though steatosis is a benign condition and NASH a progressive one, the basic mechanisms of both entities seem to be the same. This is supported by the study of Tarantino *et al*[11] who reported similar levels of transforming growth factor-β1 in serum of patients with simple steatosis and those with NASH.

PATHOGENESIS OF NAFLD

The pathogenesis of NAFLD, and especially of NASH, is not completely understood; however, a few mechanisms were proposed to explain the liver injury associated with metabolic syndrome[12]. Identification of these mechanisms has therapeutic importance, because targeted therapies might prevent the progression of NAFLD to fibrosis and cirrhosis[13].

Insulin resistance plays a central role in NASH pathogenesis. Insulin resistance is the main feature of metabolic syndrome, which embodies obesity, hypertension, diabetes, and dyslipidemia[14]. NAFLD is considered to be the hepatic component of metabolic syndrome[15-17]. Although overweight and obesity are present in the majority of patients with NASH, steatohepatitis can also occur in subjects with normal body weight[18]. A direct correlation between the degree of obesity and NASH development has been observed[19]; however, not all obese patients will have NAFLD. Hepatic steatosis evidenced by ultrasound is clearly more pronounced in cases of insulin resistance compared with healthy subjects[20]. Insulin resistance promotes disturbances in lipid metabolism, with increased delivery of free fatty acids to the liver, impaired mitochondrial β oxidation, "*de novo*" lipogenesis, and decreased β export from the liver[19], all of which result in fatty liver development. Some authors suggest that hyperinsulinemia of NAFLD is the result of the decreased insulin extraction by the liver[21,22]. NASH is also associated with mitochondrial abnormalities, such as swollen or elongated mitochondria with crystalline inclusions[18,23]. Liver overloading with lipids initiates multiple pathways including lipid peroxidation, generation of reactive oxygen species, oxidative stress, and production of inflammatory cytokines. In fact, oxidative stress is a trigger for lipid peroxidation in hepatocytes, with subsequently secretion of proinflammatory cytokines and activation of fibrosis-developing stellate cells, which are the main mediators of liver fibrosis. tumor necrosis factor (TNF)-α is increased in NAFLD patients and it has a central role in liver injury and disease progression from fatty liver to steatohepatitis and hepatic fibrosis, by activating both Kupffer and stellate hepatic cells[12,24]. Taking into account this hypothesis, targeted therapies against TNF-α might be beneficial in NASH treatment[25,26].

Another concept involves adipocytokines, which are secreted by the adipose tissue (WAT) known as white adipose tissue and are related to visceral obesity. WAT is responsible for secretion of a several adipokines and cytokines, such as adiponectin, leptin, TNF-α, and interleukin (IL)-6, which are involved in hepatic inflammatory process[27,28].

A theory concerning iatrogenic NAFLD induced by several medications has emerged. Drugs like diltiazem, amiodarone, tamoxifen, steroids, and antiretrovirals are involved in fatty liver or insulin resistance development[18].

Besides the well-recognized risk factors for NAFLD, such as type 2 diabetes, insulin resistance, hyperlipemia, and obesity, other metabolic conditions have been associated with fatty liver disease, namely polycystic ovary syndrome, and lipodystrophy[29,30]. Other rare conditions associated with NAFLD are hypobetalipoproteinemia, Weber-Christian syndrome, total parenteral nutrition, toxic exposure at organic solvents, dimethylformamide, gastric by-pass, and jejunoileal bypass[18].

DIAGNOSTIC PROCEDURES

The goal of diagnostic procedures is to identify the patients with NASH before the onset of advanced fibrosis. Liver biopsy is now considered the "gold standard" for the assessment of liver fibrosis. It is important to take into account that a needle biopsy is merely a sample of the entire liver and that fibrosis presents a diffuse pattern in chronic liver disease. The liver biopsy removes only about 1/50 000th of the liver and carries substantial interpretation errors. Liver biopsy is an invasive procedure with certain unavoidable risks and complications[3].

Therefore, the development of noninvasive tests for assessing hepatic inflammation and fibrosis has become an active area of research.

NAFLD is first of all a diagnosis of exclusion, so other

specific causes of liver diseases should be ruled out: viral hepatitis, alcoholic liver disease, Wilson disease, hemochromatosis, and autoimmune hepatitis[15]. The most challenging of them is exclusion of alcoholic liver disease, because the histological picture of both conditions is similar. It is necessary to obtain an accurate history concerning the patient's daily alcohol intake; knowing that consumption of more than 10 g/d in females and 20 g/d in males are responsible for liver injury in the absence of other risk factors, such as obesity, diabetes, and viral hepatitis[30].

The clinical presentation of patients with either NAFLD or NASH is proteiform. The majority of subjects are asymptomatic, but some of them can present with fatigue or right upper quadrant discomfort. Hepatomegaly is discovered in 50% of patients at physical examination[31]. The presence of fatigue does not correlate with the severity of liver injury[31]. These patients share a common clinical feature, obesity, and potentially other features of metabolic syndrome: hyperglycemia, dyslipidemia, and hypertension. Liver dysfunction might be discovered incidentally during a routine check-up, or a work-up for other conditions (Table 1).

Table 1 Clinical features of nonalcoholic fatty liver disease

Symptoms	Signs
None	Hepatomegaly (50% of patients)
Fatigue	Obesity
Right upper quadrant discomfort	Hypertension

Laboratory tests

Approximately 80% of patients with NAFLD have liver function tests in normal ranges; only a small proportion exhibits mild elevation of aminotransferases[32]. The ratio between aspartate aminotransferase (AST) and alanine aminotransferase (ALT) is predictive for the severity of the liver disease, with an AST/ALT ratio > 1 suggesting cirrhosis or advanced fibrosis[33,34]. The degree of aminotransferases elevation is not higher than four times of the upper limit of normal and does not correlate with the severity of steatosis or fibrosis[18]. In the majority of cases, ALT/AST ratio is > 1.

Can serum aminotransferases levels distinguish between NASH and NAFLD? Higher AST and ALT levels, and AST/ALT ratio are all significantly associated with NASH. Serum AST presents a stronger association and has a higher likelihood of discriminating NASH from other forms of NAFLD. However, a multivariable model using both AST and ALT, showed that the discriminating score for distinguishing the patients with NASH from those without NASH was only 26%. This result indicates that additional noninvasive methods are needed for an accurate diagnosis. Fracanzani *et al*[35] studied 455 patients with NAFLD, divided in two groups according to their serum ALT levels. They compared clinical and histological features of patients with and without increased serum ALT. NASH was diagnosed in 62% and 74% of patients with normal or increased ALT levels, respectively. There were no significant differences in advanced fibrosis between the two groups, underlying the need for liver biopsy for diagnosis and staging of fibrosis in NASH[35].

Laboratory signs of advanced liver disease, such as hyperbilirubinemia, hypoalbuminemia, and abnormal prothrombin time are seen only in cases associated with cirrhosis. Other biological abnormalities, such as hyperglycemia and hypertriglyceridemia are related to the coexistent metabolic conditions.

Laboratory assessment of dyslipidemia and insulin resistance should also be performed. A simple laboratory test was designed to evaluate the insulin profile. It is known as homeostasis model assessment (HOMA), and is defined as the fasting insulin level (μU/mL) multiplied by the fasting glucose level (mmol/L) and divided by 22.5[20]. Although HOMA is not a perfect measure of insulin resistance, it is an easy way to estimate insulin resistance. A possible link between HOMA and hepatic steatosis has been demonstrated[20].

The major problem remains to determine the consequences of small amounts of alcohol intake in a patient with liver disease. Taking into account the difficulty of distinguishing between alcoholic and nonalcoholic liver disease based on patient's history, many attempts have been made to assess alcohol consumption using serum markers. Over time, several surrogate markers for alcoholism have been determined: high serum concentration of γ-glutamyltransferase, increased mean corpuscular volume, increased AST levels, AST/ALT ratio > 2, and a desialylated transferrin/total transferrin ratio > 1[18,36].

NAFLD is also accompanied by changes in serum iron markers, such levels of ferritin in 20%-50% of patients and increased transferrin saturation in 5%-10% of cases[33]. Hemochromatosis gene testing is recommended if the ferritin level is significantly elevated.

Overall, none of these tests have specificity for the diagnosis of NAFLD, pointing out only a liver dysfunction. The pattern of aminotransferase elevation does not provide an etiological clue for the hepatic disease, nor does it make a distinction between simple fatty liver and NASH[18].

In fact, the differentiation between steatosis and steatohepatitis can be made only by a histological approach[33]. Besides, the amount of lipid accumulated in the liver cannot be assessed using functional liver tests; however, the degree of liver infiltration with fat can be diagnosed using a variety of imaging methods.

Imaging studies

The most common and less invasive imaging technique used for NAFLD diagnosis is ultrasonography. Ultrasonography, the first-line imaging technique, assesses the presence of steatosis, showing a hyperechogenic liver parenchyma, known as "bright liver" and "blurring of the vascular margins". The increased hepatic echogenicity is diffuse and easy to appreciate by comparison with the lower echogenicity of the kidney or spleen.

The hepato-renal contrast is an ultrasound index for quantification the liver steatosis[37,38]. Normal liver exhibits an echostructure similar to that of renal parenchyma. In fatty liver, the increased hepatic echogenicity creates hepato-renal contrast. Webb *et al*[38] assessed the severity of liver steatosis in a study of 93 patients with positive histol-

ogy for chronic liver disease, according to the discrepancy in ultrasonographic liver-kidney densities. They reported that the hepato-renal index could quantify the severity of liver steatosis to a lower limit of 5%.

A simple parameter, noninvasive and easy to perform, is spleen longitudinal diameter. As the study of Tarantino *et al*[17] recently showed, spleen diameter could differentiate between NAFLD and NASH better than both IL-6 and vascular endothelial growth factor, with values greater than 116 mm predicting NASH[17].

Another technique that might be helpful in the diagnosis of steatosis is Doppler ultrasound. NAFLD is associated with hepatic parenchyma perfusion abnormalities. Several parameters have been described that reflect altered hepatic hemodynamics, among them, the most important is the hepatic vein Doppler pattern[3]. Recently, a new parameter was used in NAFLD evaluation: Doppler perfusion index (DPI), a ratio between hepatic arterial blood flow and total liver blood flow. DPI has been used in the detection of overt liver metastatic disease[39]. In a small trial, Dugoni *et al*[40] reported that DPI was highly predictive of fatty liver in patients with NAFLD. Larger studies are required to evaluate the role of DPI in the diagnosis of NAFLD.

The sensitivity of ultrasonography in detecting steatosis varies between 60% and 94%[18], and also varies depending on steatosis degree. Sensitivity is very low when the degree of steatosis is less than 30%[41]. Another difficulty consists of the impossibility of identifying the inflammatory changes of the hepatic parenchyma and to differentiate simple steatosis from steatohepatitis. It is also difficult to differentiate steatosis from liver fibrosis, because both of them have similar appearance on ultrasound[18]. This limitation was overcome by a superior technology, contrast-enhanced ultrasonography. Lim *et al*[42] studied the role of hepatic vein transit times (HVTT) using a microbubble contrast agent as a tracer and reported that HVTT can predict disease severity in patients with hepatitis C. Moreover, Iijima *et al*[43] evaluated the utility of contrast ultrasound with levovist for the diagnosis of NASH. The signal intensity from regions of interest on the contrast images was measured and estimated using time intensity curves. They found a statistically significant decrease of signal intensity in NASH, when compared with NAFLD, due to reduced uptake of levovist mediated by cell injury. Because this method has only been applied in small trials, larger studies are needed to establishing the role of contrast ultrasonography in the diagnosis of NASH in clinical practice.

The sensitivity of ultrasonography decreases in morbid obesity, because the ultrasonographic examination is difficult to perform in such circumstances[3]. Ultrasonography is inexpensive, simple, easily reproducible, and can be used repetitively to assess steatosis changes over the time, in conjunction with ALT fluctuations and BMI variation. The specificity of the method in detecting fatty infiltration of the liver is high, around 90%[18] (Table 2).

Computed tomography and magnetic resonance imaging are other alternatives, but their use is limited because they are expensive and the information they provide is limited. Compared with ultrasound, CT scans and MRI are superior when the fat deposition is focal[18], otherwise, abdominal ultrasound is more sensitive in diagnosing fatty liver disease[18,44,45] (Table 2).

Table 2 Noninvasive diagnosis of non-alcoholic fatty liver disease (adapted from Lewis *et al*[45]) (%)

Imaging	Sensitivity	Specificity	PPV	NPV
Ultrasound	91-100	93-100	62-89	94
Ultrasound (levovist)	100	95-100	N/A	N/A
Ultrasound (elasticity)	91	84	47	97
CT	93	N/A	76	N/A
MRI	N/A	N/A	N/A	N/A
MR (spectroscopy)	N/A	N/A	N/A	N/A
MR (elastography)	85	86	73	94

N/A: Not available; PPV: Positive predictive value; NPV: Negative predictive value; MRI: Magnetic resonance imaging; MR: Magnetic resonance; CT: Computed tomography.

CT scan technology can be also used to evaluate thickened abdominal subcutaneous adipose tissue and to measure the liver fat[46]. Non enhanced CT can identify steatosis using changes in signal intensity. The density of the liver, as visualized by CT, decreases as the severity of steatosis increases. CT can also visualize splenomegaly in the presence of portal hypertension, which is suggestive for advanced fibrosis in patients with NAFLD. CT allows grading of steatosis, by calculating the liver-to-spleen attenuation ratio[47]. Noncontrast CT is preferred for detecting steatosis because the images appear enhanced[48]. Focal fatty lesions can be identified by dual-energy CT scans. The limitations of CT consist of the difficulty to identify intermediate stages of fibrosis and its use in follow-up purposes, owing to the radiation exposure.

Magnetic resonance imaging (MRI) provides an accurate and rapid assessment of hepatic steatosis to a lower limit of 3%[49]. Phase-contrast imaging correlates with the quantitative assessment of fatty infiltration across the entire range of liver diseases. Loss of intensity on T1-weighted images can be useful in identifying focal fat deposition[49].

A new MRI technique, proton magnetic resonance spectroscopy (MRS), measures the fat proton fraction and hepatic triglyceride levels (HTGC). HTGC > 5% is the diagnostic level of hepatic steatosis[50,51]. MRS characterizes metabolic processes involved in cellular regeneration, and thus it can evaluate the disease severity in NASH. An increased ATP/phosphate ratio might be a signal for progression to an advanced stage of fibrosis in NASH. MRS is probably more accurate than previous imaging procedures for the diagnosis of NAFLD but it needs *in vivo* human validation.

Multi-echo magnetic resonance (MR) imaging, acquired at in-phase and out-of-phase echo times, allows simultaneous fat content and T2 quantification. This technique could be used to determine the fat-to-water ratio and the T2 values[52].

None of these imaging techniques is able to distinguish between liver steatosis and steatohepatitis; thus liver biopsy is required for definitive assessment of the hepatic disorder[3].

Unfortunately, the new imaging procedures, magnetic resonance spectroscopy, and contrast enhanced ultrasound cannot, as yet, be used routinely.

Table 3 Serological markers for nonalcoholic steatohepatitis and fibrosis

Serological markers	Advantage	Disadvantage
C reactive protein[53]	Independent risk factor for progression of NAFLD	Lack of specificity for NASH
Plasma pentraxin 3[54]	Can differentiate between NASH and non progressive NAFLD Fibrosis marker in NAFLD	Lack of specificity for NASH
Hyaluronic acid[34]	Identify fibrosis at a cut-off value of 45 ng/mL	Cannot differentiate NASH from simple steatosis
Tissue inhibitor of metalloproteinases[34]	Fibrosis marker	Cannot differentiate NASH from simple steatosis
Cytokeratin 18[3]	Marker of hepatocyte apoptosis Independent predictor of NASH and severity of disease	Limited utility in clinical practice
Polypeptide specific antigen[57]	Marker in differentiating NASH from pure fatty liver	Marker for various cancers
Endothelin 1[59]	Can differentiate NASH from simple steatosis	Lack of specificity for NASH

NAFLD: Non-alcoholic fatty liver disease; NASH: Nonalcoholic steatohepatitis.

BIOMARKERS FOR ASSESSMENT OF STEATOHEPATITIS AND FIBROSIS

Over time, several biological markers have been studied for evaluating the extent of steatosis, the presence of necroinflammation, and the development of fibrosis to avoid performing liver biopsy, an invasive procedure that still represents the gold standard of diagnosis. The most important parameter to be identified through non-invasive methods is inflammation, as it plays a central role in NAFLD progression.

Several biomarkers of inflammation were extensively studied in relation to fatty liver disease. The C reactive protein (CRP) is an acute-phase reactant produced by the liver and has an increased serum concentration in a variety of inflammatory conditions. The assessment of plasma levels of CRP proved to be useful in differentiating between simple steatosis and NASH. Moreover, it seems that high concentrations of high-sensitivity CRP are associated with extensive liver fibrosis in NASH[53].

Plasma pentraxin 3 (PTX3) is a novel marker that seems to be promising in distinguishing between NASH and non-NASH patients, and also in assessing the severity of fibrosis[54]. Plasma pentraxin 3 is an acute-phase reactant and together with CRP is a member of the pentraxin family of proteins[54]. The PTX 3 level is increased in NASH, but also in other diseases, such as vasculites, cardiovascular, and inflammatory conditions[54].

Another biomarker with a significant role in the fatty liver is IL-6. IL-6 is a chemokine that rises in NAFLD, and it is synthesized by hepatocytes and by immune cells, endothelial cells, and adipocytes[12,55]. Plasma levels of IL-6 vary in proportional with the hepatic concentration and indicate inflammatory activity and the degree of fibrosis[55].

TNF-α has long been recognized for its proinflammatory properties, and its role in NASH progression is clearly established, as well as in other inflammatory diseases. TNF-α is highly expressed in NASH and it has been shown that anti-TNF therapy with pentoxifylline is associated with improvement of liver histology and normalization of aminotransferases[56].

Cytokeratin 18 is a relatively new marker that derives from the caspase-3 pathway; however, to date, it has limited utility in clinical practice and is used only for research purposes. Cytokeratin-18 represents a marker of hepatocyte apoptosis, and its value as a potential biomarker for NASH is based on the observation that apoptosis is prominent in NASH and absent in simple steatosis[3].

Tarantino *et al*[57] found that the polypeptide specific antigen, a protein released during apoptosis, is an important marker of fibrosis, and is more accurate than ALT levels. Tissue polypeptide specific antigen seems to have a clinical utility in the follow-up of obese patients with NASH, because a significant decrease in serum concentration of this marker was associated with weight loss[58].

Oxidative stress has been documented to play a part in NASH pathogenesis, and several parameters have been assessed in different studies: glutathione peroxidase activity, superoxide dismutase activity, and vitamin E levels[3]. None of these markers seemed to have a significant value in evaluating the histological picture of NASH[3]. The clinical usefulness of these biomarkers is yet not established, and their accuracy in noninvasive assessment of steatohepatitis is under debate.

Fibrosis assessment is crucial in NASH because it represents an advanced stage of liver injury. Several studies evaluated certain matrix components, such as transforming growth factor β, hyaluronic acid, tissue inhibitors of metalloproteinases, and others[33]; however, none of them have entered routine use. Endothelin-1 is another mediator of fibrosis in NASH, with an established correlation between serum levels and the degree of fibrosis[59].

Serological markers for NASH and fibrosis are shown in Table 3.

DIAGNOSTIC PANELS FOR ASSESSMENT OF STEATOSIS, STEATOHEPATITIS AND FIBROSIS

Noninvasive panels of serological markers have been developed to evaluate the presence of steatosis and hepatic necroinflammation to avoid liver biopsy. Avoiding liver biopsy is desirable because it has certain disadvantages: it is an invasive procedure, is prone to sampling errors, and suffers from inter-observer variability[60]. The NASH-test imagined by BioPredictive was validated for the assessment of

steatohepatitis in patients without significant alcohol consumption and takes into account the following parameters: total bilirubin, GGT, α2-macroglobulin, apolipoprotein A1, haptoglobin and ALT, and is adjusted for age and gender plus[61] weight, height, AST, serum glucose, triglycerides, cholesterol and SteatoTest. The NASH test should be performed only if the SteatoTest is positive. The SteatoTest is a quantitative test that estimates liver steatosis, particularly in cases of associated metabolic syndrome[62]. The NASH test is a variation of the SteatoTest-ActiTest for the differentiation of steatosis from NASH. The Acti Test was designed for staging necroinflammation in viral hepatitis C and B[61]. Performing these biomarker tests should reduce the need for liver biopsy[63].

Serological markers for fibrosis assessment are frequently used in Europe, in contrast with the United States where liver biopsy is preferred. Different tests have been used for evaluating fibrosis, such as the AST/ALT ratio and the APRI test, which assesses platelets and AST levels[64]. At the moment, the most commonly used are the FibroTest (BioPredictive) in Europe, and FibroSpect and FibroSure in the United States[64]. FibroTest was first developed for patients with viral hepatitis C, and was then extended for NAFLD[33]. The advantages over liver biopsy are: entire examination of the liver and lack of risks due to the noninvasive procedure. FibroSpect evaluates liver fibrosis by analyzing the following markers: hyaluronic acid, tissue-inhibited matrix metalloproteinase inhibitor-1, and α-2 macroglobulin[64]. FibroTest takes into account GGT, haptoglobin, bilirubin, apolipoprotein A, and α-2-macroglobulin. The most important deficiency of these types of tests is their inability to distinguish between mild and moderate fibrosis, knowing that early detection of fibrosis is valuable for preventing disease progression[64]. The utility of these tests is limited in cases with advanced fibrosis.

Fibroscan

Fibroscan, or transient elastography, is a noninvasive method that evaluates liver stiffness using pulse-echo ultrasound[33,64]. Transient elastography measures liver stiffness in a painless and reproducible manner. It has several advantages over liver biopsy: it is noninvasive, evaluates a larger part of the liver, and seems to be more sensitive than serological markers[64]. The main weakness of Fibroscan is interference by steatosis with the wave velocity, as liver stiffness due to fibrosis might be counterbalanced by the presence of fatty infiltration[33]. Some authors state a positive correlation between liver stiffness assessed by Fibroscan and the degree of fibrosis in NAFLD[65,66]. When liver elasticity is used for fibrosis measurement in NAFLD, it is important to take into account that fatty liver can make the liver less stiff and therefore the reference ranges might be different. Fibroscan might also be unreliable in obese people because of technical reasons[67].

Acoustic radiation force impulse (ARFI) sonoelastography has recently been proposed as an alternative method to Fibroscan to assess liver elasticity. This alternative technique utilizes acoustic waves to interrogate the mechanical stiffness properties of the liver. One advantage of ARFI imaging is that it is integrated into a conventional ultrasonography (US) system and can thus be performed during standard US examinations of the liver, which are routinely performed in patients with chronic liver disease. Preliminary results indicate that ARFI imaging technology can be applied for the diagnosis of significant liver fibrosis[68,69]. The role of ARFI elastography for the diagnosis of NAFLD has not yet been established.

Another technique used for detecting moderate to severe hepatic fibrosis in obese individuals with NAFLD is magnetic resonance elastography. It has a higher diagnostic accuracy in fibrosis staging that is not related to BMI[70]. Further studies are needed to clearly define the role of liver elastography in patients with fatty liver disease.

Total overnight salivary caffeine assessment test

An interesting idea concerning the assessment of liver function in chronic liver diseases was elaborated by a working group conducted by Tarantino *et al*[71]. Systemic caffeine clearance, evidenced by measuring salivary caffeine concentration can be used as a hepatic function test in compensated cirrhosis. The total overnight salivary caffeine assessment is a reliable test for evaluating liver function and it can also differentiate between cirrhosis type, such as viral and cryptogenic (likely metabolic) cirrhosis.

Dynamic breath tests

Dynamic breath tests can detect specific alterations in different metabolic pathways. Braun *et al*[72] combined two tests to assess the extent of hepatic injury in patients with NAFLD: the ^{13}C-methacetin breath test (MBT) and the ^{13}C-octanoate breath test (OBT), which evaluate cytochrome P450 activity and mitochondrial dysfunction. Both mechanisms increase oxidative stress, which is clearly implicated in NASH pathogenesis. The noninvasive OBT reliably distinguish between fatty liver and NASH, and the MBT can predict the extent of liver fibrosis.

Additional studies are required to establish the role of these tests as an alternative to liver biopsy in the diagnosis and follow-up of hepatic injury in patients with NAFLD.

CONCLUSION

Currently, the standard procedure for evaluating the degree of necroinflammation and fibrosis, and for quantifying hepatic steatosis remains liver biopsy. However, this is an invasive procedure with unavoidable risks and limitations. Moreover, in most cases of NAFLD, the results of liver biopsy are not relevant to the choice of treatment, which remains that of metabolic syndrome. Hence the need for noninvasive strategies to cover the whole spectrum of NAFLD. Noninvasive investigations, such as various biomarkers, fibrosis scoring panels, and imaging techniques, offer considerable promise in their ability to detect steatosis and to stage liver fibrosis. Further testing and validation are needed for these noninvasive procedures to refine their role of clinical practice and supplant the need for liver biopsy in NAFLD.

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REVIEW

Current developments in natural orifices transluminal endoscopic surgery: An evidence-based review

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Abstract

Tremendous advances have been made in recent years addressing the key obstacles to safe performance and introduction of human natural orifice transluminal endoscopic surgery (NOTES). Animal studies have focused on identifying optimal solutions to these obstacles, in particular methods of creating transluminal access, safe closure of the point of access, and development of a multitasking platform with dedicated instruments. Whether the performance data generated from these animal studies can be reproduced in humans has yet to be determined. Reports of human NOTES procedures are emerging, and the possibility of accomplishing human NOTES based on existing technology has been demonstrated. However, dedicated platforms and devices are still lacking to allow for pure NOTES procedures, and whether NOTES can deliver the postulated benefits of earlier recovery and improved cosmesis remains uncertain.

Key words: Natural orifice transluminal endoscopic surgery; Endoscopic surgery; Minimally invasive surgery; Vaginal surgery

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INTRODUCTION

The natural orifice transluminal endoscopic surgery (NOTES) white paper released in 2005 stated that a number of key issues had to be overcome before NOTES could be fully implemented in human subjects[1] (Table 1). Since then, there has been an exponential growth in the number of NOTES-related publications in the literature, from less than 10 articles in 2006 to over 180 in 2009 (through a PubMed search). NOTES has been demonstrated to be a feasible approach for the performance a wide variety of procedures in animal studies, and reports of human studies are emerging[2-16]. This paper aims to provide an evidence-based review of the current developments in NOTES, with particular emphasis on recent advances in tackling the key obstacles, and to provide an update on the latest development in human NOTES procedures.

METHODS

Search strategy

Studies were identified by performing electronic searches of MEDLINE, EMBASE, Current Contents, the Cochrane Library, and Entrez PubMed from January 2000 to January 2010. The search terms "natural orifice" or "transluminal or translumenal" and "endoscopy or endoscopic surgery or surgery" were used. Additional articles were identified by a manual search of the references in key articles. Amongst the identified studies, articles in English describing randomized controlled trials (RCTs) were considered first. In areas with limited or no RCTs, nonrandomized comparative studies and case series were also included. Only fully peer-reviewed articles were selected.

Inclusion and exclusion criteria

Only studies reporting the outcomes of NOTES-related procedures or devices were selected. Case reports were excluded except in human studies, where only a limited selection of studies was available.

ANIMAL STUDIES

Methods of access to the thoracic or peritoneal cavity

The thoracic and peritoneal cavity can be accessed by the transluminal approach and the method of transluminal access represents the first barrier to NOTES. In mediastinal/thoracic NOTES, the only site of access is through the thoracic esophagus. While for the abdominal cavity, NOTES accesses can be made *via* the transgastric, transcolonic, transvaginal, or transvesical approach.

Note that the thoracic esophagus is surrounded by a number of critical structures, including the descending thoracic aorta, the azygous vein, the pulmonary veins, and the heart. Locating a point of safe access is of paramount importance to avoid catastrophic vascular complications. Endoscopic ultrasound (EUS) has been shown to be a valuable tool for locating sites of safe accesses[17-19]. In the mediastinum, EUS can help identify landmarks such as the aortic arch, which facilitates optimal entrance sites for forward-viewing exploration and intervention[17].

With the site of entry located, one then needs to consider the method of creating a transluminal incision. In early transgastric NOTES procedures, published by Kalloo *et al*[20] and other authors between 2004 and 2005, the majority of transgastric gastrotomies were created by a needle-knife, followed by progressive enlargement of the incision using a pull-type sphincterotome or dilating balloon[20-24]. Both methods are effective ways of creating a transgastric gastrotomy. Nevertheless, using the sphincterotome is quicker than balloon dilation, and it also prevents spontaneous closure of the gastrotomy[22]. Thus, the sphincterotome method is more advantageous if repeated gastric crossing is required. In an attempt to further improve the ease of creating direct transgastric accesses, a prototype one-step needle sphincterotome has been developed by the authors' unit (Figure 1)[25]. The instrument consists of a retractable needle knife and a pull-type sphincterotome on the same instrumental shaft, which allows extension of the gastrotomy incision created by the needle knife without the need of changing instruments. It has been shown to allow significantly quicker creation of a gastrotomy than the balloon dilation method without increasing the risk of complications.

Though direct incision on the gut wall is a simple method of creating transluminal accesses, concerns regarding peritoneal contamination and the difficulty in closure of the opening have prompted the development of a submucosal tunneling technique[26-30]. This technique is the preferred method of transluminal access in mediastinal NOTES and it is also feasible in transgastric procedures[30]. In brief, it involves an initial mucosal incision, followed by creation of a submucosal tunnel using either high-pressure carbon dioxide inflation and balloon dilation, or by submucosal dissection, in a manner similar to endoscopic submucosal dissection. The length of the tunnel is reported to be between 5 and 10 cm. At the end of the tunnel, a myotomy is made to gain access into the peritoneal cavity or the mediastinum. This method was shown to be least susceptible to immediate leakage after

Table 1 Summary of human natural orifice transluminal endoscopic surgery procedures

Authors	Type of procedure	No. of patients	Organ of access
Marescaux *et al*[2] (2007)	Cholecystectomy	1	Transvaginal
Zorrón *et al*[3] (2007)	Cholecystectomy	1	Transvaginal
Gettman *et al*[4] (2007)	Peritonoscopy	1	Transvesical
Hazey *et al*[5] (2008)	Peritonoscopy	10	Transgastric
Lacy *et al*[6] (2008)	Sigmoidectomy	1	Transvaginal
Ramos *et al*[7] (2008)	Sleeve gastrectomy	1	Transvaginal
Zorrón *et al*[8] (2008)	Peritonoscopy	1	Transvaginal
Zornig *et al*[9] (2009)	Cholecystectomy	68	Transvaginal
Decarli *et al*[10] (2009)	Cholecystectomy	12	Transvaginal
Gumbs *et al*[11] (2009)	Cholecystectomy	4	Transvaginal
Auyang *et al*[12] (2009)	Cholecystectomy	1	Transgastric
Horgan *et al*[13] (2009)	Cholecystectomy	1	Transvaginal
Kaouk *et al*[14] (2009)	Nephrectomy	1	Transvaginal
Fischer *et al*[15] (2009)	Sleeve gastrectomy	1	Transvaginal
Lacy *et al*[16] (2009)	Sleeve gastrectomy	1	Transvaginal

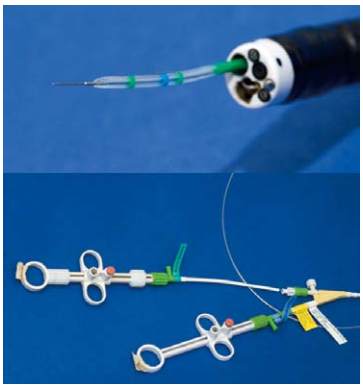

Figure 1 The one-step needle sphincterotome.

closure, with a leak pressure rivaling hand-sewn sutures in study involving 34 *ex vivo* porcine stomachs[31]. However, in one study, partial necrosis of the overlying mucosa was observed in up to four out of eight surviving swine (50%), and one swine suffered from severe peritonitis[27]. The cause of necrosis might be due to the high-pressure CO_2 bursts used for dissection, leading to impairment of blood supply. Other groups using the same technique without the device did not report such a complication[28,29]. Pauli *et al*[29] also reported that submucosal tunneling might increase the ease of in-line endoscope positioning to predetermined abdominal positions.

On the other hand, a number of hollow viscera are available for making accesses to the abdominal cavity. These include the stomach, colon, vagina, and the urinary bladder[2,20,32,33]. To avoid the limited maneuverability of a retroflexed endoscope, one will need to consider the access organ and the effect on the in-line position of the endoscope. Theoretically, the transgastric approach should facilitate in-line positioning of the endoscope to pelvic organs, while the transrectal, transvaginal, or transvesical routes provide good forward views of the upper abdominal structures. In an *in vivo* study by Voermans *et al*[34] involving 12 swine, transgastric peritoneoscopy was found to be inferior to laparoscopy in detecting simulated peritoneal metastasis, in particular for those located in the liver. In another study, they also found that both transgastric and transcolonic routes provided similar degrees of visualization and access efficacy to the liver and the peritoneal cavity[35]. More studies are required to determine the best access route for performing a particular abdominal NOTES procedure, and it is likely that the preference is governed by the nature of the procedures.

GASTRIC (LUMENAL) CLOSURE AND DEVELOPMENT OF SUTURING AND ANASTOMOTIC DEVICES

The ability to achieve secure lumenal closure is pivotal to the success of NOTES. Postoperative leakage is not only associated with morbidity and mortality, but it also bears potential legal and social consequences. Most of the novel designs of NOTES instruments published in the literature have attempted to address this issue. Technological advances in lumenal closure can be divided into four groups, namely the clipping, stitching, stapling, and occluding systems (Table 2).

Clipping systems

Jumbo endoclips *vs* over-the-scope clips: The jumbo endoclip was the earliest described method for lumenal closure[20]. It is achieved by applying clips to approximate the mucosal edges of an opening[20,36,37]. However, the technique can sometimes be difficult, especially if the luminal opening is too large to allow application of the jaws of the clips for mucosal apposition[38]. The fact that only the mucosal edges (and perhaps some submucosa) are recruited in these types of clips renders the security of approximation in large defects very much in doubt[36].

Table 2 Summary of devices for luminal closure in natural orifice transluminal endoscopic surgery

Mechanism of closure	Device name	Outcomes from comparative studies?	Tested in a survival study?
Clipping	Jumbo clips	Yes	Yes
	Over-the-scope clips	Yes	Yes
Stitching	Eagle claw	No	Yes
	T-tags	Yes	Yes
	Loop anchor purse-string	Yes	No
	G-prox needle	Yes	No
	Flexible endostitch	Yes	No
Stapling	SurgASSIST	Yes	No
Occluding	Nitinol cardiac occluder	No	Yes

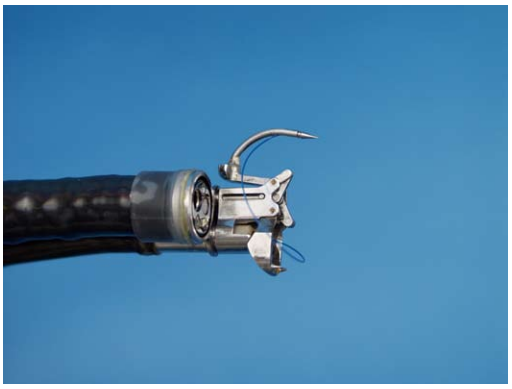

Figure 2 The Eagle claw.

Jumbo endoclips *vs* over-the-scope clips (OTSC)'s are nitinol clips that return to their original shape after release and allow approximation of large defects similarly to a surgical clamp[38]. The additional use of a twin grasper that allows inversion of the seromuscular layers has been shown to enhance approximation of all layers of the bowel wall. Randomized animal studies comparing OTSC's and hand-sewn closures have shown comparable leak pressures[39-41]. In another survival study, endoclip closure was found to be associated with significantly higher risk of leakage as compared to OTSC[42].

Stitching systems

A number of stitching systems have been developed, with none being commercially produced. These include the Eagle claw, T-tags, loop anchor purse-string, G-prox needle, and the Flexible endostitch.

Eagle claw: The Eagle claw (Olympus Medical Systems, Tokyo, Japan) was first developed by the Apollo group as an endoscopic suturing device to simulate surgical plication for hemostasis of bleeding peptic ulcers (Figure 2)[43]. The device consists of an opposing jaw that opposes tissue on closing and allows passage of a mounted 3O nylon stitch with a detachable needle. A metal pusher then

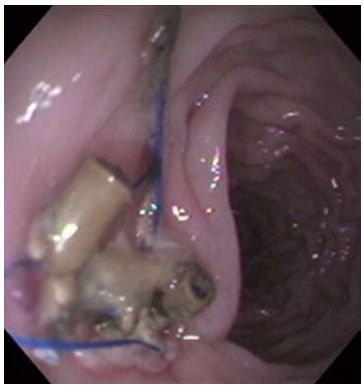

Figure 3 Appearance of the T-tags 1 wk after placement for repair of a colonic perforation in an animal model (Courtesy of Professor A Fritscher-Ravens).

tightens the stitch at the two edges of the stomach wall. In our survival study using the swine model, 10 gastrotomies were successfully closed after bilateral fallopian tubal ligation, and none of the animals suffered from suture line leakages upon post-mortem after 2 wk[44]. The device is now under development by Apollo Endosurgery and a newer version of the device has been shown to allow both interrupted and running suture placement[45].

T-tags: T-tags were first proposed by Fritscher-Ravens *et al*[46] in 2003 and is a device consisting of a series of double tags that are deployed by a transmural needle puncture through the two sides of the gastrotomy. The double tags are then tightened and locked, allowing opposition of the edges of the muscle wall (Figure 3). Other groups have also reported devices with similar design[27,47,48]. The device has been shown to produce fluid- and air-tight closures in the porcine model, and full thickness healing was observed. However, a few complications have been reported, including inadvertent injury to surrounding organs during transmural puncture of the needle[27,47].

Loop anchor purse-string: This is a variation of the T-tags where the anchors (loop anchors) are modified by adding a small metal wire loop to the crosspiece[49]. These anchors are then loaded onto a needle and deployed by using an inner stylet. To achieve gastrotomy closure, a transmural puncture is performed at the edges of the gastrotomy and anchors are deployed sequentially. The stitch is then tightened by pulling on the free ends of the suture and this leads to a purse-string closure of the defect. The device has been shown to achieve significantly higher leak pressures than endoclips in an *ex vivo* model.

G-Prox needle: G-prox has an operating mechanism similar to the T-tags. Closure of an enterotomy is achieved by puncturing the two edges of a defect with a 19-gauge needle, after which two pre-loaded expandable baskets connected by a non-absorbable suture are released[50]. One end of the suture is then tightened and this causes approximation of the baskets and closure of the defect. The device has been shown to create closures comparable to hand sewn sutures in an *ex vivo* model.

Flexible endostitch: The Flexible endostitch (Covidien, North Haven, USA) was adapted from a rigid laparoscopic version of the device. The jaws of the device holds a double-ended needle attached to a suture thread. The needle is toggled back and forth between the two jaws of the device to create a running suture[51]. In the *in vitro* model, it has been shown to produce leak pressures comparable to that of hand sewn sutures.

Stapling systems

SurgASSIST: Long before the advent of NOTES, stapling systems were shown to be reliable methods of creating anastomosis and closure of enterotomy in both open and laparoscopic surgery[52]. Flexible stapling systems based on the same technology should theoretically produce a low rate of leakage comparable to their rigid counterparts. SurgASSIST is a mechanically driven flexible linear stapler available from Power Medical Interventions (Langhorne, Pennsylvania, USA), which has been recently acquired by Covidien. The device has an automated firing system that aligns and approximates the staple arms and creates four linear rows of staples with closure of the enterotomy[53]. The problem with the device, however, is the difficulty in navigating the two staple jaws into a correct position before closure. Nevertheless, the device has been shown to produce burst pressures comparable to running sutures[36].

Occluding systems

Nitinol cardiac occluder: This occluder was originally designed for closure of atrial septal defects and was proposed to be a possible alternative method for closure of a gastrotomy. Animal survival studies have shown that prolonged closures up to 6 wk were possible with no evidence of leakage[54]. Results from comparative studies, however, are still lacking.

DEVELOPMENT OF A MULTITASKING PLATFORM

It is generally agreed that a multitasking flexible endoscope-based platform designated for NOTES is essential for replication of complex laparoscopic surgical manoeuvres, including dissection and suturing. This has spurred the development of a number of different platforms including the EndoSAMURAI (Olympus Corp, Tokyo, Japan) (Figure 4), the Anubis (Karl Storz, Tuttlingen, Germany), the Direct Drive Endoscopic system (DDES) (Boston Scientific, Massachusetts, USA), and the TransPort™ Multi-lumen Operating Platform (USGI medical, California, USA)[55-57]. The aim of these platforms is to provide a flexible, yet stable, system through which NOTES procedures can be performed universally through any of the transluminal approaches. Furthermore, these systems should provide a stable image of the operating field comparable to that in laparoscopic surgery

Figure 4 The EndoSAMURAI (Courtesy of Olympus Co., Tokyo, Japan).

and be independent of the movements of the working arms. More importantly, ergonomic user interfaces are available to control the movements of the arms (some of them capable of five degrees of freedom).

In a bench top simulation setting, both the EndoSAMURAI and the DDES have been shown to significantly enhance performance times and accuracy in complex surgical tasks as compared to using the double-channelled endoscope[55,56]. Twelve participants, who included experienced surgeons, medical students, and research assistants, were able to complete a suture using the EndoSAMURAI. The DDES system was also shown to allow performance of complex tasks, such as cutting, grasping, suturing, and knot tying[57]. An added advantage of DDES is that it can be operated by a single operator. Performance data of the other multitasking platforms, however, are still lacking and outcomes from human studies are still awaited.

DEVELOPMENT DEDICATED INSTRUMENTS

Flexible instruments and hemostatic appliances

Another obstacle to performing NOTES in a flexible system is the inferior properties of the endoscopic forceps or coagulation devices currently available when compared to their laparoscopic counterparts. In a recent study comparing the use of monopolar forceps, endoscopic suturing, and argon plasma coagulation in controlling bleeding from a major arterial branch, argon plasma coagulation was shown to be the quickest modality in achieving hemostasis[58]. In another study, the use of novel flexible bipolar forceps was shown to be comparable to laparoscopic bipolar forceps in stopping bleeding from blood vessels ranging from 1.5 to 6 mm in diameter. Delayed bleeding was observed in 3% of the blood vessels when blood pressure was raised to more than 200 mmHg for 10 min[59]. The development of other flexible instruments has also been announced but their performance data are still pending[60].

HUMAN NOTES PROCEDURES

Despite the tremendous amounts of research being directed towards NOTES, reports of human NOTES procedures are still limited. The majority of the publications were case series or single case reports, and only one study was comparative (Table 2)[2-16]. The most reported human NOTES procedure was a cholecystectomy and these procedures were performed *via* the transvaginal or transgastric routes[2,3,10-13]. NOTES peritoneoscopy, sleeve gastrectomy, sigmoidectomy, and nephrectomy have also been reported[4-9,14-16]. The NOTES appendectomy performed in India have been widely cited as a personal communications, but published data is still being awaited.

In fact, most NOTES cholecystectomies reported to date are hybrid procedures[2,3,5,10-13]. A 2 to 5 mm transumbilical port was first inserted for insufflation of pneumoperitoneum and also to allow for monitoring of the procedure. Most studies achieved transluminal access *via* the transvaginal route but the transgastric approach has also been described. In transvaginal cholecystectomy, a posterior colpotomy was made under direct laparoscopic view through the umbilical port and one to two trocars were inserted[10]. Both a flexible and an ultra-long rigid system have been used to perform the procedure. Retraction of the gallbladder was achieved by the umbilical port or additional transvaginal ports. In cases where a flexible endoscope was used, dissection was performed using instruments inserted through channels of the endoscope and clipping of the cystic artery and duct were done with either endoscopic hemoclips or surgical clips through the transumbilical or transvaginal trocars. In cases where a rigid system was used, the procedure was performed in a manner similar to traditional laparoscopic cholecystectomy, using ultra-long rigid instruments introduced through the transvaginal trocars.

In the human series describing NOTES cholecystectomy (Table 1), three out of 86 operations were unsuccessful and none required conversion. These three patients suffered from severe pelvic adhesions that prevented transvaginal insertion of trocars. The mean time to completion of the operation ranged from 51 to 135 min and no major complications were reported. In the largest series including 68 patients, the patients were also interviewed at 3 to 10 mo after surgery and none of them had abdominal or gynecological complaints in relation to sexual intercourse[9].

In the only human NOTES comparative study, transgastric peritoneoscopy was compared to diagnostic laparoscopy in evaluating patients with a pancreatic mass. Transgastric peritoneoscopy confirmed the decision to proceed to open laparotomy in nine out of ten patients, and the procedure was found to be safe and feasible. However, the authors also commented that the accesses to the right lobe of the liver and right upper quadrant structures were inadequate endoscopically and that attempted biopsies were unsuccessful due to inability to reach these areas[5].

On the other hand, there have also been case reports describing transvaginal nephrectomy, sleeve gastrectomy, and sigmoidectomy[6,7,9,15,16]. All these procedures were hybrid procedures where a transumbilical port was inserted for monitoring and retraction of the tissues, while the transvaginal ports were used for dissection and retrieval of

the specimen. All procedures were successfully performed and none reported major complications.

COMMENTS

The NOTES white paper in 2005 identified a number of fundamental obstacles to implementation of NOTES in humans[1]. Since then, these issues have become key areas of rigorous research in the laboratory setting and many findings have been published in the literature. Of interest is that, with the exception of case series and reports of human NOTES described in this paper, all of the studies performed so far were in animals. It is obvious that the intrinsic differences in physiology and anatomy between animals and humans do have significant impacts on the outcomes of the procedures, and whether results obtained in animals can be replicated in humans remains uncertain. More importantly, implementation of NOTES in human is still severely impaired by the availability of reliable devices specific for the procedure. The majority of devices that were described in this review remain as prototypes that are available to only a few exclusive centers. This limits the ability of researchers to compare different devices and procedures, let alone document the safety profiles and efficacy over a large study population.

In terms of the methods of gaining transluminal accesses, several problems remain to be solved. Firstly, the optimum method of creating the transluminal enterotomy is still uncertain. To some extent, the type of procedure being performed governs the methods of creating the opening. The submucosal tunneling method might be more appropriate when access to a particular organ is required. Likewise, the optimal access organ that provides the best in-line positioning when performing NOTES procedures on a specific region within the abdominal cavity will need to be determined. These issues remain to be resolved in future studies.

The NOTES white paper also states that a closure device that allows 100% reliability is a must before NOTES could be more widely implemented in humans. Along this line, many novel closure devices have been developed over the years. However, none of the reports have included a sufficiently large sample size to determine the exact risk of leakage. Direct head-to-head comparison of these devices has only been performed in one *in vitro* study, and there is a paucity of literature concerning the difference in *in vivo* efficacy of these devices[52]. Without these data, it is unlikely that any of the manufacturers will agree to undergo clinical human trials.

Besides closure devices, another area with exciting development is the research on flexible endoscope based multitasking platforms and instruments. The EndoSAMURAI, DDES, and the TransPort Multi-lumen Operating Platform™ were developed with an aim to perform complex transluminal procedures[55-57]. At present, most of these devices are still cumbersome to use and have been tested only in an *in vitro* setting. Size, ease of introduction, maneuverability *in vivo*, and lack of tactile feedback are some of the problems of the current platforms, which need to be addressed before they can be put into use in human subjects. It is also not certain how well they actually perform in a surgical operation, when grasping, dissecting, ligating, and suturing movements are performed in conjunction.

For the above reasons, the emergences of human NOTES procedures have largely been based on rigid platforms. This has been made possible by the adoption of ultra-long laparoscopic instruments introduced transvaginally, which allows replication of the steps of a laparoscopic surgical procedure. This may well be an intermediate form of NOTES before more reliable and steady platforms become available. Thus far, the outcomes of these NOTES procedures using laparoscopic instruments have been encouraging, and results from comparative studies are eagerly awaited to determine whether NOTES can truly offer earlier recovery and improve cosmesis.

CONCLUSION

Significant advances have been made in recent years in addressing the key obstacles to safe performance and introduction of human NOTES. However, most studies to date are still largely experimental, and whether these performance data can be repeated in humans remains uncertain. On the other hand, reports of human NOTES procedures are beginning to emerge. These studies have demonstrated the feasibility of performing human NOTES using existing technology. Dedicated devices are still lacking to allow for pure NOTES. Whether NOTES can deliver the postulated benefits of earlier recovery and improved cosmesis has yet to be confirmed.

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ORIGINAL ARTICLE

Role of *CYP2E1* gene polymorphisms association with hepatitis risk in Northeast India

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Abstract

AIM: To investigate hepatitis virus, genetic and environmental factors, and their interactions in predisposing patients to liver diseases in Northeast India.

METHODS: A total of 104 jaundice patients and 124 community controls were included. Serological analysis was performed by routine enzyme-linked immunosorbent assay, and nucleic acid testing for hepatitis viruses was done by polymerase chain reaction (PCR), followed by PCR direct sequencing for viral genotyping. Cytochrome P450 2E1 (*CYP2E1*) polymorphism was studied by PCR-restriction fragment length polymorphism. Nitrite and volatile nitrosamines in indigenous foods consumed routinely by the Northeast Indian ethnic population were estimated by Griess's reagent and GC-MS, respectively.

RESULTS: Hepatitis A virus (HAV) infection was predominantly prevalent (36.5%) in our cohort, followed by hepatitis B virus (HBV), hepatitis E virus (HEV) and hepatitis C virus. HBV genotype D and HEV genotype 1 were the most dominant. *CYP2E1* c1/c2 genotype frequency was comparatively higher in alcoholic ($P < 0.0001$, OR = 30.5) and cryptogenic ($P = 0.014$, OR = 8.714) patients, and was associated with significantly higher hepatitis risk ($P = 0.0.007$, OR = 6.489). Mutant C allele of Cyp2E1 *Dra* I frequency was comparatively higher in HAV ($P = 0.006$), alcoholic ($P = 0.003$) and cryptogenic ($P = 0.014$) cases, and was associated with overall hepatitis risk ($P = 0.026$, OR = 5.083). Indigenous foods, Gundruk, Kharoli, betel leaf and nuts were found to have the highest nitrite content.

CONCLUSION: Apart from viral factors, *CYP2E1* polymorphism might be associated with increased risk of liver diseases in Northeast India. Indigenous foods that contain nitrite and nitrosamine might be an associated risk factor.

Key words: Viral hepatitis; Cytochrome P450 2E1; Gene polymorphism; Nitrites; Nitrosamines

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INTRODUCTION

Application of molecular genetic techniques in human can-

cer risk assessment will likely emerge as a method of identifying subpopulations with different sensitivities to carcinogen exposure[1]. Liver diseases and cancer show a marked worldwide geographic and ethnic distribution[2]. Etiological factors that have been associated with liver diseases and cancer include hepatitis virus infection[3], liver flukes[4], aflatoxins[5], alcohol[6], smoking[6] and dietary nitroso compounds[7]. Differences in hepatitis B virus (HBV)[8] and hepatitis C virus (HCV)[9] genotypes are linked to various degrees of liver disease severity and rate of disease progression towards hepatocarcinogenesis. Unfortunately, there are no data available on these important aspects in liver disease patients from Northeast India, who are ethnically distinct from those in other parts of India, and have the incidence of cancer of various etiologies in the country, according to a survey done by the National Cancer Registry Program of the Indian Council for Medical Research (ICMR).

According to epidemiological studies, 90% of cancers are associated with environmental factors, including nitrosamines, which are acquired through tobacco smoke, vehicle exhaust and foodstuffs[10]. It has been shown that nitrite alone can cause cancer; however, an even more serious cause of concern is the well-documented potential of nitrites/nitrates to cause cancer through the formation of nitrosamines[11].

Cytochrome P450 2E1 (*CYP2E1*) is an N-nitrosodimethyl-amine demethylase that is expressed primarily in the liver. It takes part in the metabolism of drugs, but also activates many pre-carcinogens and pre-toxins[12]. Cyp2E1 activates N-nitrosamines, which are contained in tobacco smoke and foodstuffs[13] and several industrial[14] and endogenous carcinogens[15]. Cyp2E1 activity is mediated by various determinants, such as obesity, fasting and liver dysfunction, and by a number of environmental factors[16]. Cyp2E1 activity is accompanied by generation of a significant amount of an active oxygen form, which damages cell membranes and macromolecules and leads to formation of DNA adducts. Polymorphism in the Cyp2E1 gene is associated with malignancies of different cellular origins, including the liver[17]. *CYP2E1* polymorphism in the 5'regulatory region with C→T replacement at position -1019 and *Rsa* I restriction site loss (*CYP2E1*5B*) is one of the most important polymorphisms identified. Homozygous c2/c2 genotype is associated with a 10-fold increase in *CYP2E1* gene transcription[18]. Another important *CYP2E1* polymorphism is located in intron 6, revealed by *Dra* I and identified as C (minor) and D (common) alleles[19].

Here, we present the results from Guwahati (the capital of Assam, and hub and gateway of Northeast India) of a case-control study designed to explore the viral, environmental and genetic risk factors for liver diseases. The study was approved by the Institutional Biosafety and Ethics Committee.

MATERIALS AND METHODS

Blood samples were obtained from 104 acute hepatitis patients with clinical jaundice and liver disease during the non-rainy season, who were receiving care at the Central Hospital, NF Railway, Maligaon, Guwahati. One hundred and twenty-four sex, age and residence pair-matched community controls, with similar ethnicity and food habits were also recruited for this study. Cases and controls were evaluated on the basis of history (including their food, drinking, smoking and tobacco chewing habits), clinical examination, liver function profile, and serological test of hepatitis A, B, C and E using commercially available IgM enzyme-linked immunosorbent assay (ELISA) kits (therefore including acute cases).

Viral DNA isolation and genotyping

Viral DNA isolation of HBV for hepatitis B surface antigen (HBsAg)-positive cases was performed using the standard phenol-chloroform method using 150 μL of patient plasma, followed by ethanol precipitation. The viral DNA thus isolated was resuspended in an adequate amount of nuclease-free water. HBV genotyping was performed by multiplex polymerase chain reaction (PCR) using specific primers for each genotype (A-F) of HBV[20], and validated by sequencing of representative cases for the basal core promoter, precore and core region of HBV genome.

RNA extraction and HCV and hepatitis E virus genotyping

Viral RNA was extracted from 140 μL of serum with QIAamp@Viral RNA Kit (Qiagen, Germany) according to the manufacturer's instructions. RNA pellets were reconstituted in 60 μL elution buffer and stored at -20℃ until use. One-tube nested reverse transcription PCR (RT-PCR) amplification was performed using specific primers for the conserved 5'UTR region as described earlier for genotyping of HCV[21]. Amplification of the specific 256-bp product was achieved for the anti-HCV-positive cases. Briefly, 10 μL RNA was mixed with 0.2 μL (20 pmol) of antisense primer, incubated for 1 min at 94℃ and 1 min at 56℃, and then stored on ice. Fifty microliters of the reaction mixture that contained 10 × reaction buffer, dNTPs (10 mmol/L), $MgCl_2$ (2.5 mmol/L), 20 pmol primers AS1 and S1, 0.5 U *Taq* Polymerase (New England Biolabs, Ipswich, MA, USA) and 2.5 μL (20 U/μL) MMuLV Reverse Transcriptase (New England Biolabs) was added to the pre-cooled RNA mix for one-step RT-PCR. The conditions were 60 min at 42℃ for reverse transcription, 2 min at 95℃ for denaturation of the RT, followed by 35 cycles of 30 s at 95℃, annealing for 30 s at 54℃, and extension for 30 s at 72℃. After the last cycle, a final extension was made at 72℃ for 7 min. The second round of PCR was performed with the same master mix that contained AS2 and S2 primers using 5 μL of the first product as a template under the same reaction conditions. Positive and negative controls were included in every PCR amplification experiment. This was followed by direct sequencing and comparison with the standard NCBI Genbank database. Hepatitis E virus (HEV) genotyping was performed by RT-PCR amplification using the primers for the HEV ORF1 region reported by Jilani *et al*[22], which gave a PCR-amplified product of 343 bp; followed by direct sequenc-

Table 1 Demographical, biochemical and serological profiles of liver disease patients

Parameter	HAV	HBV	HCV	HEV	HAV + HBV	Alcoholic	Cryptogenic
n	38	22	4	10	2	12	16
Male:female	22:16	16:6	2:2	6:4	2:0	11:1	12:4
Mean age (yr)	23 ± 16	40 ± 28	45 ± 8	38 ± 15	55	44 ± 5	43 ± 21
Mean SGOT	241 ± 168	333 ± 108	86 ± 46	778 ± 336	40 ± 5	323 ± 212	109 ± 66
Mean SGPT	265 ± 198	243 ± 148	73 ± 28	513 ± 366	39 ± 8	283 ± 155	85 ± 43

HAV: Hepatitis A virus; HBV: Hepatitis B virus; HEV: Hepatitis E virus; HCV: Hepatitis C virus; SGOT: Serum glutamic oxaloacetic transaminase; SGPT: Serum glutamic pyruvic transaminase.

ing and comparison with the available genotype database for HEV from the NCBI Genbank database.

PCR-restriction fragment length polymorphism analysis of CYP2E1 gene polymorphism

*CYP2E1*5B* (5' flanking region, -1019 bp site) genotyping was performed by PCR-fragment length polymorphism (RFLP) analysis with the primers reported by Hayashi *et al*[23]; and *Rsa* I restriction enzyme. An allele with an *Rsa* I site (characterized by two bands of 360 and 50 bp on agarose gel electrophoresis) was defined as wild-type and designated c1, and an allele without this site, as a variant or rare type and designated c2 (characterized by presence of a single band at 410 bp)[24].

Dra I digestion detects a polymorphism in intron 6 of the *CYP2E1* gene. Genomic DNA was PCR-amplified with primers reported by Kato *et al*[25], which yielded a 995-bp fragment that was subjected to *Dra* I restriction enzyme digestion. Two *Dra* I restriction enzyme recognition sites exist in this amplified DNA sequence but only one is known to be polymorphic. The presence of the polymorphic *Dra* I restriction site yielded three fragments of 572, 302 and 121 bp (type D, major allele), whereas the absence of the polymorphic site was determined by the presence of 874-bp and 121-bp fragments (type C, minor allele)[26].

To improve the genotyping quality and validation, 20% of samples were re-genotyped by other laboratory personnel and results were reproducible with no discrepancy in genotyping. Genotyping of 10% of samples was confirmed by DNA sequencing.

Nitrite determination and analysis of volatile nitrosamines

Several indigenously prepared fermented food products and the raw material used to prepare them were short-listed and collected, based on a questionnaire of the food habits of jaundice patients and community controls enrolled in the present study. Nitrite estimation in fresh and fermented foods from Northeast India was done using the standard Griess's reagent method followed by spectrophotometric detection at 540 nm. The presence of volatile N-nitrosamines in fresh and fermented foods was done by GC-MS analysis following the protocol of Mitacek *et al*[27], followed by detection using a Hewlett-Packard Model 5890 GC coupled to a model 610 Thermal Energy Analyzer (TEA; Thermo Electron, Waltham, MA, USA).

Statistical analysis

Results were expressed as mean ± SD. Serum aspartate aminotransferase and alanine aminotransferase levels in each group were analyzed by student's *t* test. ORs were calculated using logistic regression. Statistical analysis was carried out for *CYP2E1* genotypes in liver disease and hepatitis subgroups (specific for viral hepatitis groups, and alcoholic and cryptogenic cases) and compared to community controls using SPSS version 13.0 software. An adjusted two-tailed *P* value (corrected) less than 0.05 at 95% CI was considered statistically significant.

RESULTS

Blood samples were obtained from patients with liver disease who were receiving care in a regional referral hospital in Guwahati. These patients had a median age of 41 ± 16 years and showed a male to female ratio of 2.15:1. The majority of the liver disease patients were male (71/104, 68.27%). The hepatitis virus infection spectrum analyzed based on IgM ELISA results was, hepatitis A virus (HAV, 38/104, 36.5%), HBV (22/104, 21.15%), HCV (4/104, 3.8%), HEV (10/104, 9.6%), and HAV-HBV co-infection (2/104, 1.92%). Others had alcoholic (12/104, 11.53%) and cryptogenic (16/104, 15.38%) liver disease etiology (Table 1).

Viral genotyping

Viral genotyping was performed for HBV, HCV and HEV samples. HBV genotyping was performed by multiplex PCR. HBV genotype D (13/22, 59.1%) was the most prevalent in the HBV-positive cases, followed by HBV genotype A (4/22, 18.2%), mixed genotype A + D (4/12, 18.2%) and genotype C (1/22, 4.5%) (Figures 1A and 2A). A few of the randomly selected genotyped samples were cross-checked and validated by direct sequencing of the core region of HBV followed by phylogenetic analysis.

HCV genotype was determined by direct sequencing of the PCR amplicon generated from the conserved 5'UTR region of the HCV genome. The nucleotides that were sequenced by direct sequencing were aligned using ClustalW, and version 1.6 of the tree view program from Expasy (POWER) was then used to construct an uprooted phylogenetic tree (Figures 1B and 2B). After comparison with known sequences from the NCBI Genbank database, the distribution of the genotypes based on four isolated

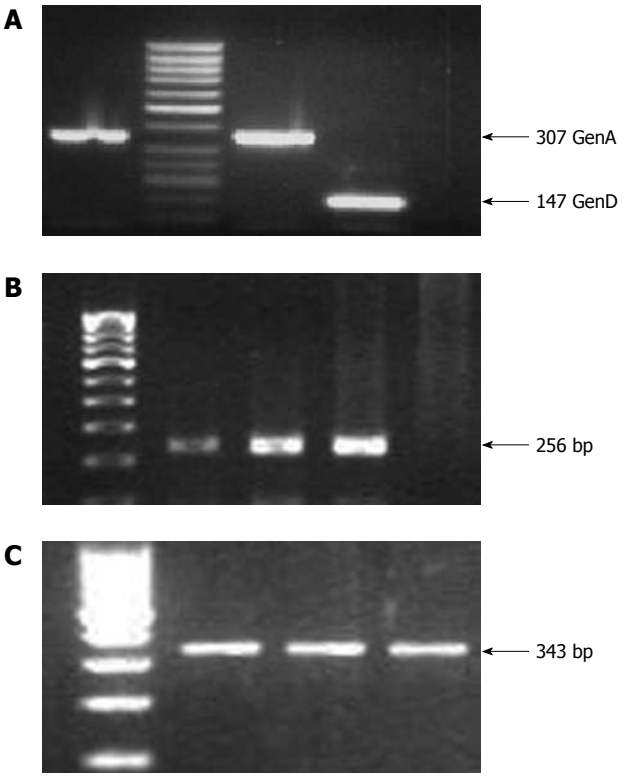

Figure 1 Polymerase chain reaction amplification results. A: Hepatitis B virus (HBV) genotyping results, where an amplicon of 307 bp represents HBV genotype A whereas an amplicon of 147 bp represents HBV genotype D; B: Hepatitis C virus (HCV) amplicon of 256 bp for the 5'UTR region; C: Hepatitis E virus (HEV) amplicon of 343 bp for the ORF1 region. The HCV and HEV amplicons were purified by gel extraction and subjected to direct sequencing for genotype determination on comparison with the standard NCBI representative sequences for HCV and HEV.

HCVs were found to be one each from genotypes 4 (*asm-hcv*-4), 3 (*asm-hcv*-3), 2 (*asm-hcv*-1) and 6 (*asm-hcv*-2).

HEV genotype was determined by amplification of the ORF1 region and subjecting the amplified product to direct sequencing, and then comparing the sequences with the standard NCBI Genbank sequences for HEV. HEV genotype 1 was the only genotype found in our cohort.

PCR-RFLP analysis of CYP2E1 gene polymorphism

The distribution of *CYP2E1*5B* c1c1, c1c2 and c2/c2 genotypes in liver disease cases were 90.38%, 9.62% and 0%, respectively compared to 98.39%, 1.61% and 0% in healthy controls (Figure 3). The *CYP2E1*6* DD, DC and CC genotype frequencies in liver disease cases were 92.3%, 3.85% and 3.85%, respectively, compared to 98.39%, 1.61% and 0% in healthy controls (Tables 2 and 3). Cyp2E1 *Rsa* I genotype distributions were consistent with Hardy-Weinberg equilibrium, but Cyp2E1 *Dra* I genotype was only consistent for the control population. The c1/c2 variant genotype was significantly more prevalent in alcoholic [$P < 0.0001$, OR = 30.5 (4.835-192.418)] and cryptogenic hepatitis [$P = 0.014$, OR = 8.714 (1.137-66.784)] cases. The prevalence of mutant C allele of *Dra* I was also predominant in alcoholic [$P = 0.003$, OR = 12.220 (1.550-96.035)] and cryptogenic hepatitis [$P = 0.014$, OR = 8.714 (1.137-66.784)] cases.

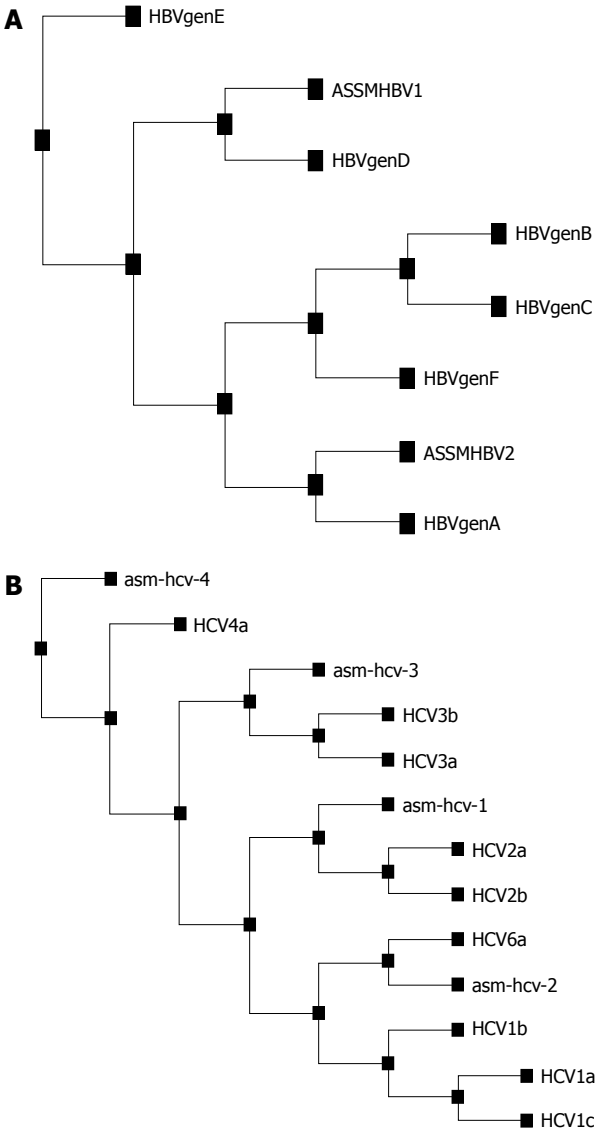

Figure 2 Phylogenetic analysis using the Expasy software tool. A: Randomly sequenced hepatitis B virus (HBV) cases representing HBV genotype D (*ASSMHBV1*) and A (*ASSMHBV2*) by multiplex polymerase chain reaction genotyping, therefore confirming and validating our genotyping results; B: Hepatitis C virus (HCV) genotype base on direct sequencing of 5'UTR for isolate from Guwahati.

Nitrite determination and analysis of volatile nitrosamines

To study the correlation of environmental factors with severity of liver disease, nitrite concentration in various fermented food products widely used in the households of Assam and Northeast India were estimated using Griess reagent. The concentration of nitrites in raw and fermented food products produced by indigenously developed fermentation techniques, as well as a few common supplementary food products consumed routinely, are shown in Figure 4 and Table 4. The highest amount of nitrite was found in mustard (fresh and fermented), followed by another fermented food product, Gundruk (fermented radish leaf) and betel leaf (commonly known as Pan, and taken in combination with betel nut).

Based on the case histories of the liver disease patients, which included their food habits, as well as considering

Table 2 Distribution of Cytochrome P450 2E1 genotype in hepatitis cases compared to controls

	Number of individuals (% of group)			Less common allele frequency	χ^2 value (*P* value)	OR (95% CI)
	Homozygous-more common allele	Heterozygous	Homozygous-less common allele			
Rsa I polymorphism	c1/c1	c1/c2	c2/c2			
Controls (*n* = 124)	122 (98.39)	2 (1.61)	0	1.61	ref.	
Hepatitis (*n* = 104)	94 (90.39)	10 (9.71)	0	9.61	0.007	6.489 (1.389-30.326)
Dra I polymorphism	DD	DC	CC			
Controls (*n* = 124)	122 (98.39)	2 (1.61)	0	1.61	ref.	
Hepatitis (*n* = 104)	96 (92.31)	4 (3.84)	4 (3.84)	7.69	0.026	5.083 (1.055-24.492)

Data represented as number of subjects showing respective genotype (%); $P < 0.05$ was considered to be statistically significant, control group was considered as reference group.

Table 3 Detail distribution of Cytochrome P450 2E1 genotypes in different underlying etiology of hepatitis

	Number of individuals (% of group)			Less common allele frequency	χ^2 value (*P* value)	OR (95% CI)
	Homozygous-more common allele	Heterozygous	Homozygous-less common allele			
Rsa I polymorphism	c1/c1	c1/c2	c2/c2			
Controls (*n* = 124)	122 (98.4)	2 (1.6)	0	1.6	ref.	
HAV (*n* = 38)	36 (94.7)	2 (5.3)	0	5.3	0.160	5.229 (0.840-32.537)
HBV (*n* = 22)	20 (90.9)	2 (9.1)	0	9.1	0.080	2.905 (0.252-33.484)
HCV (*n* = 4)	4 (100)	0 (0)	0	0	0.798	0.968 (0.938-1.0)
HEV (*n* = 10)	10 (100)	0 (0)	0	0	0.686	0.924 (0.880-0.971)
HAV + HBV (*n* = 2)	2 (100)	0 (0)	0	0	0.856	0.984 (0.962-1.006)
Alcoholic (*n* = 12)	8 (66.66)	4 (33.33)	0	33.33	< 0.0001	30.500 (4.835-192.418)
Cryptogenic (*n* = 16)	14 (87.5)	2 (12.5)	0	12.5	0.014	8.714 (1.137-66.784)
Dra I polymorphism	DD	DC	CC			
Controls (*n* = 124)	122 (98.4)	2 (1.6)	0	1.6	ref.	
HAV (*n* = 38)	34 (89.5)	2 (5.25)	2 (5.25)	10.5	0.006	7.176 (1.260-40.863)
HBV (*n* = 22)	22 (100)	0	0	0	0.514	0.847 (0.790-0.908)
HCV (*n* = 4)	4 (100)	0	0	0	0.798	0.968 (0.938-1.0)
HEV (*n* = 10)	10 (100)	0	0	0	0.164	0.924 (0.880-0.971)
HAV + HBV (*n* = 2)	2 (100)	0	0	0	0.856	0.984 (0.962-1.006)
Alcoholic (*n* = 12)	10 (83.3)	2 (16.7)	0	16.7	0.003	12.220 (1.550-96.035)
Cryptogenic (*n* = 16)	14 (87.5)	0	2 (12.5)	12.5	0.014	8.714 (1.137-66.784)

Data represented as number of subjects showing respective genotype (%); $P < 0.05$ was considered to be statistically significant, control group was considered as reference group. HAV: Hepatitis A virus; HEV: Hepatitis E virus; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

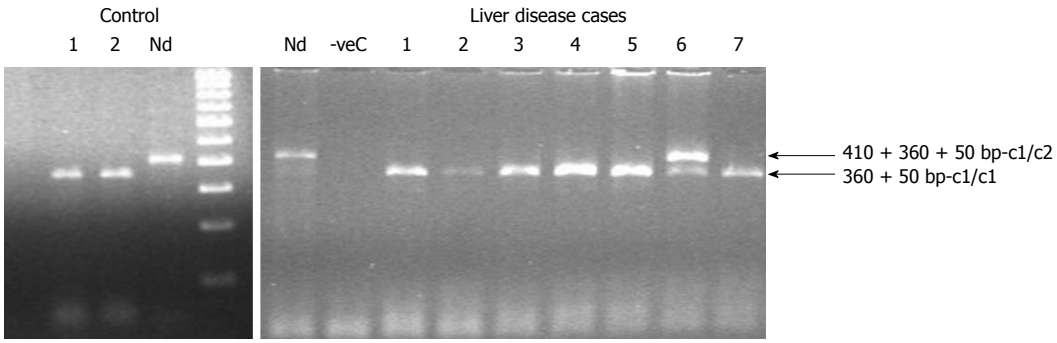

Figure 3 Polymerase chain reaction-restriction fragment length polymorphism results for Cytochrome P450 2E1 *rsa* I genotyping for the control and liver disease cases. Lane 1 and 2 in the control section and lane 1-5 and 7 of the liver disease section represents c1/c1 genotype (360 + 50 bp); whereas lane 6 of the liver disease section repre-sents c1/c2 genotype (410 + 360 + 50 bp). Nd: Non-digested samples of 410 bp.

the food habits of the general Northeast Indian population, we analyzed raw and fermented bamboo shoots and fish (including dried fish) for the presence of volatile N-nitrosamines by GC-MS. The GC-MS/TEA analysis revealed the presence of detectable amounts of N-nitrosamines in raw fish (data not shown), whereas there was

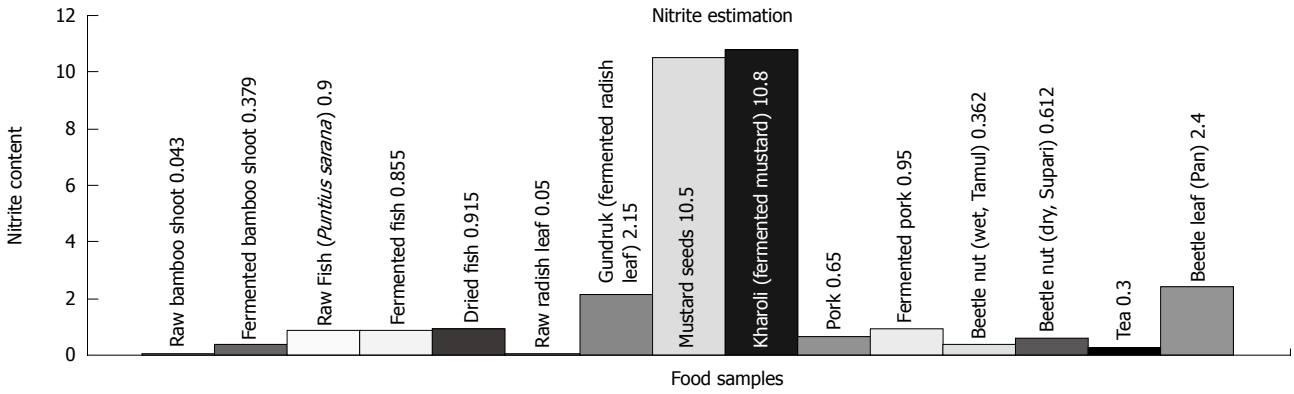

Figure 4 Presence of nitrite in (μg/mL) in different food products routinely consumed in Northeast India.

Table 4 Nitrite estimation in food products routinely consumed in Northeast India

Sl. No.	Name of foodstuff	Amount of nitrite present (μg/mL)
1	Raw bamboo shoot	0.043
2	Fermented bamboo shoot	0.379
3	Raw fish (*Puntius sarana*)	0.900
4	Fermented fish	0.855
5	Dried fish	0.915
6	Raw radish leaf	0.050
7	Gundruk (fermented radish leaf)	2.150
8	Mustard seeds	10.500
9	Kharoli (fermented mustard)	10.800
10	Pork	0.650
11	Fermented pork	0.950
12	Beetle nut (wet, Tamul)	0.362
13	Beetle nut (dry, Supari)	0.612
14	Tea	0.300
15	Betel leaf (Pan)	2.400

no detectable amount of N-nitrosamines in fermented and dry fish or raw and fermented bamboo shoots.

DISCUSSION

Along with age, sex and viral factors, alterations in host genetic factors and environmental factors are also considered to be important in the development of liver disease, but unfortunately, there are very limited data available on these important aspects in liver disease patients from Northeast India, who are ethnically distinct from those in other parts of the country. More importantly, the population in the northeast region is vulnerable to cancer of different etiology, as shown by the ICMR National Cancer Registry Program of. In this study, we determined the prevalence of systemic hepatotropic viruses and their genotypes, Cyp2E1 polymorphism, and the presence of dietary toxic environmental carcinogens such as nitrites and nitrosamines in fresh and fermented food from Northeast India, and evaluated the role that they might play in the severity of the liver disease or its predisposition.

The highest prevalence of fecal-oral infection occurs in regions where low standards of sanitation promote virus transmission[28]. In most industrialized nations, where hepatitis A is no longer considered a childhood disease, infections with HAV are increasingly contracted by adults[29]. Despite the high prevalence of antibody in highly endemic populations, the virus perpetuates in the region due to its high physical stability. In our study, HAV infection was found in 36.5% of the population and most of the infected individuals were children and young people. This was followed by HBV infection in 21.2% of the study population, which is relatively on the higher side compared to other reports from different parts of India[30-33]. This is a major concern because HBV infection is associated with a high rate of hepatocellular carcinoma[34]. HBV genotype D was the most predominant genotype in our cohort, which is contrary to a report from the sister state in Northeast India, Arunachal Pradesh[35].

HEV and HCV infection was found in 9.6% and 3.8% of the cases, respectively. Viral hepatitis is a major public health problem in India, which is hyperendemic for HAV and HEV. HEV is also the major cause of sporadic adult acute viral hepatitis and acute liver failure, and many epidemics of HEV have already been reported in India. HCV infection in India has a population prevalence of around 1%, and occurs predominantly through transfusion and the use of unsterile glass syringes. HCV genotypes 2 and 3 are found in 60%-80% of the population[36]. Our results showed the prevalence of different HCV genotypes, namely, 2, 3, 4 and 6, which warrants further study, including large cohort populations from all Northeast Indian states. Here, to the best of our knowledge, we reported the presence of HEV genotype 1 in Northeast India for the first time, which is similar to reports published from other parts of India.

CYP2E1 enzymes belong to the phase I group of drug-metabolizing enzymes that are involved in the metabolic activation and detoxification of various potential genotoxic compounds. *CYP2E1* is involved in metabolism of more than 80 low-molecular-weight, hydrophobic, toxicologically dangerous compounds and contributes to activation of many pro-carcinogens and several drugs to highly reactive metabolites[14]. Hepatic *CYP2E1* has been shown to activate various carcinogens, therefore, there has been interest in whether certain *CYP2E1* polymorphisms

might predispose to liver diseases and cancer[37]. The functional polymorphism in these genes exhibits inter-individual variations in susceptibility towards various diseases and differences in therapeutic response. The variant sequences of these genes differ considerably between ethnic groups. Therefore, the objective of the study was to assess the prevalence of *CYP2E1* gene variants in healthy volunteers and compare them with the liver disease patients from Guwahati.

The most important polymorphisms identified in 5' regulatory region with C→T replacement in position -1019 and *rsa* I restriction site loss (*CYP2E1*5B*) (77, 25). Variant *c*2 allele is expressed *in vitro* at a higher rate compared to wild-type, and homozygous *c*2/*c*2 genotype is associated with a 10-fold increase in *CYP2E1* gene transcription. The functional significance of *CYP2E1*5B* polymorphism might be due to its localization in presumed binding sites for hepatic transcription factor, hepatocyte nuclear factor-1[18,23]. Rare *c*2 allele frequency constitutes 24%-30% for Asian populations[25], 2%-3% for Caucasians[23], 0.3%-7% for Afro-Americans[23,38], 15% for Mexican Americans[39], and 18% for Taiwanese[40]. The *Dra* I polymorphism is also associated with altered activity of *CYP2E1*, although *Dra* I is located in intron 6 and is not thought to affect transcription of the gene[19].

Our study showed that the prevalence of mutant C1/C2 Cyp2E1 *Rsa* I allele and the mutant C allele of *Dra* I was significantly higher in liver disease patients. The presence of mutant Cyp2E1 *Rsa* I allele ($P = 0.007$, OR = 6.489 at 95% CI: 1.389-30.326) and mutant C allele of *Dra* I ($P = 0.026$, OR = 5.083 at 95% CI: 1.055-24.492) was significantly associated with hepatitis risk in Northeast Indian patients.

The prevalence of the *c*1/*c*2 genotypes was lower than that reported in other Asian countries, but amongst the highest reported in the Indian population[41]. The serum glutamic oxaloacetic transaminase (SGOT) levels were also significantly higher in HAV cases that contained wild-type Cyp2E1 *Dra* I allele ($P = 0.019$). The prevalence of mutant *Dra* I allele among patients with viral hepatitis was found to be significantly more only in cases of HAV infection ($P = 0.006$). The presence of underlying mutant *Dra* I allele might play a role in liver damage caused by acute HAV infection, but this also augments more indebt studies to conclude on the molecular interactions influenced by HAV on *CYP2E1* genes functionality or activity.

Induction of cytochrome P450 2E1 by ethanol is believed to be one of the central pathways by which ethanol generates a state of oxidative stress and causes hepatotoxicity. Hepatic *CYP2E1* enzyme activity is significantly higher in alcoholic patients with liver disease than in those without signs of liver disease[42]. In our study, Cyp2E1 *c*1/*c*2 ($P < 0.0001$) and mutant *Dra* I ($P = 0.003$) allele was significantly associated with alcoholic liver disease. Mutant *c*1/*c*2 and *Dra* I ($P = 0.014$) was also found to be associated with cryptogenic hepatitis in liver disease patients from Northeast India.

There is a concern to maintain the levels of nitrite as low as possible because of suspected adverse effects on oxygenation of the blood, and/or indirect carcinogenic effects, through formation of nitrosamines. Nitrites have been known to cause cancer directly. Although there is little correlation between nitrate/nitrite and nitrosamine content of food, nitrates and nitrites are agents in endogenous nitrosamine formation in the gastrointestinal tract[11]. Therefore, the presence of high nitrite concentration in raw and fermented mustard, radish and betel leaf and nut is also a high risk factor for adverse health effects, along with genetic and viral factors. Addition of nitrite-containing salts for storage of some dried fish products and fermented pork also adversely affects the quality of the food. The European Commission Scientific Committee for Food (document 111/5611/95) has recommended that nitrate and nitrite should be limited to an acceptable daily intake of 0.06 mg/kg. Therefore, ingestion of the above food products that contain high nitrite concentrations is a high risk factor, especially for children.

Case-control studies have suggested that exposure to exogenous and possibly endogenous nitrosamines in food or tobacco in betel nut and cigarettes plays a role in the development of liver disease and cancer. There is evidence that endogenous nitrosation of areca nut alkaloids can occur in animals and humans, and areca-nut-derived nitrosamines, including 3-(methylnitrosamino) propionitrile, have been detected in the saliva of betel quid chewers which is a common practice in Guwahati and throughout Northeast India. Epidemiological data have linked the use of areca nut with other cancers such as liver cancer[43]. The presence of volatile nitrosamines (N-diethylnitrosamine and N-dimethylnitrosamine) in raw fish has been detected using the protocol followed by Mitacek *et al*[27]. The presence of volatile nitrosamines could be an indication of increasing pollution of the River Brahmaputra, which is one of the life lines of Northeast India, and its tributaries, from where the fish *Puntius sarana* is caught and fermented and dried. Our results is of grave importance as case-control studies conducted in Thailand have implicated traditional lifestyle and especially consumption of fermented-style fish and fermented vegetables[44]. Fortunately, the nitrosamine levels were undetectable in fermented and dried fish, contrary to what has been reported in other countries[27]. The non-detection of nitrosamines in fermented fish, irrespective of its presence in raw fish, could be attributed to the activities of lactic acid bacteria during fermentation.

To conclude, the diversity of etiological factors associated with liver disease burden in Northeast India is enormous with respect to the high prevalence of certain hepatitis viruses, such as HBV, as well as the various HBV and HCV genotypes found in our study cohort. Moreover, strict vigilance and upgrading of overall hygiene standards is mandatory to investigate epidemics of HAV or HEV, which are also prevalent in Northeast India. *CYP2E1* polymorphism is supposedly associated with the risk of liver disease, especially in non-viral hepatitis patients, and the presence of higher nitrite concentration in fermented dietary products in Northeast India, and nitrosamines in *Areca catechu* (betel nut) and raw fish, have clinical signifi-

cance, because these environmental factors can act as additional risk factors in liver disease susceptibility, by virtue of the gene-environment interaction.

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COMMENTS

Background

Molecular epidemiology of risk factors such as viral (e.g. hepatitis A, B, C and E viruses), host genetic [e.g. Cytochrome P450 2E1 (*CYP2E1*) gene polymorphism] and environmental factors such as toxic components in food and alcohol, is important for liver disease assessment, which might help to identify subpopulations with different sensitivities and predisposition to different grades or severity of liver disease. Scanty or no data are available on the above aspects from Northeast India, which has an ethnically distinct and different population from the rest of the country. The authors present the results of a case-control study from Guwahati (the capital of Assam, and hub and gateway of Northeast India), which was designed to explore the viral, environmental and genetic risk factors for liver diseases.

Research frontiers

The authors identified patient subgroups by serological profiling based on standard enzyme-linked immunosorbent assay techniques, followed by a molecular-genotyping-based approach using polymerase chain reaction-restriction fragment length polymorphism direct sequencing for identifying hepatitis B, C and E genotypes, as well as *CYP2E1* polymorphisms. Biochemical assays (Griess's method) and GC-MS were used for analyzing and quantifying nitrites and nitrosoamines.

Innovations and breakthroughs

For the first time, all three critical parameters, that is, viral, host genetic and environmental risk factors were evaluated in a case-control study from Northeast India. The study analyzed hepatitis virus genotypes, the role of *CYP2E1* polymorphisms, and the presence of toxic carcinogenic components in routinely consumed indigenous food products of Northeast India.

Applications

The molecular genotyping data on viral hepatitis could be useful for clinicians because viral genotypes have been shown to influence disease progression and antiviral therapies, and therefore, will helpful for clinical interventions and patient care. *CYP2E1* genotyping data are useful as a prognostic marker for assessing the predisposition of patients towards liver disease. The safety aspects of the food products exclusively consumed in Northeast India were elucidated. Information about this is important for the public in Northeastern states of India.

Peer review

The results of this paper are interesting and well presented.

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ORIGINAL ARTICLE

A new index for non-invasive assessment of liver fibrosis

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Abstract

AIM: To construct and evaluate a new non-invasive fibrosis index for assessment of the stage of liver fibrosis.

METHODS: A new fibrosis index (Fibro-Stiffness index) was developed in 165 of 285 patients with chronic hepatitis C, and was validated in the other 120 patients where liver biopsy was performed. Its usefulness was compared with liver stiffness (LS) measured by FibroScan, the aminotransferase-to-platelet ratio index, the Forns index and the FibroIndex.

RESULTS: The Fibro-Stiffness index consists of LS, platelet count and prothrombin time. The values of the Fibro-Stiffness index differed significantly between neighboring fibrosis stages except F0-F1. The area under the receiver operating characteristics curves of the Fibro-Stiffness index for prediction of F $\geqslant$ 2 (0.90), F $\geqslant$ 3 (0.90) and F = 4 (0.92) in the estimation group and those for F $\geqslant$ 3 (0.93) and F = 4 (0.97) in the validation group were the highest among the 5 methods examined. The accuracy of the Fibro-Stiffness index had highest values for F $\geqslant$ 2, F $\geqslant$ 3 and F = 4 in both the estimation and validation groups. The diagnostic performance for F = 4 was improved by a combination of the Fibro-Stiffness index with serum hyaluronic acid level.

CONCLUSION: The Fibro-Stiffness index was constructed and validated. It showed superior diagnostic performance to other indices for F $\geqslant$ 2, 3 and 4.

Key words: Non-invasive fibrosis index; Fibro-Stiffness index; Chronic hepatitis C; Liver stiffness; Liver fibrosis

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INTRODUCTION

The stage of liver fibrosis is important in the clinical

management of chronic hepatitis C, since the treatment and prognosis of chronic hepatitis depend on the fibrosis stage[1]. In chronic viral hepatitis, the presence of significant fibrosis (F ⩾ 2) indicates the need for antiviral therapies, and the outcome of therapy should be assessed by the improvement in fibrosis stage. Furthermore, the risk of hepatocellar carcinoma or bleeding from esophageal varices is high in patients with advanced fibrosis[2,3]. Liver biopsy is the gold standard for the assessment of fibrosis stage in chronic hepatitis. However, liver biopsy is an invasive and expensive procedure, and its accuracy is sometimes questionable because of sampling errors, inadequate specimens and the subjectivity of diagnosis[4,5].

Non-invasive assessment of liver fibrosis is a major objective that has been encouraging many approaches, such as routine laboratory tests and serum markers of fibrosis[6-12]. The aminotransferase-to-platelet ratio index (APRI)[11], the Forns index[6], the FibroTest[7] and the FibroIndex[12] have been proposed for use as non-invasive fibrosis indices. Transient elastography with the use of a new apparatus, FibroScan (EchoSens, Paris, France) for measurement of liver stiffness (LS) has been developed[13]. LS measured by FibroScan has been reported to correlate with stage of fibrosis in various liver diseases[13-24]. It was used for assessing the effect of treatment in chronic hepatitis C[25].

In the present study, we developed a new fibrosis index, the Fibro-Stiffness index, consisting of LS, platelet count and prothrombin time from 165 patients with chronic hepatitis C (estimation group) to improve the diagnostic efficacy of LS. We also tried a combination of Fibro-Stiffness index and routinely available laboratory tests to improve its diagnostic performance. These results in the estimation group were validated in 120 patients with chronic hepatitis C (validation group).

MATERIALS AND METHODS

Patients

In 285 consecutive patients with chronic hepatitis C virus infection, liver biopsy was performed at Fujita Health University Hospital from July 2004 to February 2009 (Table 1).

From July 2004 to September 2007, 165 of these patients (estimation group) were used to develop the Fibro-Stiffness index. From October 2007 to February 2009, the other 120 patients (validation group) were used to validate the diagnostic performance of the Fibro-Stiffness index. The usefulness of the Fibro-Stiffness index was compared with LS, the APRI, the Forns index and the FibroIndex.

Clinical data were collected within 3 d of liver biopsy. Sections were stained with hematoxylin-eosin stain and Azan stain. Liver biopsy specimens were assessed by 2 hepatologists (Yoshioka K and Kawabe N). When fibrosis stages evaluated by 2 hepatologists differed, the higher fibrosis stage was adopted. Fibrosis stage, determined according to the METAVIR score, was classified as F0, no fibrosis; F1, portal fibrosis without septa; F2, few septa; F3, numerous septa without cirrhosis; and F4, cirrhosis.

Liver stiffness measurement

LS measurement by transient elastography was performed with FibroScan (EchoSens, Paris, France) within a week of liver biopsy. FibroScan is equipped with a probe including an ultrasonic transducer and a vibrator. A vibration of mild amplitude and low frequency is transmitted from the vibrator placed on the body surface toward the liver through the intercostal space. The vibration induces an elastic shear wave that propagates through the liver tissue. The pulse-echo ultrasound acquisitions follow the propagation of the shear wave and determine its velocity. The velocity is directly related to tissue stiffness; the harder the tissue, the faster the shear wave propagates. LS is calculated from velocity and expressed in kilopascals (kPa). Ten successful acquisitions were performed on each measurement, and the median value was adopted as representative of LS.

Statistical analysis

The end point was the discrimination between F0 and F1-4, between F0-1 and F2-4, between F0-2 and F3-4 and between F0-3 and F4, using a combination of LS and relevant biochemical or hematological variables. Variables that correlated significantly with fibrosis stage in the estimation group were identified by univariate analyses (analysis of variance). Then the independent predictors of fibrosis stage were assessed by multiple regression analysis (ordinal logistic regression). A predictive index was constructed by modeling the values of the independent variables and their coefficient of regression. The difference of fibrosis indices between neighboring fibrosis stages were estimated by the Tukey-Kramer test. The optimal discriminate cut-off values of each fibrosis index were assessed from the area under the receiver operating characteristics (ROC) curves (AUCs). The optimal discriminating cut-off values were determined at the maximum total of sensitivity and specificity. The statistical analysis was performed by JMP® (SAS Institute, Cary, NC, USA).

RESULTS

Development of the Fibro-Stiffness Index

LS, platelet count, prothrombin time, aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, γ globulin, total cholesterol and hyaluronic acid were significantly correlated with fibrosis stage in the estimation group (Table 2). Among these variables, LS ($P < 0.0001$), platelet count ($P = 0.0408$), and prothrombin time ($P = 0.0066$) were identified as independent predictors of fibrosis stage by multiple regression analysis (Table 3). By multiple regression analysis, the estimated values of LS, platelet count and prothrombin time were calculated as -0.2662, 0.0749 and 0.0560, respectively. The optimum intercept was also calculated as 5.7710. Thus the Fibro-Stiffness index was constructed with these 3 variables: Fibro-Stiffness index = 5.7710 - 0.2662 [LS (kPa)] + 0.0749 [platelet count ($\times 10^4$/mL)] + 0.0560 [prothrombin time (%)].

Table 1 Characteristics of the 165 patients in the estimation group and the 120 patients in the validation group (mean ± SD)

	All patients (n = 285)	Estimation group (n = 165)	Validation group (n = 120)	P-value
Male gender, n (%)	149 (52.3)	92 (55.8)	57 (47.5)	NS
Age (yr)	52.4 ± 13.3	53.2 ± 12.6	51.5 ± 14.2	NS
Liver stiffness (kPa)	9.99 ± 6.99	10.29 ± 7.33	9.58 ± 6.51	NS
Platelet count (× 10^4/mL)	16.54 ± 5.28	16.53 ± 5.41	16.57 ± 5.13	NS
Prothrombin time (%)	9.35 ± 11.3	92.4 ± 10.2	95.1 ± 12.6	NS
AST (IU/L)	52.5 ± 34.0	53.0 ± 34.6	51.8 ± 33.4	NS
ALT (IU/L)	70.8 ± 52.6	72.4 ± 54.9	68.7 ± 49.4	NS
Total protein (g/dL)	7.81 ± 0.52	7.79 ± 0.49	7.85 ± 0.57	NS
Albumin (g/dL)	4.31 ± 0.34 (n = 283)	4.31 ± 0.31 (n = 163)	4.31 ± 0.38	NS
γ-GTP (IU/L)	59.8 ± 62.0	58.1 ± 57.9	62.1 ± 67.5	NS
γ-globulin (g/dL)	1.57 ± 0.41 (n = 276)	1.54 ± 0.38 (n = 146)	16.1 ± 0.44	NS
Total cholesterol (mg/dL)	178.1 ± 31.9	177.2 ± 31.1	179.5 ± 33.0	NS
Hyaluronic acid (ng/mL)	104.1 ± 128.3 (n = 281)	114.5 ± 140.0 (n = 161)	90.0 ± 109.3	NS
Fibrosis stage, n (%)				
F0	28 (9.8)	14 (8.5)	14 (11.7)	
F1	85 (29.8)	52 (31.5)	33 (27.5)	
F2	82 (28.8)	42 (25.5)	40 (33.3)	
F3	53 (18.6)	33 (20.0)	20 (16.7)	
F4	37 (13.0)	24 (14.5)	13 (10.8)	

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; NS: Not significant.

Table 2 Variables associated with fibrosis stage in the estimation group (165 patients) in univariate analysis (mean ± SD)

	F0 (n = 14)	F1 (n = 52)	F2 (n = 42)	F3 (n = 33)	F4 (n = 24)	P-value
Liver stiffness (kPa)	5.58 ± 1.96	5.76 ± 1.91	9.02 ± 4.11	13.40 ± 4.95	20.77 ± 10.81	< 0.0001
Platelet count (× 10^4/mL)	20.80 ± 4.77	18.79 ± 5.19	15.66 ± 4.54	15.72 ± 5.58	11.75 ± 2.63	< 0.0001
Prothrombin time (%)	101.8 ± 10.3	96.1 ± 7.9	92.5 ± 7.9	88.7 ± 10.5	83.6 ± 9.6	< 0.0001
AST (IU/L)	30.3 ± 17.3	40.1 ± 23.7	46.9 ± 31.8	73.2 ± 40.3	77.0 ± 33.3	< 0.0001
ALT (IU/L)	44.4 ± 34.2	58.1 ± 47.1	62.5 ± 42.9	103.0 ± 72.9	94.6 ± 47.9	< 0.0001
Total protein (g/dL)	7.61 ± 0.30	7.78 ± 0.58	7.74 ± 0.44	7.83 ± 0.44	7.91 ± 0.48	0.4459
Albumin (g/dL)	4.52 ± 0.26 (n = 13)	4.44 ± 0.23 (n = 51)	4.32 ± 0.32	4.15 ± 0.24	4.12 ± 0.38	< 0.0001
γ-GTP (IU/L)	40.07 ± 26.10	57.52 ± 84.73	49.29 ± 32.91	75.79 ± 47.91	61.25 ± 40.46	0.2409
γ-globulin (g/dL)	1.22 ± 0.25 (n = 12)	1.44 ± 0.32 (n = 45)	1.52 ± 0.33 (n = 36)	1.62 ± 0.35 (n = 30)	1.83 ± 0.43 (n = 23)	< 0.0001
Total cholesterol (mg/dL)	179.5 ± 29.4	186.7 ± 31.5	173.8 ± 32.0	176.4 ± 26.8	162.3 ± 30.4	0.0251
Hyaluronic acid (ng/mL)	59.6 ± 83.1	49.64 ± 41.1 (n = 50)	110.3 ± 113.3	136.0 ± 138.4	266.6 ± 217.9 (n = 23)	< 0.0001

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase.

Table 3 Multiple regression predicting liver fibrosis stage with liver stiffness and laboratory data in the estimation group

	Estimated value	Standard error	χ^2	P-value
Liver stiffness (n = 165)	-0.2661602	0.0481713	30.53	< 0.0001
Platelet count (n = 165)	0.0748652	0.0366051	4.18	0.0408
Prothrombin time (n = 165)	0.0560460	0.0206514	7.37	0.0066
AST (n = 165)	-0.0093853	0.0100069	0.88	0.3483
ALT (n = 165)	0.0007594	0.0058897	0.02	0.8974
Albumin (n = 163)	-1.1130330	0.7353340	2.29	0.1301
γ-globulin (n = 146)	-0.6349751	0.5201549	1.49	0.2222
Total cholesterol (n = 165)	0.0011393	0.0057519	0.04	0.8430
Hyaluronic acid (n = 161)	-0.0019629	0.0017064	1.32	0.2500

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase.

Comparison of Fibro-Stiffness index with LS, the APRI, the Forns index and the FibroIndex in the estimation group

Fibro-Stiffness index was compared with LS, the APRI, the Forns index and the FibroIndex in the estimation group (Figure 1). The values of Fibro-Stiffness index and LS significantly differed between neighboring fibrosis stages except F0-F1 (Figure 1A and B). The APRI did not significantly differ between any neighboring stages (Figure 1C). The Forns index significantly differed only between F1 and F2 (Figure 1D). The FibroIndex significantly differed between F1 and F2 and between F3 and F4 (Figure 1E).

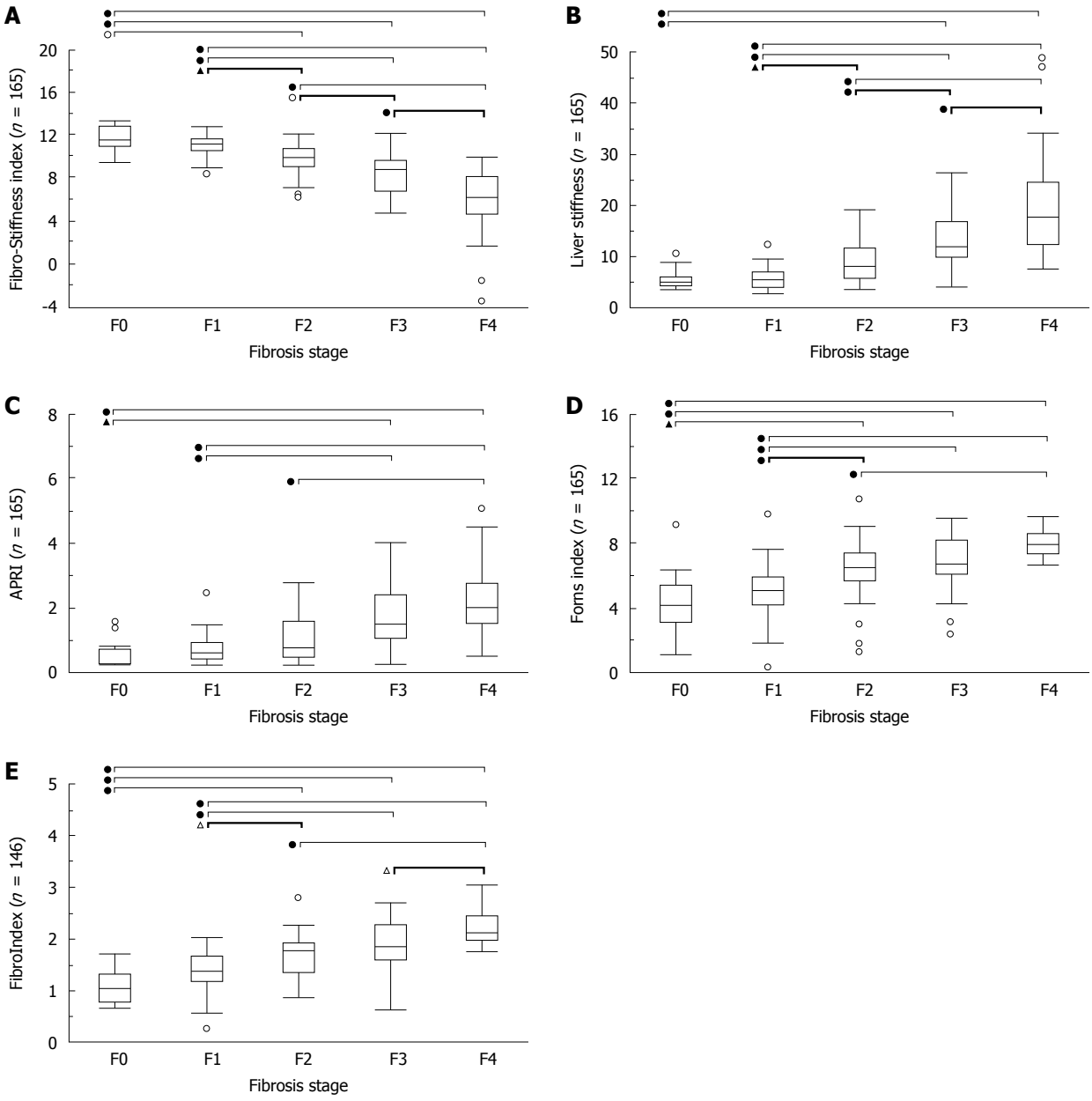

Figure 1 Correlation between 5 noninvasive methods for assessment of fibrosis and stage of fibrosis in the estimation group. A: Fibro-Stiffness index: ρ = -0.7626, *P* < 0.0001; B: Liver stiffness measured by FibroScan: ρ = -0.7340, *P* < 0.0001; C: Aspartate aminotransferase-to-platelet ratio index (APRI): ρ = 0.6008, *P* < 0.0001; D: Forns index: ρ = 0.6175, *P* < 0.0001; E: FibroIndex: ρ = 0.6496, *P* < 0.0001. The top and bottom of each box represent the 25th and 75th percentiles, giving the interquartile range. The line through the box indicates the median, and the error bars indicate the 10th and 90th percentiles. The closed circles, the closed triangles, the open circles and the open triangles indicate *P*-values of 0.001, 0.005, 0.01 and 0.05, respectively.

ROC analysis for comparison of diagnostic performance of the Fibro-Stiffness index with LS, the APRI, the Forns index and the FibroIndex in the estimation group

The ROC analysis of the Fibro-Stiffness index, LS, the APRI, the Forns index and the FibroIndex was performed to discriminate between fibrosis stages (Table 4). The AUC of the Fibro-Stiffness index was the highest for discriminating F ≥ 2, F ≥ 3 and F = 4 among the 5 examined methods. The AUC of the FibroIndex was the highest for discriminating F ≥ 1.

Optimal discriminating cut-off values of the Fibro-Stiffness index, LS, the APRI, the Forns index and the FibroIndex were determined by ROC analysis. The cut-off values of the Fibro-Stiffness index for F ≥ 1, F ≥ 2, F ≥ 3 and F = 4 were 11.09, 10.12, 9.87 and 8.51, respectively (Table 4). The diagnostic performance was assessed by sensitivity, specificity, accuracy, positive and negative predictive values, and likelihood ratio. Regarding accuracy, the values of the Fibro-Stiffness index for F ≥ 2, F ≥ 3 and F = 4 were the highest among the 5 examined methods. The value of the APRI was the highest for F ≥ 1.

Improvement of diagnostic performance by combination of the Fibro-Stiffness index with laboratory tests

The negative predictive value for F ≥ 1 and the positive

Table 4 Assessment of liver fibrosis stages classification by liver fibrosis indices

	AUCs (95% CI)	Optimal cutoff value	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio
F ⩾ 1 (F0 *vs* F1-2-3-4)								
Fibro-Stiffness index	0.82 (0.715-0.928)	11.07	77.5	78.6	77.6	97.5	24.4	3.62
Fibro-Stiffness index and AST	Non	11.07 and 51 (IU/L)	85.4	78.6	84.8	97.7	33.3	3.99
Liver stiffness	0.77 (0.666-0.867)	6.6 (kPa)	64.2	85.7	66.1	97.8	18.2	4.49
APRI	0.81 (0.680-0.934)	0.44	88.1	64.3	86.1	96.4	33.3	2.47
Forns index	0.77 (0.640-0.908)	4.72	82.1	71.4	81.2	96.9	27.0	2.87
FibroIndex	0.85 (0.757-0.932)	1.19	82.1	75.0	81.5	97.3	27.3	3.28
F ⩾ 2 (F0-1 *vs* F2-3-4)								
Fibro-Stiffness index	0.90 (0.847-0.943)	10.12	78.8	89.4	83.0	91.8	73.8	7.43
Liver stiffness	0.88 (0.826-0.929)	7.1 (kPa)	80.8	80.3	80.6	86.0	73.6	4.10
APRI	0.78 (0.714-0.851)	1.06	64.6	84.8	72.7	86.5	61.5	4.27
Forns index	0.82 (0.757-0.890)	6.22	76.8	83.3	79.4	87.4	70.5	4.61
FibroIndex	0.82 (0.754-0.888)	17.00	74.2	80.7	76.7	85.7	66.7	3.84
F ⩾ 3 (F0-2 *vs* F3-4)								
Fibro-Stiffness index	0.90 (0.851-0.953)	9.87	93.0	79.6	84.2	70.7	96.7	4.56
Liver stiffness	0.90 (0.856-0.952)	9.6 (kPa)	87.7	82.4	84.2	72.5	92.7	4.98
APRI	0.84 (0.767-0.904)	1.13	80.7	80.6	80.6	68.7	88.8	4.15
Forns index	0.80 (0.732-0.874)	6.36	82.5	70.4	74.5	59.5	88.4	2.78
FibroIndex	0.85 (0.749-0.901)	1.85	73.6	80.6	81.4	68.4	84.3	3.80
F = 4 (F0-1-2-3 *vs* F4)								
Fibro-Stiffness index	0.92 (0.871-0.965)	8.51	91.7	83.7	84.8	48.9	98.3	5.62
Fibro-Stiffness index and HA	Non	8.51 and 68 (ng/mL)	91.3	87.9	88.4	55.3	98.4	7.26
Liver stiffness	0.90 (0.844-0.957)	11.6 (kPa)	91.7	78.0	80.0	41.5	98.2	4.17
APRI	0.84 (0.759-0.915)	1.30	91.7	74.5	77.0	37.9	98.1	3.59
Forns index	0.87 (0.816-0.923)	7.07	95.8	75.9	78.8	40.4	99.1	3.97
FibroIndex	0.89 (0.830-0.943)	1.90	91.3	78.0	80.1	43.8	98.0	4.16

AUCs: Area under the receiver operating characteristics; CI: Confidence interval; HA: Hyaluronic acid; AST: Aspartate aminotransferase; APRI: Aminotransferase-to-platelet ratio index.

predictive value for F4 with the Fibro-Stiffness index were rather low. Thus a combination of the Fibro-Stiffness index with AST, ALT, albumin, γ globulin, total cholesterol and hyaluronic acid, which were correlated with fibrosis stages and not included in the Fibro-Stiffness index was examined to improve diagnostic performance in each fibrosis stage. Optimal discriminating cut-off values of these laboratory tests for F ⩾ 1 and F = 4 were calculated by ROC analysis (not shown). In F ⩾ 1, the best combination for improvement of diagnostic performance was the Fibro-Stiffness index ⩽ 10.09 or AST ⩾ 51 IU/L. The negative predictive value for F ⩾ 1 was improved by this combination compared to the Fibro-Stiffness index alone, although it was same as that of the APRI (Table 4). The combination of the Fibro-Stiffness index ⩽ 8.51 and serum hyaluronic acid ⩾ 68 ng/mL was the best combination for F = 4. The negative predictive value for F4 was improved by this combination, and was the highest among the 6 examined methods.

Validation of performance of the Fibro-Stiffness index, its combination with AST for F ⩾ 1, and its combination with hyaluronic acid for F4

The results in the estimation group were validated in the validation group of 120 patients with chronic hepatitis C (Table 5). The AUC of the Fibro-Stiffness index was the highest for F ⩾ 3 and F = 4 among the 5 examined methods. The AUC of the FibroIndex was the highest for F ⩾ 1 and that of LS was the highest for F ⩾ 2.

The accuracy of the Fibro-Stiffness index for F ⩾ 3 and F = 4 was 86.7% and 85.8%, respectively, similar to the values in estimation group, and the highest value among all the 5 methods. For F ⩾ 1, the accuracy of the FibroIndex was the highest. For F ⩾ 2, the accuracy of LS was the highest.

The combination of the Fibro-Stiffness index and AST for F ⩾ 1 improved the negative predictive value, although it was lower than that of the FibroIndex, and the accuracy was lower than those of the APRI and the FibroIndex. The combination of the Fibro-Stiffness index and hyaluronic acid for F = 4 improved the positive predictive value, and its accuracy and positive predictive value were the highest among all the 6 examined methods.

DISCUSSION

In the present study, we constructed a new fibrosis index for non-invasive assessment of liver fibrosis, the Fibro-Stiffness index, using LS, platelet count and prothrombin time. LS measured by FibroScan has been reported to correlate with stage of liver fibrosis in various liver diseases[13-24]. Previous studies also confirmed that platelet count and prothrombin time also correlated with stage of liver fibrosis[6,11,26-29]. A decrease in the platelet count is caused by splenomegaly and reduced production of thrombopoietin, accompanied by the advance of liver fibrosis. Prolongation of prothrombin time is caused by reduced production of coagulation factors by the liver

Table 5 Validation of liver fibrosis stages classification by liver fibrosis indices

	AUCs (95% CI)	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)	Positive likelihood ratio
F ≥ 1 (F0 *vs* F1-2-3-4)							
Fibro-Stiffness index	0.80 (0.712-0.878)	73.6	78.6	74.2	94.0	27.7	3.52
Fibro-Stiffness index and AST	Non	78.3	78.6	78.3	96.5	32.4	3.64
Liver stiffness	0.82 (0.727-0.907)	63.2	85.7	65.8	97.1	23.5	4.42
APRI	0.80 (0.704-0.899)	87.7	35.7	81.7	91.2	27.8	1.36
Forns index	0.77 (0.659-0.880)	72.4	71.4	72.3	95.0	25.6	2.53
FibroIndex	0.83 (0.723-0.937)	89.6	50.5	85.8	93.1	38.9	1.79
F ≥ 2 (F0-1 *vs* F2-3-4)							
Fibro-Stiffness index	0.82 (0.750-0.898)	65.7	96.6	76.7	90.6	63.8	10.31
Liver stiffness	0.85 (0.787-0.920)	76.7	83.0	79.2	87.5	69.6	4.51
APRI	0.82 (0.741-0.893)	50.7	89.4	65.8	88.1	53.8	4.76
Forns index	0.78 (0.700-0.864)	58.3	80.9	67.2	82.4	55.9	3.05
FibroIndex	0.79 (0.713-0.875)	69.5	83.0	74.2	86.2	62.9	4.02
F ≥ 3 (F0-2 *vs* F3-4)							
Fibro-Stiffness index	0.93 (0.876-0.986)	93.9	83.9	86.7	68.9	97.3	5.84
Liver stiffness	0.92 (0.871-0.977)	81.8	82.8	82.5	64.3	92.3	4.75
APRI	0.83 (0.753-0.922)	75.8	81.6	80.0	61.0	89.9	4.12
Forns index	0.84 (0.759-0.912	78.1	71.3	73.1	50.0	90.0	2.72
FibroIndex	0.85 (0.772-0.928)	76.6	80.5	80.0	60.5	90.9	4.03
F = 4 (F0-1-2-3 *vs* F4)							
Fibro-Stiffness index	0.97 (0.934-0.997)	100	84.1	85.8	43.3	100	6.29
Fibro-Stiffness index and HA	Non	100	86.9	88.3	48.1	100	7.63
Liver stiffness	0.97 (0.942-0.999)	100	83.2	85.0	41.9	100	5.94
APRI	0.92 (0.867-0.972)	100	75.7	78.3	33.3	100	4.12
Forns index	0.88 (0.798-0.968)	83.3	72.9	73.9	25.6	97.5	3.07
FibroIndex	0.92 (0.874-0.971)	100	77.6	80.0	35.0	100	4.46

AUCs: Area under the receiver operating characteristics; CI: Confidence interval; HA: Hyaluronic acid; AST: Aspartate aminotransferase; APRI: Aminotransferase-to-platelet ratio index.

with advanced fibrosis. The Fibro-Stiffness index, which combines these 3 factors, was shown to be a highly accurate index to estimate fibrosis stage in chronic hepatitis C.

So far, several non-invasive fibrosis indices such as the APRI[11], the Forns index[6], the FibroIndex[12], and the FibroTest[7] have been developed. The Fibro-Stiffness index showed its superior correlation with fibrosis stage compared with the APRI, the Forns index and the FibroIndex. The Fibro-Stiffness index and LS showed a significant difference between neighboring fibrosis stages except between F0 and F1 in the estimation group. The AUC of the Fibro-Stiffness index was the highest among the 5 examined methods for F ≥ 2, F ≥ 3 and F = 4 in the estimation group, and for F ≥ 3 and F = 4 in the validation group. The AUCs of the APRI, the Forns index and the FibroIndex for predicting F4 in the present study were similar to the values reported in their respective original manuscripts (APRI, 0.88; Forns index, 0.81; FibroIndex, 0.86)[6,11]. Therefore, the results of the present study can be considered to be appropriate. The superiority of the Fibro-Stiffness index was further demonstrated by the accuracy values. The accuracy of the Fibro-Stiffness index was highest for F ≥ 2, F ≥ 3 and F = 4 in both the estimation group and validation group.

Although the Fibro-Stiffness index was shown to be a highly accurate index, the positive predictive value was rather low for F4. A combination of the Fibro-Stiffness index and hyaluronic acid was shown to improve the diagnostic performance. Serum hyaluronic acid has been reported to be useful for diagnosis of liver fibrosis and cirrhosis[8,30]. In the estimation group and in the validation group, both the accuracy and positive predictive value of the combination of the Fibro-Stiffness index and hyaluronic acid were higher than those of the Fibro-Stiffness index alone, and were the highest among all the 6 examined methods. The fact that a combination of the Fibro-Stiffness index and hyaluronic acid enables us to diagnose F4 with a sensitivity of 91%-100% and positive predictive value of 48%-57% is important, because the risk of hepatocellar carcinoma or bleeding from esophageal varices is high in patients with F4[2,3].

For predicting F ≥ 1, the Fibro-Stiffness index was inferior to the other fibrosis indices in terms of sensitivity, accuracy and negative predictive value. The combination of Fibro-Stiffness index with AST improved sensitivity, accuracy and negative predictive value in both the estimation group and the validation group. However, the combination of Fibro-Stiffness index with AST was still inferior to the APRI in the estimation group, and inferior to the FibroIndex and the APRI in the validation group. Further investigation is necessary to improve the diagnostic efficacy of the Fibro-Stiffness index for F ≥ 1.

In chronic viral hepatitis, the presence of significant fibrosis (F ≥ 2) indicates the need for antiviral therapies. The Fibro-Stiffness index showed a highly accurate diagnostic performance for F ≥ 2 in both the estimation group and validation group. Thus the patients with a Fibro-Stiffness index of ≥ 10.12 which indicate F ≥ 2

will be candidates for liver biopsy or interferon treatment.

In conclusion, a new fibrosis index for non-invasive assessment of liver fibrosis, the Fibro-Stiffness index, was constructed using LS measured by FibroScan, platelet count and prothrombin time and was validated. The Fibro-Stiffness index demonstrated superior diagnostic performance to LS alone, the APRI, the Forns index and the FibroIndex for F $\geqslant$ 2, F $\geqslant$ 3 and F = 4. The diagnostic performance of the Fibro-Stiffness index for F4 was further improved by combination with hyaluronic acid levels.

COMMENTS

Background

The stage of liver fibrosis is important for clinical management of chronic hepatitis C, since the treatment and prognosis of chronic hepatitis depend on the fibrosis stage. Liver biopsy is the gold standard for the assessment of fibrosis stage. However, it is an invasive and expensive procedure, and its accuracy is sometimes questionable.

Research frontiers

A number of non-invasive fibrosis indices, such as the aminotransferase-to-platelet ratio index (APRI), the Forns index and the FibroIndex have been proposed for assessment of liver fibrosis. Transient elastography with the use of a new apparatus, FibroScan, for measurement of liver stiffness (LS) was developed. LS has been reported to correlate with liver fibrosis in various liver diseases. So far no fibrosis indices incorporating LS have been reported. In the present study, we developed a new non-invasive fibrosis index, the Fibro-Stiffness index, which incorporated LS.

Innovations and breakthroughs

The Fibro-Stiffness index consists of LS, platelet count and prothrombin time. In the present study, its usefulness was compared with LS, the APRI, the Forns index and the FibroIndex. The diagnostic performance of the Fibro-Stiffness index was superior to other indices. Furthermore, the diagnostic performance of the Fibro-Stiffness index for F4 was further improved by combination with hyaluronic acid.

Applications

Using the Fibro-Stiffness index, it is possible to assess the stage of liver fibrosis of patients with chronic hepatitis C non-invasively, accurately and quantitatively. Therefore, the Fibro-Stiffness index is useful not only for the diagnosis of stage of liver fibrosis but also for the assessment of regression of liver fibrosis by interferon treatment in patients with chronic hepatitis C.

Terminology

Fibro-Stiffness index: a new non-invasive fibrosis index which we developed in the present study and consists of LS, platelet count and prothrombin time. Its diagnostic performance is superior to other indices.

Peer review

The authors proposed a novel index for non-invasive assessment of hepatic fibrosis. Its reliability was validated on another group of patients. I think the index is clinically useful and significant.

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BRIEF ARTICLE

Helicobacter species and common gut bacterial DNA in gallbladder with cholecystitis

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Abstract

AIM: To analyze the association between *Helicobacter* spp. and some common gut bacteria in patients with cholecystitis.

METHODS: A nested-polymerase chain reaction (PCR), specific to 16S rRNA of *Helicobacter* spp. was performed on paraffin-embedded gallbladder samples of 100 cholecystitis and 102 control cases. The samples were also analyzed for some common gut bacteria by PCR. Positive samples were sequenced for species identification.

RESULTS: *Helicobacter* DNA was found in seven out of 100 cases of acute and chronic cholecystitis. Sequence analysis displayed *Helicobacter pullorum* (*H. pullorum*) in six cases and *Helicobacter pylori* in one; *H. pullorum* was only found in cases with metaplasia. Control samples were negative for *Helicobacter* spp. and some common gut bacteria. There was a significant difference (P = 0.007) between cholecystitis and control samples for *Helicobacter* DNA.

CONCLUSION: A possible relationship was detected between *Helicobacter* DNA and cholecystitis. Further serological and immunohistochemical studies are needed to support these data.

Key words: *Helicobacter*; Gallbladder; Cholecystitis; 16S rRNA; Polymerase chain reaction

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INTRODUCTION

The most well-known member of the *Helicobacter* genus, *Helicobacter pylori* (*H. pylori*), is classified as a type 1 carcinogen[1], and infects the human stomach and causes gastritis, peptic ulcer disease and gastric cancer. Besides *H. pylori*, the genus *Helicobacter* contains more than 25 species[2], many of which cause extragastric diseases in humans and animals[3-10]. These are named enterohepatic *Helicobacter* species (EHS) or EHS and colonize the hepatobiliary tract of humans, and include *Helicobacter hepaticus* (*H. hepaticus*), *Helicobacter bilis* (*H. bilis*), *Helicobacter rappini* (*H. rappini*), *Helicobacter ganmani* (*H. ganmani*) and *Helicobacter pullorum* (*H. pullorum*). Several of these EHS are associated with the pathogenesis of chronic biliary disorders, such as cholecystitis, cholelithiasis, gallbladder carcinoma and bile tract carcinoma and some liver diseases, such as primary

sclerosing cholangitis, primary biliary cirrhosis and hepatocellular carcinoma[11-14]. Moreover, chronic pancreatitis and pancreatic cancer, as well as inflammatory bowel diseases in humans have also been reported to be positive for EHS in various polymerase chain reaction (PCR)-based studies[15-17].

Chronic cholecystitis is the most prevalent disease in various populations in industrialized countries[18]. During the 20 years from 1965-1969 to 1985-1989, the mortality from gallbladder cancer increased by 30% in Sweden. However, not all high-risk European countries showed such an increase and the mortality decreased in some countries[19]. Chronic cholecystitis is commonly associated with gallstone disease[20] and some studies have shown that cholecystitis and gallstones can cause epithelial hyperplasia of the gallbladder mucosa or cancer, and various bacterial genomes have been detected in gallbladder carcinoma tissue[21]. Moreover, a recent study has shown that *H. pylori* can damage human gallbladder epithelial cells *in vitro*, and could be the key factor that leads to clinical cholecystitis[22]. Some studies have revealed the presence of bile-resistant EHS in the gallbladder mucosa and in gallstones. It has been shown that the presence of *H. pylori* and EHS in bile might represent a risk factor for bile stone formation[4,23-26]. One study has clearly demonstrated the presence of a mixed bacterial population in gallstones[4]. *Salmonella typhi* is another bacterial pathogen of the biliary tree in human gallstones and gallbladder cancer[27,28]. *Salmonella* biofilm has been shown on human gallstones[29]. Moreover, *Campylobacter* spp. have also been detected in bile and epithelial samples in cholecystolithiasis[30].

H. pylori, *H. pullorum* and *H. bilis* have been isolated from humans with gallbladder disease such as cholecystitis, cholelithiasis[9,31,32], gallbladder carcinoma and bile tract carcinoma[33]. A possible relationship between chronic cholecystitis and *Helicobacter* DNA has been shown by some investigators[9,31,32,34] but, as far as we are aware, there has been no study published on Scandinavian patients with cholecystitis. Therefore, we examined the relationship between *Helicobacter* spp. and some common gut bacteria in Swedish patients with cholecystitis.

MATERIALS AND METHODS

Patients and histological methods

We re-examined the gallbladders from 100 cholecystitis patients from 2006-2007 (mean age: 48 years; range: 20-84 years; 35 male, 65 female) and 102 control patients (mean age: 58 years; range: 11-85 years; 54 male, 48 female) from 1999 to 2009, taken from the files of the Department of Pathology, Lund University Hospital. Of the 100 cholecystitis samples, 50 were acute (mean age: 55 years; range: 23-81 years; 22 male, 28 female), and 50 were chronic (mean age: 44 years; range: 20-84 years; 13 male, 37 female). Among the 50 patients with acute cholecystitis, 34 cases (median age: 56 years; range: 23-79 years; 15 male, 19 female) were without metaplasia and 16 (median age: 54 years; range: 36-81 years; 7 male, 9 female) had metaplasia. Among the 50 patients with chronic cholecystitis, 27 cases (median age: 45 years; range 20-84 years; 8 male, 19 female) were without metaplasia and 23 (median age: 42 years; range: 20-71 years; 5 male, 18 female) had metaplasia. As control samples, we used 18 normal gallbladders from patients with pancreatic malignancies reported elsewhere[17], and 84 consecutive patients with normal gallbladders from 1999 to 2009 (median age: 61 years; range: 11-85 years; 44 male, 40 female). There was no metaplasia in these gallbladders. The diagnosis was: six hepatocellular carcinoma, 40 liver metastases (mainly colorectal), four intestinal carcinoids, three liver carcinoid metastases, seven focal nodal hyperplasias, three bile duct cysts, one gallbladder adenoma, three splenomegalies, two pancreatic neuroendocrine malignancies, one benign pancreatic cyst, one adrenal carcinoma, and 13 normal gallbladders with no other diagnosis.

Two to five sections were taken from each case, and one section from the ductus cysticus. Sections that showed mucosal metaplasia were stained with Alcian blue-periodic acid Schiff (AB-PAS), pH 2.5, and Warthin-Starry silver stain for *Helicobacter* spp. One section was immunostained with anti-*H. pylori* antibody (DAKO, Glostrup, Denmark; diluted 1:300) according to Apostolov *et al*[9]. Mucosa was cut from the paraffin blocks with the tip of a scalpel by careful comparison with the slides. Areas with gastric metaplasia, if present, were included in the samples. The Research Ethics Committee at Lund University approved this study (permit number 588/2006).

DNA extraction

DNA was extracted from approximately 5 mg of each paraffin-embedded gallbladder tissue sample. To ascertain that epithelium was included, two pieces, each of 2-3 mg, were taken from each case. Paraffin-embedded gallbladder samples were de-embedded as previously described[10]. Gallbladder tissue samples were de-embedded by heating at 60℃ for 10 min, followed by washing in xylene for 2 × 5 min. The specimens were rehydrated through graded ethanol (99% and 95% for 2 × 5 min and 70% for 5 min), and finally washed for 5 min in double-distilled water. DNA was extracted by a QIAamp DNA Mini Kit tissue protocol (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The extracts (200 μL total volume) were combined, and 5 μL of the mixtures was analyzed by PCR.

Helicobacter-specific PCR

DNA extracts were amplified in a GeneAmp 2700 Thermocycler (Applied Biosystems, Foster City, CA, USA) using a semi-nested PCR assay specific for *Helicobacter* 16S rDNA, as previously described[11], using primers 1F (5'CTATGACGGGTATCCGGC3'), 1R (5'CTCACGACACGAGCTGAC3') and 2R (5'TCGCCTTCGCAATGAGTATT3'). Primers 1F and 1R were used in the first step, whereas primers 1F and 2R were used in the second step. The reaction mixture of the first step (25 μL) contained 0.5 μmol/L each primer (1F and 1R), 0.8 mmol/L

each dNTP (Amersham Biosciences, Uppsala, Sweden), 1 × chelating buffer, 2.5 mmol/L $MgCl_2$, 0.05% casein, 0.05% formamid, 1.25 U r*Tth* DNA polymerase (Applied Biosystems), and 5 μL extracted DNA. *H. pylori* (CCUG 17 874) was used as a positive control in all PCR reactions. The amplification conditions for the first step were 94℃ for 2 min; 30 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s; and finally 72℃ for 5 min. The reaction mixture of the second step (25 μL) contained 0.5 μmol/L each primer (1F and 2R), 0.2 mmol/L each dNTP, 1 × buffer Ⅱ, 2.5 mmol/L $MgCl_2$, 1.0 U Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and 2 μL 10 × diluted PCR product from the first step. The 416-bp PCR products were visualized by 1.3% agarose gel electrophoresis.

Amplification of non-Helicobacter bacteria

Enterobacteriaceae-, *Bacterioides-Prevotella* group- *and Enterococcus*-specific PCRs were performed. The reaction mixture and amplification conditions, except for annealing temperatures, for non-*Helicobacter* PCR assays were the same as in the first step of the semi-nested *Helicobacter* PCR. The annealing temperatures and primers used for detection of Enterobacteriaceae, *Bacterioides-Prevotella* group and *Enterococcus* were as described before[11]. Primers Eco1457F (5'CATTGACGTTACCCGCAGAAGAAGC3') and Eco1652R (5'CTCTACGAGACTCAAGCTTGC3') were used to amplify Enterobacteriaceae and primers Ent1F (5'TACTGACAAACCATTCATGATG3') and Ent2R (5'AACTTCGTCACCAACGCGAAC3') were used to amplify *Enterococcus,* whereas primers Bac303F (5'GAAGGTCCCCCACATTG3') and Bac708R (5'CAATCGGAGTTCTTCGTG3') were used to amplify the *Bacteroides-Prevotella* group. As positive controls, *Escherichia coli* (CCUG 17620), *Bacteroides fragilis* (CCUG 4856), and *Enterococcus faecalis* (CCUG 9997) were used in all PCR reactions. The 112-bp PCR product of *Enterococcus,* 418-bp product of *Bacteroides* and 195-bp product of Enterobacteriaceae were visualized by 1.3% agarose gel electrophoresis.

DNA sequence analysis

Helicobacter-specific PCR products were purified from agarose gels using the Montage DNA Gel Extraction Kit (Millipore, Bedford, MA, USA) according to the manufacturer's instructions. DNA sequence reactions were performed using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit version 3.0 (Applied Biosystems), as described by Tolia *et al*[10]. Products of the sequence reaction were aligned and the closest homologous DNA was identified by BLASTn-analysis.

Statistical analysis

Statistical analyses were done by χ^2 and Fisher's exact tests. $P < 0.05$ was considered to be significant.

RESULTS

Histology

Little metaplasia was detected in the sections and only a few glands or a few cells displayed gastric (antrum) metaplasia and/or acid mucin. Acid or neutral mucins were often seen only in parts of the epithelial cell cytoplasm. The AB-PAS staining method for metaplasia revealed among the chronic cases three with only gastric metaplasia (neutral mucosubstances), five with only non-gastric metaplasia (acid mucosubstances), and 15 with both types. For acute cholecystitis, these figures were two, five and nine, respectively (Table 1). The two types of metaplasia are displayed in Figure 1. Whartin-Starry staining and immunohistochemistry for *H. pylori* were negative in all studied specimens. The *H. pylori*-positive specimen was from a case of acute cholecystitis with extensive necrosis, but with a small area of preserved epithelium without metaplasia, from which the sample was taken.

Table 1 Number of cases with metaplasia in patients with cholecystitis

	Acute	Chronic
Gastric	2	3
Non-gastric	5	5
Both	9	15
None	34	27
Total	50	50

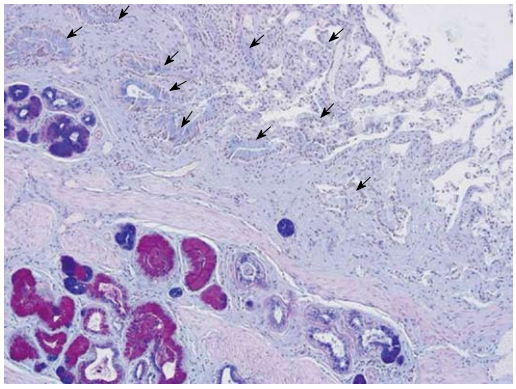

Figure 1 Histological section of ductus cysticus from a patient with chronic cholecystitis. Low-power view displaying antrum-type (red) and intestinal-type (blue) mucous metaplasia in glands. The ductus lumen is seen in the opposite corner of the photo with folds of the mucosa layer covered by epithelium without metaplasia, arrows (no intense color). Alcian blue-periodic acid Schiff staining.

Helicobacter-specific PCR assay and sequencing results

Using the *Helicobacter*-specific PCR assay and agarose electrophoresis, *Helicobacter* DNA was detected in 7/100 of gallbladder specimens of patients with cholecystitis. There were 4/50 (8%) and 3/50 (6%) samples positive for *Helicobacter* spp. among acute and chronic cholecystitis patients, respectively. Six samples showed 98-99% sequence similarity to *H. pullorum* and one to *H. pylori* (Table 2). *H. pullorum* was only found in cases with metaplasia, in six out of 39, as compared to none out of 61 without metaplasia. The difference was statistically significant ($P = 0.002$). All control samples were negative for *Helicobacter* spp. The difference between *Helicobacter* DNA prevalence in gallbladder

Table 2 Prevalence of *Helicobacter* spp. and some common gut bacteria *n* (%)

Patient group	*Helicobacter* PCR	Gut bacteria PCR	Sequencing results (No. of samples)
Acute cholecystitis	4/50 (8)	0/50 (0)	*H. pylori* (1) *H. pullorum* (3)
Chronic cholecystitis	3/50 (6)	0/50 (0)	*H. pullorum* (3)
Controls	0/102 (0)	0/102 (0)	-

Results are shown as the number of positive patients and the number of all patients in the group followed by the percentage in parenthesis. PCR: Polymerase chain reaction; *H. pylori*: *Helicobacter pylori*; *H. pullorum*: *Helicobacter pullorum*.

Table 3 Prevalence of *Helicobacter* DNA in cholecystitis mucosa in different studies from various geographical regions

Region	Prevalence (%)	Patients (*n*)	Ref.
Germany	2	1/57	Bohr *et al*[35] 2007
Japan	12-13	2/16	Murata *et al*[36] 2004
	27	4/15	Fukuda *et al*[37] 2002
China	27.2	22/81	Chen *et al*[34] 2007
Chile	39	9/23	Fox *et al*[31] 1998
Ukraine	73	16/22	Apostolov *et al*[9] 2005

of cholecystitis patients and controls was also significant ($P = 0.007$).

PCR and sequence detection of bacterial DNA other than Helicobacter

None of the tested patients' samples with acute and chronic cholecystitis and control samples was positive using the *Bacteroides*-, Enterobacteriaceae- and *Enterococcus*-specific PCR assays.

DISCUSSION

Helicobacter DNA was found in 7% of cholecystitis mucosa (8% acute, 6% chronic cholecystitis); none of the control samples was positive for *Helicobacter*. There are several reports on the presence of *Helicobacter* DNA in cholecystitis mucosa (Table 3). The studies in Germany, China and Japan with a prevalence of 2%-27% were more similar to our study[34-37] than was the study in Chile (39% prevalence)[31]. However, in a study from Ukraine (73%) the prevalence was much higher than in our study[9].

Six samples (three from acute and three from chronic cholecystitis) were positive for *H. pullorum*. Fox *et al*[31] have reported a link between EHS infections and chronic cholecystitis. *H. bilis* was the most common but *H. pullorum* was also reported[31]. Apostolov *et al*[9] have developed a first generation of enzyme immunoassays and immunoblotting to serodiagnose EHS infections in mice and humans. *H. pullorum* was found in 18% of patients with hepatitis C virus by immunohistochemistry in one of our previous studies[38]. However, *H. pullorum* is most commonly seen in poultry[39]. There is most likely a zoonotic transmission between humans and chickens by undercooked chicken.

One sample with a similar sequence to *H. pylori* was detected. Other studies on gallbladders or gallstones from patients with cholecystitis and cholelithiasis have shown the presence of *H. pylori*[9,32,40]. Other *Helicobacter* species have also been detected in different studies such as, *H. rappini*, *H. ganmani*[35] and *H. hepaticus*[41].

Kawaguchi *et al*[42] were the first to demonstrate *Helicobacter* spp. in cholecystits mucosa that displayed gastric metaplasia. Metaplasia was seen in all cases of cholecystitis in a Chilean study[31], in 15% of cases in a British study[43], and in 14% of cases in a Ukrainian study[9]. In the British study, no *Helicobacter* was found by immunostaining. Our results confirm the importance of gastric metaplasia for detection of *Helicobacter* DNA. Misra *et al*[40] have detected *Helicobacter* only in areas with gastric metaplasia, with a prevalence of 45%, but could not detect *Helicobacter* DNA in paraffin blocks or formalin-fixed mucosal tissue.

None of the gallbladder samples was positive for *Bacteroides* and *Enterococcus* spp. in our study. Enteric bacteria have been detected from gallstones and bile samples by culturing and PCR methods in some studies[44-47], but not by fluorescence *in situ* hybridization[48].

Apart from geographical differences, the variation in *H. pylori*, EHS and some gut pathogens between countries could be due to the use of different PCR methods. Our PCR technique was evaluated as a highly reliable method for genus level identification of *Helicobacter* spp.[49], and inhibitors that might influence the PCR results have been discussed in our other studies[50]. Moreover, some of the studies have used inappropriate control groups. We selected 102 normal control gallbladders from patients diagnosed with diseases other than cholecystitis.

In cholecystitis, *Helicobacter* DNA might preferentially or only be found in epithelial cells or on their surface, thus, much care has to be taken when selection the samples from the paraffin blocks.

In conclusion, several *Helicobacter* spp. infect a range of hosts (most probably, certain species are pathogens in some animals and humans). Divergent results might be due to different geographical areas, different PCR methods, using different control groups or lack of control groups, and sampling from different areas of the biopsy. The present study shows the possible relationship between *Helicobacter* spp. and cholecystitis in Swedish patients. Further studies are needed to determine the possible role of EHS and other pathogens in biliary tract infections and the possible relationship to various hepatobiliary malignancies such as cholangiocarcinoma.

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COMMENTS

Background

Helicobacter genus has nearly 25 species and many of them cause extragastric diseases in humans and animals.

Research frontiers

Helicobacter DNA in gallbladder mucosa has been reported with different prevalence and is associated with several biliary tract diseases, but there are still doubts about the relationship between enterohepatic *Helicobacter* species (EHS), *Helicobacter pylori* and hepatobiliary diseases.

Innovations and breakthroughs

Recent reports have highlighted the presence of *Helicobacter* in the biliary tract in different regions. However, this is believed to be the first study to report the possible relationship between chronic cholecystitis in Scandinavian patients.

Applications

By understanding the relationship between *Helicobacter* and cholecystitis, this study could represent a future strategy for further pathological studies of patients with cholecystitis.

Terminology

EHS are species in the genus *Helicobacter* that colonize the hepatobiliary tract and can cause extragastric diseases in humans or in animals.

Peer review

The authors have tackled a newly developing area of interest to many researchers. The work is a contribution to the study of the association between *Helicobacter* spp. and some common gut bacteria in patients with cholecystitis. They concluded that there is a possible relationship between *Helicobacter* DNA and cholecystitis, and recommended further serological and immunohistochemical studies to support their data.

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BRIEF ARTICLE

Tumor budding predicts response to anti-EGFR therapies in metastatic colorectal cancer patients

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Abstract

AIM: To investigate whether the evaluation of tumor budding can complement K-RAS analysis to improve the individualized prediction of response to anti-epidermal growth factor receptor based therapies in metastatic colorectal cancer (mCRC) patients.

METHODS: Forty-three patients with mCRC treated with cetuximab or panitumumab were entered into this study. According to the Response Evaluation Criteria in Solid Tumors criteria, 30 patients had stable or progressive disease (non-responsive), while 13 patients had a partial response. Tumor buds were evaluated from whole tissue sections stained for pan-cytokeratin, evaluated in the densest region using a 40 × objective and "high-grade" tumor budding was defined as 15 buds/high-power field.

RESULTS: Tumor buds and K-RAS mutation both correctly classified 68% of patients. All patients with K-RAS mutation (n = 7) or high-grade tumor budding (n = 11) were non-responsive, of which 4 patients had both features. All 13 partial responders were K-RAS wild-type with low-grade tumor budding. Combined, the predictive value of K-RAS and tumor budding was 80%. Additionally, high-grade tumor budding was significantly related to worse progression-free survival [HR (95% CI): 2.8 (1.3-6.0, P = 0.008)].

CONCLUSION: If confirmed in larger cohorts, the addition of tumor budding to K-RAS analysis may represent an effective approach for individualized patient management in the metastatic setting.

Key words: Anti-epidermal growth factor receptor therapy; Colorectal cancer; K-RAS; Prognosis; Tumor budding

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INTRODUCTION

Between 40%-50% of all patients with colorectal cancer are diagnosed with metastatic colorectal cancer (mCRC)[1]. Overall 5-year survival rates for these patients are still less than 10% despite improvements in treatment and com-

bined systemic chemotherapies. Monoclonal antibodies targeting the epidermal growth factor receptor (EGFR) such as cetuximab and panitumumab have recently been approved for the treatment of mCRC patients, however, response rates in general vary from 10%-20%[2,3]. Several molecular and protein biomarkers are currently being intensively investigated for their potential predictive value including K-RAS, B-RAF, PIK3CA and PTEN. Although recent randomized clinical trials have not been unanimous concerning the predictive value of K-RAS on outcome, the vast majority of studies to date do support a lack of responsiveness in patients with mutation[4-9]. These data have led the American Society of Clinical Oncology, Food and Drug Administration and European Medicines Agency to recommend that patients with mCRC be tested for K-RAS gene mutation before administration of EGFR-targeted therapies[10]. It is, however, clear that not all patients with wild-type K-RAS tumors achieve a response to anti-EGFR therapies and the results concerning other genetic alterations are not conclusive, suggesting that continued efforts on predictive biomarkers are warranted.

In colorectal cancer, tumor buds, defined as dedifferentiated single cells or clusters of up to 5 cells at the invasive tumor front, are considered the histological hallmark of epithelial mesenchymal transition and are thought to be responsible for the subsequent steps in invasion and metastasis[11]. Although tumor buds can be observed using regular hematoxylin and eosin (HE) slides, evaluation is facilitated by pan-cytokeratin stains[12]. Tumor budding has consistently been linked to higher tumor grade, vascular and lymphatic invasion and is highly predictive of both lymph node and distant metastasis stage[13-25]. Moreover, most studies confirm that high-grade tumor budding is an independent prognostic factor and recognized as such by the American Joint Committee on Cancer and International Union against Cancer (AJCC/UICC)[26]. In addition, we have previously shown that tumor budding is not related to mutation of K-RAS, leading to the hypothesis that this histomorphological feature could perhaps be used to complement the assessment of response in mCRC patients treated with anti-EGFR-based therapies[27].

Therefore, the aim of this study was to evaluate the predictive and prognostic value of tumor budding and determine its complementary value to K-RAS gene status in mCRC patients treated with cetuximab or panitumumab-based regimens.

MATERIALS AND METHODS

Patients and specimen characteristics

Forty-three consecutive patients with histologically confirmed mCRC treated at the Oncology Institute of Southern Switzerland, Bellinzona, Switzerland with cetuximab or panitumumab-based regimens were entered into this retrospective study. Cetuximab was administered at a standard loading dose of 400 mg/m^2 over 2 h, followed by a weekly dose of 250 mg/m^2 over 1 h. Single agent panitumumab 6 mg/kg every 2 wk was administered to 2 patients who were refractory to oxaliplatin- and irinotecan- based regimens. With the exception of 2 patients who received cetuximab as frontline therapy, the others had failed at least one prior chemotherapy regimen. For those who progressed on irinotecan-based regimens, cetuximab was administered in combination with these regimens given at the same dose and schedule. Therefore, patients were selected based on evidence that treatment outcome was attributable only to the administration of cetuximab or panitumumab. Treatment was continued until progressive disease (PD) or toxicity occurred. Response was assessed every 6-8 wk by means of computerized tomodensitometry or nuclear magnetic resonance. The Response Evaluation Criteria in Solid Tumors were adopted for evaluation and objective tumor response was classified into complete response (CR), partial response (PR), stable disease (SD) and PD[28]. Accordingly, only patients who achieved either CR or PR were considered as responders.

Assay methods

K-RAS, B-RAF and PIK3CA mutational status: Formalin-fixed paraffin-embedded surgical resection specimens were available for all patients. We searched for point mutations in K-RAS exon 2 (including codons 12 and 13), *BRAF* exon 15 (including codon 600) and PIK3CA exons 9 and 20 (including codons 542, 545 and 1047). All samples were subjected to automated sequencing by ABI PRISM 3100 (Applied Biosystems, Foster City, CA, USA) and analysed with appropriate software (Applied Biosystems). Each sequence reaction was performed at least twice, starting from independent PCR reactions. In each case, the detected mutation was confirmed in the sequence as sense and antisense strands.

Epidermal growth factor receptor: fluorescent in situ hybridization

The *EGFR* gene status evaluation was performed by fluorescent *in situ* hybridization (FISH) on 3-μm thick tissue sections. Tissue sections were treated using Paraffin Pretreatment Kit Ⅱ (Abbott Molecular AG, Baar, Switzerland) according to the manufacturer›s instructions. Dual-colour FISH assay was performed using LSI EGFR/CEP7 probes (Vysis). The LSI EGFR probe is labelled in SpectrumOrange and covers an approximately 300 kb region that contains the entire *EGFR* gene at 7p12. The CEP7 probe, labelled in SpectrumGreen, hybridises to the α satellite DNA located at the centromere of chromosome 7 (7p11.1-q11.1). Target sections and probe were co-denatured at 75℃ for 5 min and allowed to hybridise overnight at 37℃. Post-hybridisation stringency wash was carried out in a water bath at 72℃ for 5 min. After washing twice and drying at room temperature for 10 min, slides were mounted with 4'6-diamidino-2-phenylindole (DAPI Ⅱ, Abbott Molecular). Fluorescent *in situ* hybridization signals were evaluated with a Zeiss Axioscope equipped with single and triple band pass filters. Images for documentation were captured using an AxioCam camera and processed using the AxioVision system. Patients

showing two of chromosome 7 in the vast majority of cells were classified as eusomic. Patients with an aberrant number of chromosome 7, defined as more than 4 in at least 50% of cells, were classified as markedly polysomic. Patients with a ratio more than 3 between the *EGFR* gene and chromosome 7 centromere signals in at least 10% of cells were classified as having *EGFR* gene amplification[29].

Immunohistochemistry

Immunohistochemistry staining was performed for both CK22 (an epithelial cell marker facilitating the visualization of tumor buds) and PTEN. Paraffin-embedded tissue blocks were cut at 3 μm. Whole tissue sections were de-waxed and re-hydrated in dH_2O. Following pressure cooker-mediated antigen retrieval in 0.001 mol/L ethylenediaminetetraacetic acid pH 8.0, endogenous peroxidase activity was blocked using 0.5% H_2O_2. Sections were incubated with 10% normal goat serum for 20 min. After incubation with primary antibody (PTEN Ab-4, Neomarkers, Fremont, CA, USA; 1:50 and CK22 polyclonal, Genetex, Inc, 1:100), sections were incubated with HRP-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark) for 30 min at room temperature, immersed in 3-amino-9-ethylcarbazole+substrate-chromogen (DakoCytomation) for 30 min, and counterstained with haematoxylin. PTEN protein expression was detected mainly at the cytoplasmic level, although occasional nuclear positivity was present. PTEN negative tumors were those showing a dramatic reduction or absence of immunostaining in at least 50% of cells, as compared with the internal control. The evaluations were performed without knowledge of clinical data or the results of other analyses.

Assessment of tumor budding

Tumor budding was defined as dedifferentiated single cells or clusters of < 5 cells at the invasive tumor front. In all cases, the tumor invasive front was scanned at low power using a 5 × objective lens and the region of densest tumor budding was identified. The number of tumor buds within this region was counted using a 40 × objective lens. Evaluation was performed blinded to clinical endpoints. Inter-observer agreement was assessed between independent observers (Lugli A, Vlajnic T, Zlobec I). Discordant cases were discussed until agreement was reached. High-grade tumor budding was defined as 15 tumor buds/HPF.

Study design

The study was designed as a retrospective analysis. The main objective was to correlate response to anti-EGFR-based therapies with pathological and molecular findings. The secondary endpoint was represented by the correlation of tumor budding with progression-free survival (PFS) and overall survival.

Statistical analysis

Threshold values for determining high-grade *vs* low-grade tumor budding were assessed using receiver operating characteristic curve analysis with 100- bootstrapped replications of the data. The sensitivity, specificity, positive predictive value and negative predictive value (NPV) for high-grade tumor budding, EGFR amplification or copy number gain, K-RAS, B-RAF, PIK3CA and loss of PTEN as well as their association with response were evaluated by simple logistic regression analysis. Inter-observer variability of tumor budding (low-grade/high-grade) was assessed by the κ statistic and by investigating the percentage of concordance between independent observers. Univariate and multivariable PFS time differences stratified by tumor budding and after adjustment for K-RAS mutational status were evaluated using simple and multiple Cox regression analysis, respectively, after verification of the proportional hazards assumption. The Kaplan-Meier method was used to illustrate PFS time differences by tumor budding grade. Fisher's Exact test was used to determine the association of tumor budding for response in subgroup analysis. Finally, classification and regression tree analysis (CART) methods were used to determine the features best predicting response to treatment[30]. The CART trees were fitted using DTREG statistical software. To assess the amount of overfitting, 100 10-fold cross-validation experiments were performed[31]. In each of those 100 experiments, the data set was randomly split into 10 smaller data sets and a pruning method was used to choose the best number of nodes for the original tree pruned with respect to 90% of the data according to the misclassification rate for the other 10% of the data. To resolve uncertainty in assessing the optimal number of terminal nodes for the full data set, we conducted a two-tailed Fisher's exact test to test for a relationship between tumor budding, K-RAS mutation and response to therapy. Given the significant association of both these features with response, CART analysis was performed for patients with low-grade tumor budding and K-RAS wild-type gene status only. A second CART analysis was performed conditioning only on K-RAS wild-type patients.

RESULTS

Patient characteristics

The present study analyzed forty-three patients, 26 men (60%) and 17 women (40%). Patient characteristics and response by treatment with anti-EGFR monoclonal antibodies are summarized in Table 1. Median survival time was 37.3 mo (range 3.6-180) and PFS time was 16.0 mo (range 1-171). Thirteen patients (30%) achieved PR after cetuximab- or panitumumab-based therapy.

Association of tumor budding with molecular features

The percentage of concordance between observers was 88% with a κ value of 0.6. Tumor budding and K-RAS gene status was evaluable in all cases. High-grade tumor budding occurred in 11 cases (25.6%) while low-grade tumor budding was found in the remaining 32 patients (74.4%). Tumor budding was not significantly associated with either EGFR status (P = 0.95), K-RAS (P = 0.43),

Table 1 Characteristics of metastatic colorectal cancer patients treated with anti-epidermal growth factor receptor therapy (*n* = 43) *n* (%)

Clinico-pathological feature	Frequency
Age (yr), median (range)	64 (26-82)
Gender	
Male	26 (60.5)
Female	17 (39.5)
Response	
Progressive disease	19 (44.2)
Partial response	13 (30.2)
Stable disease	11 (25.6)
EGFR	
No Amplification/gene copy number gain	4 (10.5)
Amplification/gene copy number gain	34 (89.5)
K-RAS	
Wild-type	32 (74.4)
Mutation	11 (25.6)
B-RAF	
Wild-type	38 (88.4)
Mutation	5 (11.6)
PIK3CA	
Wild-type	41 (95.4)
Mutation	2 (4.7)
PTEN	
Negative	12 (27.9)
Positive	31 (72.1)
Overall survival time (mo), median (range)	37.3 (3.6-180)
Progression-free survival (mo), median (range)	16.0 (1-171)
Number of tumor buds, median (range)	9.0 (1-44)
Tumor budding	
High	11 (25.6)
Low	32 (74.4)

EGFR: Epidermal growth factor receptor.

B-RAF (P = 0.598), PIK3CA (P = 0.451) or PTEN expression (P = 0.241) (data not shown).

Association of tumor budding with response

The predictive ability of each feature for response is shown in Table 2. High-grade tumor budding was significantly associated with no objective response (P = 0.011). In fact, all patients with PR had low-grade tumor budding (sensitivity 100%) and all patients with high-grade tumor budding were PD or SD (negative predictive value, NPV: 100%). The overall accuracy of tumor budding for response was 68.3%.

K-RAS gene status was evaluated in all 43 patients and 11 (25.6%) were identified as mutated while the remaining 32 cases (74.4%) were wild-type. A significant association of K-RAS mutation with no objective response was observed (P = 0.011). Moreover, all patients achieving PR were K-RAS wild-type (sensitivity 100%) while K-RAS mutated cases were all non-responders (NPV 100%). As for tumor budding, the overall accuracy of K-RAS for response was 68.3%.

Of the 19 patients with wild-type K-RAS and no response, high-grade tumor budding was able to identify an additional 7 non-responder patients (Table 3). Together, the combined overall accuracy of tumor budding and K-RAS increased from 68.3% to 80% with a sensitivity of 100% for PR and an improvement in specificity to 72.1% with only 12/43 cases in this series misclassified with these two parameters alone.

Algorithm for patient classification using tumor budding, EGFR, K-RAS, B-RAF, PIK3CA and PTEN

Since the predictive accuracy for response using tumor budding combined with K-RAS mutation was 80%, the classification of wild-type K-RAS/low-grade tumor budding patients was further investigated using the remaining molecular parameters and analyzed by CART (Figure 1A). For the remaining 25 patients, negative expression of PTEN occurred in 6 cases and 5/6 (83%) were not responders. Of the remaining 16 patients with positive PTEN expression and available EGFR status, all cases with amplification or copy number gain (n = 13, three were not evaluable for EGFR gene status) had a PR. PIK3CA and B-RAF gene status did not contribute predictive information in this setting which included tumor budding. Moreover, of the 43 patients, 4 cases were misclassified, leading to 90.7% of patients being classified into appropriate response groups.

In order to compare the performance of this algorithm conditioned on K-RAS and tumor budding to an algorithm conditioned only on K-RAS, we performed a second CART analysis to classify patients with wild-type K-RAS gene status into response groups using the remaining molecular features, as described above (Figure 1B). Using this approach, PTEN expression, followed by B-RAF mutation and EGFR amplification or copy number gain were included in the analysis. PIK3CA was not a predictive factor here, most likely due to the low frequency (n = 2) of patients with mutation in this cohort. Seven of the 43 patients were incorrectly classified leading to an overall classification rate of 83.7%.

An overview of the predictive accuracies of K-RAS, tumor budding, K-RAS plus tumor budding, as well as the two algorithms including and excluding tumor budding is presented in Figure 2. In particular, the accuracy of either tumor budding alone or K-RAS analysis alone was 68.3%. This value improved to 80% when analyzing the combined accuracy of budding with K-RAS gene status. The predictive ability of a 4-panel combination of features including K-RAS/PTEN/B-RAF/EGFR was 83.7%. Among the features evaluated, the combined analysis of K-RAS/tumor budding/PTEN/EGFR demonstrated an overall accuracy of 90.7% for response to anti-EGFR agents.

Tumor budding, K-RAS and PFS

When evaluating PFS, high-grade tumor budding was significantly linked to an increased relative risk [HR (95% CI): 2.8 (1.3-6.0), P = 0.008] (Figure 3). In addition, when evaluating both tumor budding and K-RAS mutation status in multivariable analysis, high-grade tumor budding maintained its negative effect on clinical outcome [HR (95% CI): 2.78 (1.3-6.0), P = 0.022],

Table 2 Predictive ability of each feature for partial response

Feature	Total No. of patients	No. of correctly predicted PR	No. of correctly predicted PD + SD	No. of PD + SD predicted as PR	No. of PR predicted as PD + SD	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)	*P*-value
Budding (≤ 15 cells)	43	13	11	19	0	100	40.6	37	100	68.3	0.011
EGFR (no AMP/CNG)	38	10	4	24	0	100	29.4	14	100	57.1	0.556
K-RAS (wild-type)	43	13	11	19	0	100	40.6	37	100	68.3	0.011
B-RAF (wild-type)	43	13	5	25	0	100	34.2	17	100	58.3	0.301
PIK3CA (wild-type)	43	13	2	28	0	100	31.7	7	100	53.3	1.0
PTEN (positive)	43	12	11	19	1	91.7	38.7	37	92.3	64.5	0.07

PD: Progressive disease; SD: Stable disease; PR: Partial response; EGFR: Epidermal growth factor receptor; PPV: Positive predictive value; NPV: Negative predictive value; AMP/CNG: Amplification or copy number gain.

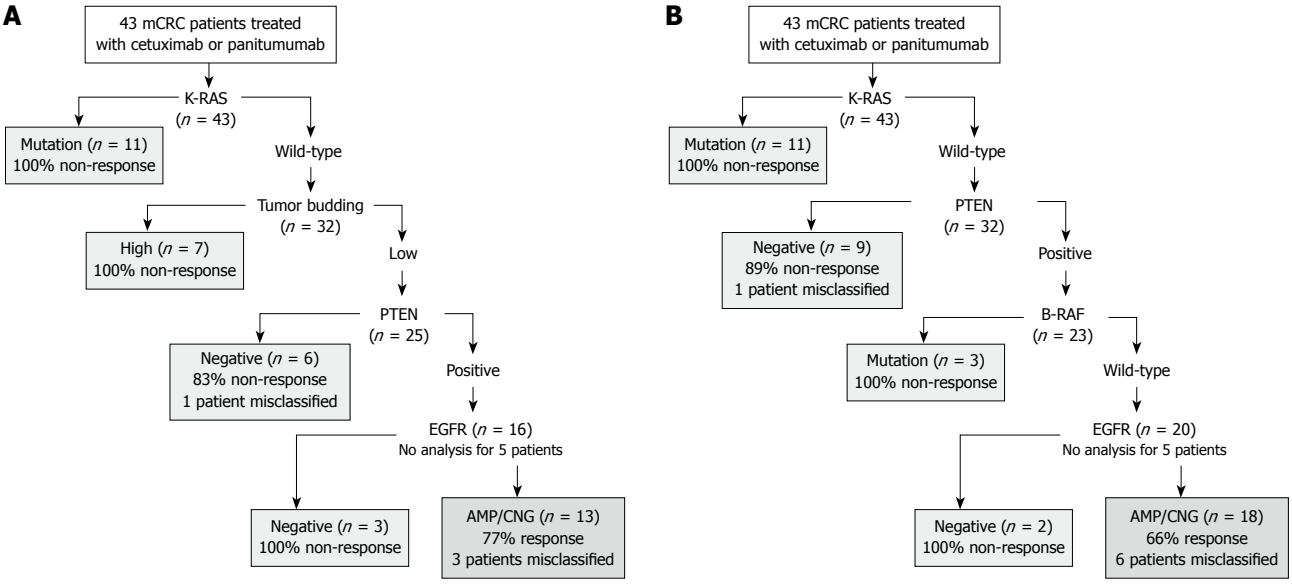

Figure 1 Algorithm illustrates the classification of patients into response groups. Non-response: Patients with progressive disease or stable disease. A: Classification and regression tree (CART) analysis was performed for patients with K-RAS wild-type/low tumor budding. CART identified a significant contribution of PTEN and epidermal growth factor receptor (EGFR) to the classification of responsive and non-responsive patients. Thirty-nine patients correctly classified (90.7%); B: CART analysis performed for patients with K-RAS wild-type tumors identifying a significant contribution of PTEN, B-RAF and EGFR to the classification of responder and non-responder patients. Thirty-six patients correctly classified (83.7%). AMP/CNG: Amplification or copy number gain; mCRC: Metastatic colorectal cancer.

while K-RAS was not linked to PFS [HR (95% CI): 1.54 (0.8-3.1), $P = 0.236$].

DISCUSSION

The aim of this study was to determine whether tumor budding is a predictive or prognostic factor in mCRC patients treated with anti-EGFR-based therapies. Our results show that high-grade tumor budding predicts non-response in these patients and in combination with K-RAS mutation may correctly predict response with 80% accuracy. Additionally, high-grade tumor budding was found to lead to unfavourable PFS also in a K-RAS-independent manner.

We found no association between high-grade tumor budding and K-RAS gene mutation in this series of mCRC patients. Using two entirely independent cohorts of 88 and 117 patients, respectively, we have previously shown this lack of association between K-RAS and tumor budding, although mutation in codon 12 and 13 was observed in 38.6% of all high-grade tumor budders[27,32]. Our findings here using a third independent cohort are in agreement with these results. In contrast, Prall and Oswald documented in 95 sporadic CRC patients, a significant association between mutation and tumor budding, and moreover, independently of invasion growth patterns[20]. Our differing results may be explained by the types of tumor specimens used (paraffin-embedded *vs* fresh frozen), differences in molecular analysis (DNA sequencing *vs* PCR-RFLP) and notably by the choice of methods of evaluation (tumor buds only *vs* tumor buds plus cytoplasmic pseudo-fragments).

We document here a significant association between high-grade tumor budding and a lack of objective response in patients with mCRC treated with anti-EGFR therapies. Tumor budding has been significantly related to unfavourable clinical and histopathological features including higher tumor grade, vascular invasion, lymph node

Table 3 Tumor budding and K-RAS followed by PTEN, epidermal growth factor receptor, B-RAF and PIK3CA stratified by response group

Response	K-RAS	Tumor budding	PTEN	EGFR (AMP/CNG)	B-RAF	PIK3CA
NR	WT	Low	NEG	Yes	WT	WT
NR	WT	Low	NEG	Yes	WT	WT
NR	WT	Low	NEG	Yes	WT	WT
NR	WT	Low	NEG	Yes	WT	WT
NR	WT	Low	NEG	Yes	MUT	WT
NR	WT	Low	POS	No	WT	WT
NR	WT	Low	POS	No	WT	WT
NR	WT	Low	POS	Yes	WT	WT
NR	WT	Low	POS	Yes	WT	WT
NR	WT	Low	POS	Yes	WT	WT
NR	WT	Low	POS	NE	MUT	WT
NR	WT	Low	POS	No	MUT	MUT
NR[1]	WT	High	NEG	No	WT	WT
NR[1]	WT	High	NEG	Yes	WT	WT
NR[1]	WT	High	NEG	Yes	MUT	MUT
NR[1]	WT	High	POS	Yes	WT	WT
NR[1]	WT	High	POS	Yes	WT	WT
NR[1]	WT	High	POS	Yes	WT	WT
NR[1]	WT	High	POS	Yes	MUT	WT
NR[1]	MUT	Low	NEG	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	Low	POS	Yes	WT	WT
NR[1]	MUT	High	NEG	Yes	WT	WT
NR[1]	MUT	High	NEG	Yes	WT	WT
NR[1]	MUT	High	POS	No	WT	WT
NR[1]	MUT	High	POS	Yes	WT	WT
PR[1]	WT	Low	NEG	NE	WT	WT
PR[1]	WT	Low	POS	NE	WT	WT
PR[1]	WT	Low	POS	NE	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT
PR[1]	WT	Low	POS	Yes	WT	WT

[1]Cases which were correctly predicted using K-RAS and tumor budding. EGFR: Epidermal growth factor receptor; AMP/CNG: Amplification or copy number gain; MUT: Mutation; NE: Not evaluable; NEG: Negative; NR: Non response; POS: Positive; PR: Partial response; WT: Wld-type.

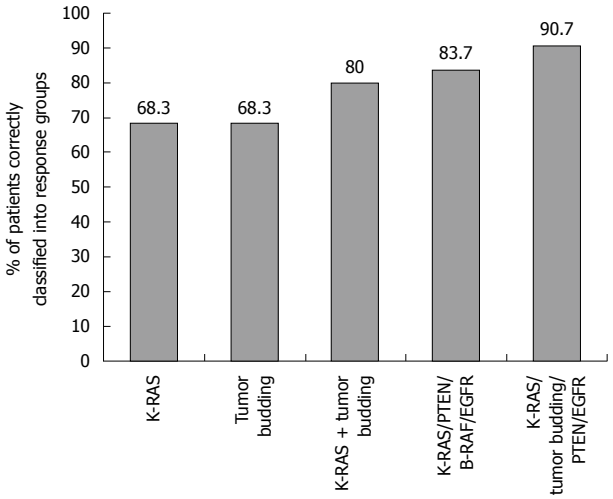

Figure 2 Overview of classification rates for various combinations of features. EGFR: Epidermal growth factor receptor.

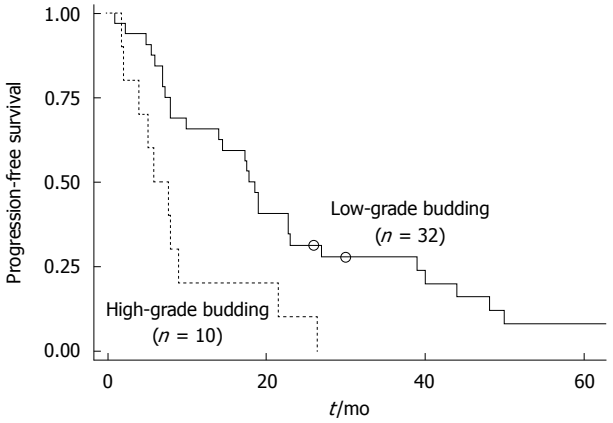

Figure 3 Kaplan-Meier survival curve showing the unfavourable progression-free survival of patients with high-grade tumor budding.

metastasis, distant metastasis, local recurrence, and poorer overall and disease-specific survival time independently of TNM stage[13-25]. Additionally, tumor budding is inversely related to dense peritumoral lymphocytic inflammation at the invasive front suggesting that the pro-budding phenotype may be tempered by specific immune responses[33]. We have recently reported that a high ratio of CD8+/tumor buds in non-metastatic CRC was found to be a more important prognostic factor than either CD8+ T-lymphocytes or tumor budding alone[27]. Although we evaluated CD8+ cells in these 43 specimens and their ratio with tumor budding, we did not find any predictive or prognostic value of CD8+ in this series, suggesting that the immune response may not play a role in conferring response in these treated, metastatic patients (data not shown). On the other hand, high-grade tumor budding was not only associated with non-response to anti-EGFR therapies but all patients with this unfavourable feature were non-responsive. In addition, we found a significantly shorter PFS in patients with high-grade tumor budding independently of K-RAS, supporting the predictive and prognostic effect of this histomorphological feature among this cohort of patients.

An association between K-RAS gene mutation and lack of response to anti-EGFR therapies has been consistently described[5,7,8,34]. Indeed, K-RAS mutational investigations are now routinely performed in molecular pathology laboratories and recommended for patients with mCRC to determine their potential benefit from anti-EGFR therapies[10]. In our study, all patients with a K-RAS mutation were non-responsive to therapy. Nonetheless, 7 patients with wild-type K-RAS were also found to be non-responders and all of these had high-grade tumor budding. In fact, all patients with an objective response to therapy

had simultaneous wild-type K-RAS and low-grade tumor budding, thereby improving the predictive accuracy for response from 68% for each biomarker alone to 80% when assessed in combination.

It is recognized that a subgroup of mCRC patients with wild-type K-RAS do not respond to anti-EGFR agents[35]. In this study, although all responders were indeed those with wild-type K-RAS and low-grade tumor budding, a considerable proportion of patients, namely 12/43 (27.9%) found with this constellation had PD or SD after treatment. In this setting, we found that loss of PTEN expression could accurately identify 83% of non-responsive cases and that *EGFR* amplification or copy number gain in PTEN-positive tumors correctly predicted 77% of responders, findings which are in line with numerous reports concerning the predictive value of these markers[36-38]. Together, 90.7% (39/43) of patients were correctly classified into response groups using these four features. Mutations in B-RAF and PIK3CA mutations have also been found to lead to non-response in mCRC patients[39-41]. Indeed, in this study, cases with either mutation were found to be patients who did not respond to therapy. However, after accounting for K-RAS and tumor budding only 3 B-RAF mutations were observed and 1 PIK3CA mutation was found, therefore the low frequency of these events may have led to their exclusion from the predictive algorithm.

Our study is constrained by several factors, the most important limitation being the sample size. To our knowledge, this is the first study evaluating tumor budding as a potential predictive or prognostic factor in mCRC patients treated with cetuximab or panitumumab, nonetheless these results need to be validated in larger cohorts. Secondly, although tumor budding is considered an additional prognostic factor by the AJCC/UICC, it has not been incorporated into standard pathological routine due to the absence of standardized methods of evaluation. Our cut-off score to define high-grade tumor budding was determined using a 40 × high-power field and found to be reproducible between independent pathologists. Not only was the threshold of 15 tumor buds defined using a valid cut-point determination method and tested using re-sampling methods, but resembles the definition of high-grade tumor budding used by Prall *et al*[19] to define the optimal threshold value for predicting metastatic disease in CRC patients (25 tumor buds observed in the densest region using a 20 × objective lens). Despite these limitations, our study is innovative, in that it appears to be the first evidence suggesting that a histomorphological feature, namely tumor budding, is both a predictive and prognostic factor in patients with mCRC treated with anti-EGFR-based therapies. Moreover, the combined analysis of K-RAS gene status and tumor budding may accurately predict both responders and non-responders with up to 80% accuracy.

These preliminary results suggest that tumor budding evaluated using pan-cytokeratin stains improves the individualized prediction of outcome in combination with K-RAS mutation for mCRC patients treated with anti-EGFR therapies. These findings warrant further investigation in large prospective studies.

COMMENTS

Background

Tumor budding is a histological feature which has consistently been linked to higher tumor grade, vascular and lymphatic invasion and is predictive of both lymph node and distant metastasis stage. Most studies confirm that high-grade tumor budding is an independent prognostic factor and recognized as such by the American Joint Committee on Cancer and International Union against Cancer. In addition, tumor budding does not appear to be related to mutation of K-RAS, leading to the hypothesis that this histomorphological feature could perhaps be used to complement the assessment of response in metastatic colorectal cancer (mCRC) patients treated with anti-epidermal growth factor receptor (EGFR)-based therapies.

Research frontiers

Monoclonal antibodies targeting the EGFR such as cetuximab and panitumumab have been recently approved for the treatment of mCRC patients, however, response rates in general vary from 10%-20%. Several molecular and protein biomarkers are being investigated as predictive factors of response including K-RAS, B-RAF, PIK3CA and PTEN. The vast majority of studies to date do support a lack of responsiveness in patients with mutation of K-RAS. It is, however, clear that not all patients with wild-type K-RAS tumors achieve a response to anti-EGFR therapies and the results concerning other genetic alterations are not conclusive, suggesting that continued efforts on predictive biomarkers are warranted.

Innovations and breakthroughs

The results show that high-grade tumor budding predicts non-response in mCRC patients who receive anti-EGFR therapies. In combination, K-RAS mutation status and tumor budding together can correctly predict response with 80% accuracy. Additionally, high-grade tumor budding was found to lead to unfavourable progression-free survival also in a K-RAS-independent manner. This study appears to be the first to show that a histomorphological feature, namely tumor budding, may be a predictive factor of response in mCRC patients treated with anti-EGFR therapy.

Applications

These preliminary results suggest that tumor budding evaluated using pan-cytokeratin stains improves the individualized prediction of outcome in combination with K-RAS mutation for mCRC patients treated with anti-EGFR therapies. These findings warrant further investigation in large prospective studies.

Terminology

Tumor budding is considered the histological hallmark of Epithelial Mesenchymal Transition. Tumor buds are defined as dedifferentiated single cells/small clusters at the invasive front of colorectal cancer.

Peer review

This is a well written and presented manuscript. The data are of major importance to the clinicians. The authors studied over more than 40 human samples and made a direct link between molecular expression, prognosis and treatment.

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BRIEF ARTICLE

Liver stiffness measurements in patients with HBV *vs* HCV chronic hepatitis: A comparative study

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Abstract

AIM: To assess the values of liver stiffness (LS) in patients with hepatitis B virus (HBV) chronic hepatitis and to compare them with those in patients with hepatitis C virus (HCV) chronic hepatitis.

METHODS: The study included 140 patients with HBV chronic hepatitis, and 317 patients with HCV chronic hepatitis, in which LS was measured (FibroScan®-Echosens®) and liver biopsy was performed in the same session (assessed according to the Metavir score).

RESULTS: According to the Metavir score of the 140 HBV patients: one had F0, 32 had F1, 67 had F2, 33 had F3 and 7 had F4. Of the 317 HCV patients: 5 had F0, 34 had F1, 146 had F2, 93 had F3 and 39 had F4. For the same severity of fibrosis, the mean values of LS in HBV patients were similar to those in HCV patients: F1, 6.5 ± 1.9 kPa *vs* 5.8 ± 2.1 kPa (P = 0.0889); F2, 7.1 ± 2 kPa *vs* 6.9 ± 2.5 kPa (P = 0.3369); F3, 9.1 ± 3.6 kPa *vs* 9.9 ± 5 kPa (P = 0.7038); F4, 19.8 ± 8.6 kPa *vs* 17.3 ± 6.1 kPa (P = 0.6574). A significant direct correlation between LS measurements and fibrosis was found in HCV patients (Spearman's r = 0.578, P < 0.0001), as well as in HBV patients (r = 0.408, P < 0.0001). The correlation was more significant in HCV than in HBV patients (Fisher's Z-test, Z = 2.210, P = 0.0271).

CONCLUSION: In our group, the mean values of LS in patients with chronic B hepatitis were similar to those in patients with chronic HCV hepatitis, for the same stage of fibrosis. Also, LS was correlated with the severity of fibrosis both in HBV and HCV chronic hepatitis patients.

Key words: Chronic B hepatitis; Chronic C hepatitis; Fibrosis; Transient elastography; Liver biopsy

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INTRODUCTION

The non-invasive assessment of fibrosis in chronic hepatitis, especially of viral etiology, is accepted more and more, partially replacing liver biopsy (LB) in some countries[1]. Guidelines from France[1] recommend that the first-line test for untreated patients with hepatitis C virus (HCV)

chronic hepatitis, with no comorbities, should be a non-invasive procedure (either FibroTest® or FibroScan®).

The non-invasive methods used for the evaluation of chronic hepatitis are: serum markers (the best known is FibroTest-ActiTest - a biochemical test which uses 6 serum biomarkers, correlated with the age and gender of the patient in a mathematical formula)[2-5]; transient elastography (TE) (FibroScan®)[6,7]; SonoElastography (Real-Time Tissue Elastography)[8-11] and magnetic resonance imaging elastography[12,13].

Recent meta-analyses[7,8] have tried to assess the practical value of TE for the evaluation of patients with chronic hepatitis. Many studies were published regarding the value of TE for evaluation of patients with HCV chronic hepatitis, but only a few studies in patients with chronic hepatitis B virus (HBV) infection. On the other hand, published data showed discordant results regarding liver stiffness (LS) in patients with HBV and HCV chronic hepatitis[14,15].

The aim of our study was to determine whether the values of LS evaluated by means of TE (FibroScan®) were similar for the same degree of fibrosis (evaluated by means of LB), in patients with chronic HBV and HCV hepatitis.

MATERIALS AND METHODS

Patients

Our study included a total of 457 successive patients, 140 with HBV chronic hepatitis and 317 with HCV chronic hepatitis. All the patients were referred to our department during a 2-year period (January 2008 to December 2009) for hepatitis assessment (according to the guidelines valid in Romania in that period, LB was mandatory for fibrosis staging). LS was evaluated in all patients by means of FibroScan, and LB was performed in the same session during the standard of care evaluation of patients with chronic hepatitis. The inclusion criteria were: (1) HCV chronic hepatitis: patients with positive anti-HCV antibodies for at least 6 mo, with or without cytolysis; detectable viral load by polymerase chain reaction (PCR); pathological lesions of chronic hepatitis demonstrated by LB; no signs of decompensated liver disease (actual or history of jaundice, ascites); and (2) HBV chronic hepatitis: patients with positive HBsAg for at least 6 mo, with or without cytolysis; positive or negative HBeAg; HBV DNA > 2000 IU/mL (> 10000 copies/mL) by PCR; pathological lesions of chronic hepatitis demonstrated by LB; no signs of decompensated liver disease (actual or history of jaundice, ascites).

TE

TE was performed in all 457 patients with the FibroScan® (Echosens®, Paris, France) by 3 experienced physicians (each having performed more than 1000 TE examinations). In each patient, 10 valid measurements were performed, after which a median value of LS was obtained, measured in kilopascals (kPa). Only patients in which LS measurements had a success rate of at least 60%, with an interquartile range (IQR) < 30%, were included in our study. The success rate was calculated as the ratio of the number of successful acquisitions over the total number of acquisitions. IQR is the difference between the 75th percentile and the 25th percentile, essentially the range of the middle 50% of the data.

LB

Echo-assisted LB was performed in all 457 patients, using Menghini type modified needles, 1.4 and 1.6 mm in diameter. Only LB fragments of at least 2 cm, including at least 8 portal tracts, were considered adequate for the pathological interpretation. All the LBs were assessed according to the Metavir score, by a senior pathologist. Fibrosis was staged on a 0-4 scale: F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa extending into lobules; F3, numerous septa extending to adjacent portal tracts or terminal hepatic venules and F4, cirrhosis.

Statistical analysis

For a statistical analysis of quantitative variables, the mean and standard deviation were calculated. Two-way ANOVA test and *t*-tests were performed, to compare mean values of LS in various fibrosis subgroups in HBV *vs* HCV patients. To compare correlations, Fisher's *Z* test was used (hypotheses about the value of the population correlation coefficient ϱ between variables X and Y can be tested using the Fisher transformation applied to the sample correlation coefficient r)[16]. The diagnostic performance of LS measurements was assessed using receiver operating characteristics (ROC) curves. ROC curves were used for the detection of significant fibrosis (F $\geqslant$ 2 Metavir) and severe fibrosis (F $\geqslant$ 3 Metavir). Optimal cut-off values for LS measurements were chosen to maximize the sum of sensitivity and specificity. The statistical analysis was performed using Microsoft Excel 2007, GraphPad Prism 5 and MedCalc programs.

RESULTS

Patients

The subgroup of HBV patients consisted of 140 subjects (31 women, 109 men; mean age 39.2 ± 12.8 years). According to the Metavir scoring system, one had F0, 32 had F1, 67 had F2, 33 had F3 and 7 had F4.

The subgroup of HCV patients consisted of 317 subjects (213 women, 104 men; mean age 49.7 ± 10.2 years). According to the Metavir scoring system, 5 had F0, 34 had F1, 146 had F2, 93 had F3 and 39 had F4.

LS measurements by TE

The mean values of LS in HBV patients were not statistically significantly different from those of HCV patients for the same degree of fibrosis (Table 1).

A significant direct correlation of LS measurements with fibrosis was found to exist in HCV patients (Spearman's correlation coefficient $r = 0.578$, $P < 0.0001$), as

Table 1 Mean values of liver stiffness according to fibrosis stage in patients with hepatitis B virus *vs* hepatitis C virus chronic hepatitis

Category	Hepatitis B virus		Hepatitis C virus		*P*
	Cases	Mean values of LS (kPa)	Cases	Mean values of LS (kPa)	
Total cases	140	8.1 ± 4.2	317	8.9 ± 5.2	0.395 (NS)
F = 0	1	7.4	5	5.2 ± 0.7	-
F = 1	32	6.5 ± 1.9	34	5.8 ± 2.1	0.0889 (NS)
F = 2	67	7.1 ± 2	146	6.9 ± 2.5	0.3369 (NS)
F = 3	33	9.1 ± 3.6	93	9.9 ± 5	0.7038 (NS)
F = 4	7	19.8 ± 8.6	39	17.3 ± 6.1	0.6574 (NS)

F: Fibrosis; LS: Liver stiffness; NS: Not statistically significant.

Table 2 Predictive value of liver stiffness for the presence of significant fibrosis (F2), severe fibrosis (F3) and cirrhosis (F4) in hepatitis B virus *vs* hepatitis C virus patients

Parameter	Hepatitis B virus			Hepatitis C virus			All		
	F2	F3	F4	F2	F3	F4	F2	F3	F4
AUROC	0.658	0.753	0.974	0.750	0.797	0.933	0.712	0.786	0.943
Cut-off (kPa)	7	8.8	13.6	6.8	8.6	13.3	6.9	8.7	13.6
Sensitivity (%)	59	53	86	60	62	77	59	60	74
Specificity (%)	70	85	99	88	81	93	78	83	95
PPV (%)	86	58	78	97	71	61	93	68	64
NPV (%)	39	82	99	23	75	96	26	77	97

AUROC: Area under the receiver operating characteristics curve; PPV: Positive predictive value; NPV: Negative predictive value.

well as in HBV patients ($r = 0.408$, $P < 0.0001$). The correlation was more significant in HCV than in HBV patients (Fisher's *Z*-test, $Z = 2.210$, $P = 0.0271$).

The predictive values of LS measurements for the presence of significant fibrosis (F2), severe fibrosis (F3) and cirrhosis (F4) are presented in Table 2.

DISCUSSION

After a number of articles were published in France regarding the value of transient elastographic LS measurement in the evaluation of fibrosis in chronic hepatitis[17-22], numerous papers have been published in other countries[15,23-29] making this method a recognized test worldwide[30]. A meta-analysis published in 2008[30] proved that TE had an excellent diagnostic accuracy for the diagnosis of cirrhosis [mean area under the ROC (AUROC), 0.94 (95% CI: 0.93-0.95)]. However, a high variation of the AUROC was found regarding the diagnosis of significant fibrosis, dependent on the underlying liver disease [AUROC for significant fibrosis, 0.84 (95% CI: 0.82-0.86)].

The vast majority of studies assessing TE as compared to LB, were performed in patients with HCV chronic hepatitis[22,24,28,31,32]. At the same time, many studies were performed to evaluate this method in other chronic hepatopathies, such as nonalcoholic steatohepatitis, hemochromatosis and primary biliary cirrhosis[6,20,23,25].

Published studies regarding the value of LS measurement by means of TE in patients with HBV chronic hepatitis have shown conflicting results.

A Korean study performed by Seo *et al*[14] included 64 patients with chronic HBV hepatitis and 27 patients with chronic HCV hepatitis who underwent LB and TE in the same session (about two-thirds male; mean age 40 years, range 14-68 years). In that study, LS measurements were better correlated with the fibrosis score in patients with chronic HCV hepatitis than in those with chronic HBV hepatitis (0.773 *vs* 0.557, $P < 0.001$). The AUROC was larger in the group of patients with chronic HCV hepatitis (0.944, 0.982, and 0.958 for F $\geqslant$ 2, F $\geqslant$ 3, and F4, respectively) than in those with chronic HBV hepatitis (0.881, 0.863, and 0.850, respectively). The optimal cut-off values for F $\geqslant$ 2 and F $\geqslant$ 3 were similar for patients with chronic HCV hepatitis (7.05 and 11.4 kPa, respectively) and chronic HBV hepatitis (7.15 and 10.75 kPa, respectively). However, sensitivity and specificity were superior in patients with chronic HCV hepatitis. The conclusion of the study was that the efficacy of LS measurement for the assessment of liver fibrosis was superior in patients with chronic HCV hepatitis than in patients with chronic HBV hepatitis.

In a study performed by Ogawa *et al*[15] in 68 patients with chronic HBV hepatitis and 161 patients with chronic HCV hepatitis, the mean values of LS measurements were 3.5 kPa for F0, 6.4 kPa for F1, 9.5 kPa for F2, 11.4 kPa for F3, and 15.4 kPa for F4 in patients with chronic HBV infection, and 6.3 kPa for F0, 6.7 kPa for F1, 9.1 kPa for F2, 13.7 kPa for F3, and 26.4 kPa for F4 in those with chronic HCV infection. The values were significantly correlated with fibrosis stage for both groups of patients (HBV, $r = 0.559$, $P = 0.0093$, and HCV, $r = 0.686$, $P < 0.0001$). This study concluded that TE was an

Table 3 Cut-off values for different stages of fibrosis in patients with hepatitis B virus chronic hepatitis, proposed by various authors (kPa)

Fibrosis	Marcellin *et al*[17]	Chang *et al*[33]	Chan *et al*[34]	Kim *et al*[35]
F0	5.1	6.9	5.9	-
F1	6.0	12.2	5.9	9.1
F2	7.0	-	7.0	-
F3	12.8	24.8	8.8	-
F4	23.7	-	14.2	14.0

efficient and simple method for the evaluation of liver fibrosis in patients with chronic viral infection, both in HBV and HCV hepatitis.

Our study, performed on a large cohort of patients (457 subjects) aimed to find out if there were significant differences in LS in patients with HBV *vs* HCV chronic hepatitis for the same degree of fibrosis, as compared to the LB. LS measurement has a well established value for staging fibrosis in HCV chronic hepatitis, proved by 2 meta-analyses[7,30]. In patients with HBV chronic infection, data regarding LS measurement for fibrosis staging are conflicting. Why? One explanation could be that the necroinflammatory activity in HBV infection can vary with time, as well as the fact that fluctuations in aminotransferases can occur. Different studies have proposed various cut-off values for different stages of fibrosis, as seen in Table 3.

In our cohort of 140 chronic HBV infected patients, the mean values for F1, F2, F3 and F4 were: 6.5, 7.1, 9.1 and 19.8 kPa, respectively, similar to those obtained in the study performed by Marcellin. Also, we must bear in mind that only the Marcellin study was performed in a Caucasian population (such as ours), the others being performed in Asian populations. In our study, the sensitivity of TE for cirrhosis prediction was better in HBV than in HCV patients, but this finding needs further confirmation since the number of F4 patients in the HBV group was small (only 7) *vs* 39 in the HCV group.

Regarding the correlation between fibrosis and LS, a significant direct correlation of TE measurements with fibrosis was found to exist in HCV patients (Spearman's correlation coefficient $r = 0.578$, $P < 0.0001$), more significant than in HBV patients ($r = 0.408$, $P < 0.0001$) ($Z = 2.210$, $P = 0.0271$). Thus it is likely that the correlation between LS and fibrosis in HBV patients can be of use in clinical practice.

As mentioned earlier, high levels of aminotransferases can influence the LS values obtained by means of TE, so that LS measurements have to be interpreted in a biochemical context, otherwise there is a risk of overestimating the severity of fibrosis. Also this is why LS measurements are not performed in acute hepatitis or during alanine aminotransferase (ALT) flares in HBV chronic hepatitis[29,36]. In order to minimize the risk of overestimating fibrosis during ALT flares, Chan *et al*[34] calculated LS cut-off values for various stages of fibrosis considering also the aminotransferase levels. In this study, the LS cut-off value for F3 was 9 kPa in patients with normal ALT and 12 kPa in patients with ALT higher than 5 times the upper limit of normal. The cut-offs for cirrhosis were 12 kPa in patients with normal ALT and 13.4 kPa in those with high ALT.

In conclusion, in our study, LS measured by TE was correlated with the degree of fibrosis both in HBV and HCV patients, the correlation being more significant in HCV patients. Our data showed that there were no statistically significant differences between the mean values of LS in HBV and in HCV patients for the same degree of fibrosis.

COMMENTS

Background

Non-invasive methods for fibrosis assessment in chronic hepatitis, such as transient elastography (TE), are being accepted more and more, replacing the invasive methods, especially in hepatitis C virus (HCV) chronic hepatitis.

Research frontiers

Many studies have been published regarding the value of TE evaluation of patients with HCV chronic hepatitis, but only a few studies in chronic hepatitis B virus (HBV) infection, showing discordant results.

Innovations and breakthroughs

This research article determined if the authors can also use liver stiffness (LS) measurement by TE for the evaluation of patients with HBV chronic hepatitis, and concluded that LS is correlated with fibrosis in both HBV and HCV patients, and that there are no statistically significant differences between the mean LS values in HBV *vs* HCV patients, for the same degree of fibrosis. These findings are concordant with previous studies by Wang *et al*, Marcellin *et al*, and Ogawa *et al*, indicating that the diagnostic accuracy of LS is comparable in HBV and HCV infection related fibrosis.

Applications

This study showed that LS evaluated by means of TE was correlated with degree of fibrosis in both HBV and HCV patients and that there were no statistically significant differences between the mean values of LS in HBV *vs* HCV patients for the same degree of fibrosis, so the authors can also use this method for the evaluation of patients with HBV chronic hepatitis in daily practice.

Terminology

TE (FibroScan) is an ultrasound-based method that uses the transmission of low frequency vibrations to create an elastic shear wave that propagates into the liver, followed by the detection of wave propagation velocity, which is proportional to the tissue stiffness, with faster wave progression occurring through stiffer tissue.

Peer review

The authors present the data from their research on whether the accuracy of LS measurement in estimating liver fibrosis differs in people with chronic HCV or HBV infection. Although many reports on small or large populations exist on the same issue, the readers of the journal may find reading the data interesting.

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BRIEF ARTICLE

Erythropoietin ameliorates early ischemia-reperfusion injury following the Pringle maneuver

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Abstract

AIM: To investigate the protective effect of erythropoietin (Epo) against ischemia-reperfusion injury (IR/I) following the Pringle maneuver (PM), in comparison with conventional steroid administration in a prospective randomized trial.

METHODS: Patients were randomized by age, sex, diagnosis, and surgical method, and assigned to three groups: (1) A steroid group (STRD, n = 9) who received 100 mg of hydrocortisone before PM, and on postoperative days 1, 2 and 3, followed by tapering until postoperative day 7; (2) An EPO1 group (n = 10) who received 30 000 U of Epo before the PM and at the end of surgery; and (3) An EPO2 group (n = 8) who received 60 000 U of Epo before the PM. Hemoglobin (Hb), hematocrit (Ht), aspartate aminotransferase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), lactate, interleukin-6 (IL-6), and tumor necrosis factor (TNF)-α were measured before and just after (Day 0) surgery, and on postoperative days 1, 3, 7 and 14.

RESULTS: There were no increases in Hb and Ht in the EPO1 and EPO2 groups. AST was significantly lower in EPO1 than in STRD on Day 0 (P = 0.041), and lower in EPO1 than in STRD and EPO2 on Day 1 (P = 0.018). ALT was significantly lower in EPO1 than in STRD and EPO2 on Day 0 (P = 0.020) and Day 1 (P = 0.004). There were no significant inter-group differences in the levels of LDH and lactate. IL-6 was significantly lower in EPO1 than in STRD and EPO2 on Day 0 (P = 0.0036) and Day 1 (P = 0.0451). TNF-α was significantly lower in EPO1 than in STRD and EPO2 on Day 0 (P = 0.0006) and Day 1 (P < 0.0001). Furthermore, hospitalization was significantly shorter in EPO1 and EPO2 than in STRD.

CONCLUSION: Epo has greater potential than steroids to ameliorate IR/I after the PM. Epo at a dose of 30 000 U, administered before PM and just after surgery, yields better results.

Key words: Erythropoietin; Hepatic resection; Pringle maneuver; Steroid; Prospective randomized study

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INTRODUCTION

The Pringle maneuver (PM) is a standard procedure used worldwide to reduce blood loss during hepatic resection. However, this procedure inevitably results in some degree of ischemia-reperfusion injury (IR/I). To ameliorate IR/I, several procedures have been used, such as steroid administration and ischemic preconditioning[1,2]. Although intravenous administration of steroids is widely used, its rationality has not been established, and major concerns have been raised regarding its possible side effects, such as infection, diabetes mellitus, and disturbance of wound healing[3].

Erythropoietin (Epo) is a hematopoietic peptide that has been used successfully for treatment of anemia in patients with end-stage renal disease. Recently, attention has been focused on the extra-hematopoietic effects of Epo, including an organ-protective effect against IR/I[4-6]. We have already reported the protective effect of Epo in a porcine liver IR/I model[7].

To elucidate and apply the protective effect of Epo against liver IR/I following the PM in a clinical setting, we performed a randomized prospective clinical trial.

MATERIALS AND METHODS

The study was performed in accordance with the principles of the Declaration of Helsinki, and was approved by the ethics committee of Dokkyo Medical University Hospital. Because Epo has been used successfully in a clinical setting for more than 15 years, we did not perform a phase I study.

Phase Ⅱ study

To determine the optimum dose, timing, and frequency of Epo administration, Epo was injected intravenously into groups of 3 patients who received each of the following doses: 6000 U (× 1), 12000 U (× 1), 18000 U (× 1), 24000 U (× 1), 30000 U (× 1), and 60000 U (× 1) at 5 min before the PM, or at 30000 U (× 2) at 5 min before the PM, and at the end of the operation. None of the patients in these groups showed any significant increase in red blood cell count or other side effects. We did not use doses of Epo exceeding 60000 U (× 1) or 30000 U (× 2), because experience with patients suffering from end-stage renal disease had shown that one or two injections of Epo did not increase hematopoiesis. Also, a study by Lipsic *et al*[8] had demonstrated the safety of a single injection of Epo at a dose of 60000 U, and this dose was the maximum for a single injection available at the time of the study. We decided to use Epo at a dose of 30000 U × 2 (at 5 min before the PM and at the end of the operation; EPO1 group), and 60000 U × 1 (at 5 min before the PM, EPO2 group).

Phase Ⅲ study

Patients treated with conventional steroids received 100 mg of hydrocortisone at 5 min before the PM, at the end of the operation, and on postoperative days 1, 2, and 3. Thus, the patients were allocated to 3 groups: a steroid group (STRD, $n = 9$), and EPO1 ($n = 10$) and EPO2 ($n = 8$) groups.

Study population and inclusion/exclusion criteria

Eligibility criteria for participation were elective primary liver surgery with written informed consent. Patients who were expected to undergo non-curative hepatectomy were excluded.

Randomization

Preoperative random allocation to the 3 groups was made on the basis of 4 parameters: age (< 65 or ⩾ 65 years), sex, indocyanine green excretion rate at 15 min (< 10% or ⩾ 10%), and disease diagnosis (hepatocellular carcinoma or others). The operative procedure was standardized so that hepatectomy was performed by a single surgeon (co-author Kubota K). The patients' clinical background factors are shown in Table 1.

Data collection

Blood samples were taken on admission (Pre), just after surgery (Day 0), and on postoperative days 1 (Day 1), 3 (Day 3), 7 (Day 7), and 14 (Day 14). Aspartate aminotransferase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), lactate, hemoglobin (Hb), and hematocrit (Ht) were measured from each sample. AST, ALT, LDH, lactate, Hb, and Ht were measured at the central laboratory of our institution. Proinflammatory cytokines, interleukin (IL)-6 and tumor necrosis factor (TNF)-α, were measured on Day 0 and Day 1. IL-6 was determined using a Human IL-6 Quantikine ELISA kit (R&D System, Minneapolis, MN) and TNF-α was determined using a Human TNF-α Quantikine ELISA kit (R&D System). All samples were measured in triplicate, in accordance with the manufacturer's recommendations.

Objectives

The primary objective of the study was to compare the protective effects of Epo and steroid against IR/I following the PM. The secondary objective was to elucidate the frequency of Epo-associated side effects.

Statistical analysis

All statistical analyses were performed with GraphPad Prism 5.0 (Graphpad Software, La Jolla, CA). Comparisons between the two groups were made using Mann-Whitney *U* test. Kruskal-Wallis test with post-hoc test (Dunn's multiple comparison test) was used for comparisons between the conventional, EPO1, and EPO2 groups. Differences at $P < 0.05$ were considered to be statistically significant.

RESULTS

Figure 1 shows changes in Hb and Ht. During the periop-

Table 1 Patients' backgrounds

	STRD (n = 9)	EPO1 (n = 10)	EPO2 (n = 8)	P-value
Age (yr)	62.7 ± 10.6	62.1 ± 15.8	69.3 ± 7.8	0.395
Male/female	6/3	7/3	6/2	0.976
ICG R15 (%)	14.0 ± 8.8	10.7 ± 4.8	11.3 ± 5.2	0.854
Diagnosis (HCC/others)	6/3	6/4	4/4	0.890
Operation time (min)	322 ± 122	245 ± 83	299 ± 57	0.105
Pringle time (min)	43.9 ± 26.8	39.2 ± 21.2	53.8 ± 15.1	0.153
Operative blood loss (mL)	674 (70-3673)	469 (66-1900)	356 (228-1362)	0.386
Operative method (anatomical/ nonanatomical)	6/3	4/6	6/2	0.321

HCC: Hepatocellular carcinoma; STRD: Steroid group; EPO: Erythropoietin.

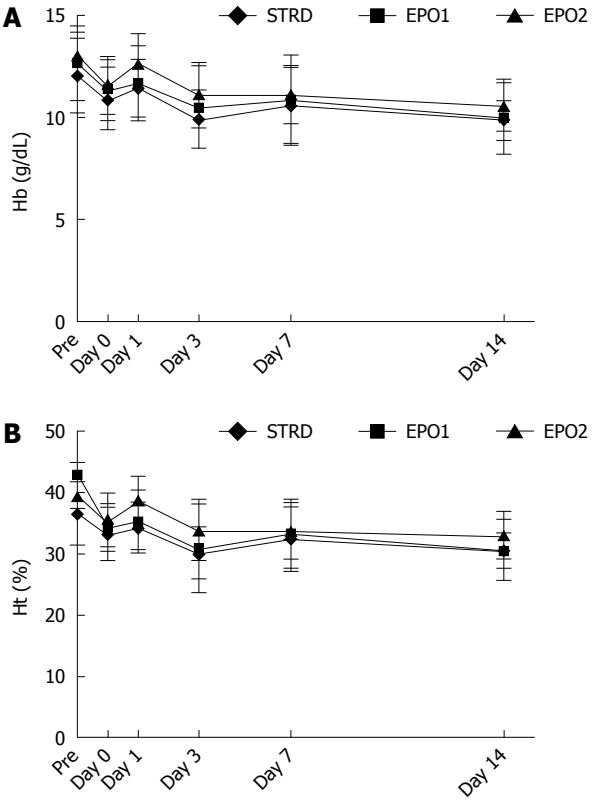

Figure 1 Changes in hemoglobin and hematocrit. Hemoglobin (Hb) (A) and hematocrit (Ht) (B) did not differ between the three groups until Day 14 (P > 0.05 for Hb and Ht, Kruskal-Wallis test). STRD: Steroid group; EPO: Erythropoietin.

erative period, there were no significant differences in Hb and Ht between the three groups, and no complications associated with the Epo treatments.

Figure 2 shows changes in the AST level at Pre, and on Days 0, 1, 3, 7 and 14. On Day 0, median values in the STRD, EPO1, and EPO2 groups were 409 (124-1481), 142 (34-552) and 205 (115-357) IU/L, respectively (P = 0.041, Kruskal-Wallis test). The AST level in the EPO1 group was significantly lower than that in the STRD group (P = 0.023). On Day 1, the corresponding median values were 275 (143.0-1389), 157 (55-552) and 342 (172-1361) IU/L, respectively (P = 0.018, Kruskal-Wallis test). The AST level in the EPO1 group was significantly lower than that in the STRD and EPO2 groups (P = 0.023 and 0.016, respectively). There were no significant inter-group differences in the AST level after Day 3.

Figure 3 shows changes in the ALT level at Pre, and on Days 0, 1, 3, 7 and 14. On Day 0, the median levels in the STRD, EPO1, and EPO2 groups were 351 (140-1390), 76 (20-319) and 141 (85-323) IU/L, respectively (P = 0.020, Kruskal-Wallis test). The ALT level in the EPO1 group was significantly lower than that in the STRD and EPO2 groups (P = 0.023 and 0.017, respectively). On Day 1, the corresponding median levels were 300 (110-1700), 112 (20-256) and 289 (200-1253) IU/L, respectively (P = 0.004, Kruskal-Wallis test). The AST level in the EPO1 group was significantly lower than those in the STRD and EPO2 groups (P = 0.008 and 0.001, respectively). There were no significant inter-group differences in the ALT level after Day 3.

Figure 4 shows the changes in LDH and lactate levels at Pre, and on Days 0 and 1. There were no significant differences in LDH or lactate levels between the three groups.

On Day 0, the median levels of the proinflammatory cytokine, IL-6, in the STRD, EPO1, and EPO2 groups were 300 (93-477), 155 (44-523) and 347 (300-414) pg/mL, respectively (P = 0.0036, Kruskal-Wallis test) (Figure 5A). The IL-6 level in the EPO1 group was significantly lower than that in the EPO2 group (P = 0.0037). On Day 1, the corresponding median levels of IL-6 were 129 (22-317), 75 (13-146) and 83 (56-240) pg/mL, respectively (P = 0.0451, Kruskal-Wallis test) (Figure 5B). The IL-6 level in the EPO1 group was significantly lower than that in the STRD group (P = 0.0185). On the other hand, on Day 0, the median levels of TNF-α in the STRD, EPO1, and EPO2 groups were 13.5 (10.1-21.0), 9.7 (5.0-11.0) and 8.0 (3.1-11.3) pg/mL, respectively (P = 0.0006, Kruskal-Wallis test) (Figure 6A), the levels in the EPO1 and EPO2 groups being significantly lower than that in the STRD group. On Day 1, the corresponding median levels of TNF-α were 17.7 (10.0-29.7), 4.6 (2.0-8.0) and 3.5 (1.4-6.0) pg/mL, respectively (P < 0.0001, Kruskal-Wallis test) (Figure 6B), the levels of TNF-α in the EPO1 and EPO2 groups being significantly lower than that in the STRD group.

Postoperative complications were observed in two cases in the STRD group, but no such cases occurred in the EPO1 and EPO2 groups (Table 2). The median periods

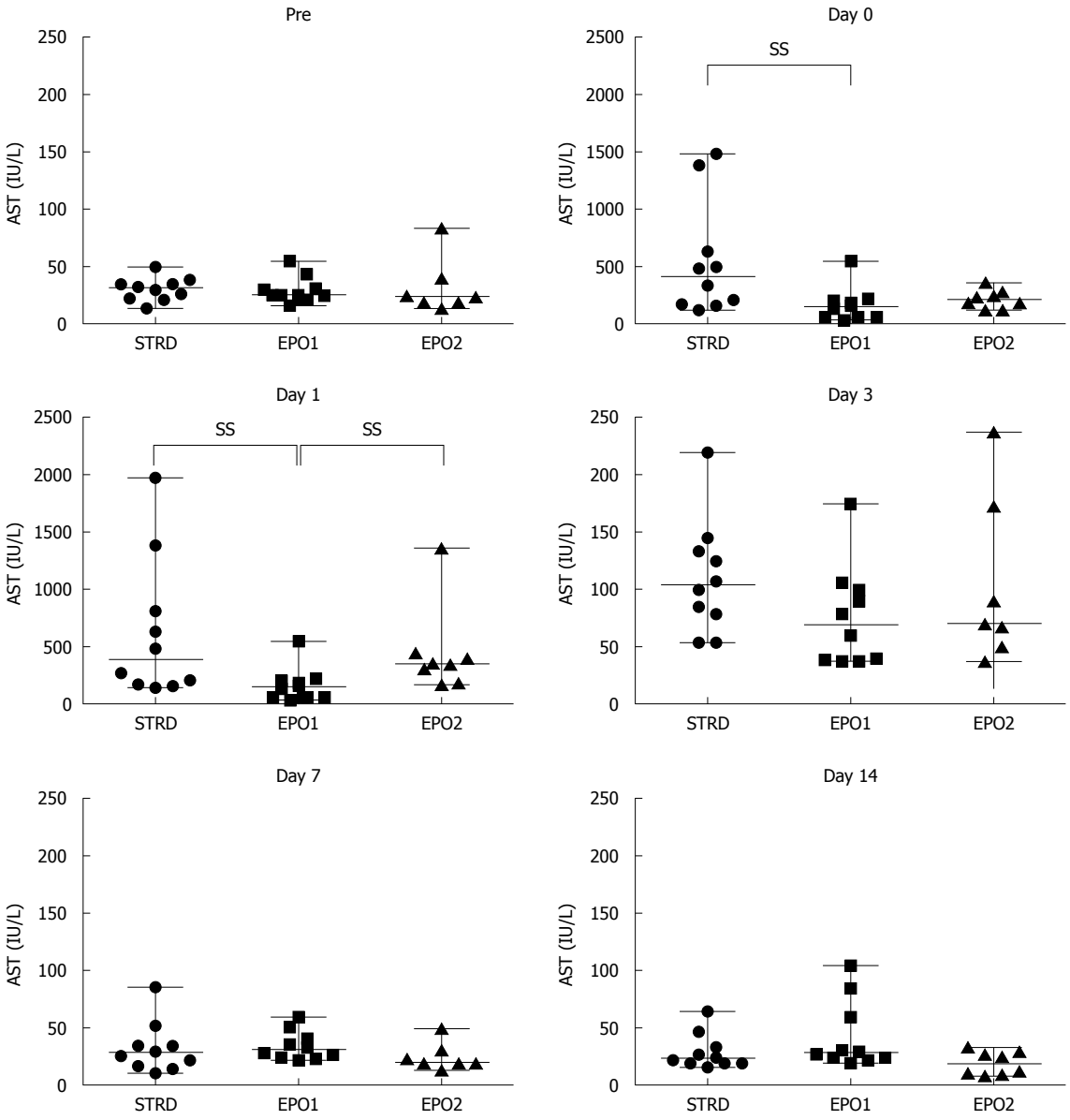

Figure 2 Changes in aspartate aminotransferase. The aspartate aminotransferase (AST) level in the erythropoietin (EPO)1 group was significantly lower than that in the steroid (STRD) group on Day 0 (P = 0.041, Kruskal-Wallis test), and also significantly lower than that in the STRD and EPO2 groups on Day 1 (P = 0.018, Kruskal-Wallis test). There were no significant differences in the AST level after Day 3. Note that the scale of the Y-axis differs in each graph. SS: Statistically significant.

Table 2 Postoperative complications

	Complication	Frequency (%)
STRD (n = 10)	n = 2 Prolonged ascites Pleural effusion	20
EPO1 (n = 10)	0	0
EPO2 (n = 8)	0	0

STRD: Steroid group; EPO: Erythropoietin.

of hospitalization in the STRD, EPO1, and EPO2 groups were 32 (10-74), 13 (8-77) and 16 (10-22) d, respectively (P = 0.0463, Kruskal-Wallis test) (Figure 7), this period being significantly longer in the STRD group than in the EPO1 and EPO2 groups.

DISCUSSION

Most patients undergoing hepatic resection have associated chronic hepatic disease and deterioration of hepatic functional reserve. A dilemma therefore arises in deciding a balance between the curativeness of hepatic resection and the likelihood of postoperative hepatic failure. There is a tendency for surgeons to aim at removing as large a portion of liver as possible to ensure that any disease, such as carcinoma, is resected completely. On the other hand, excessive removal of liver parenchyma results in a higher risk of postoperative hepatic failure. Amelioration of IR/I is therefore a crucial factor to consider in the prevention of liver function deterioration.

The present study demonstrated that Epo potently ameliorated IR/I following the PM. The AST level in

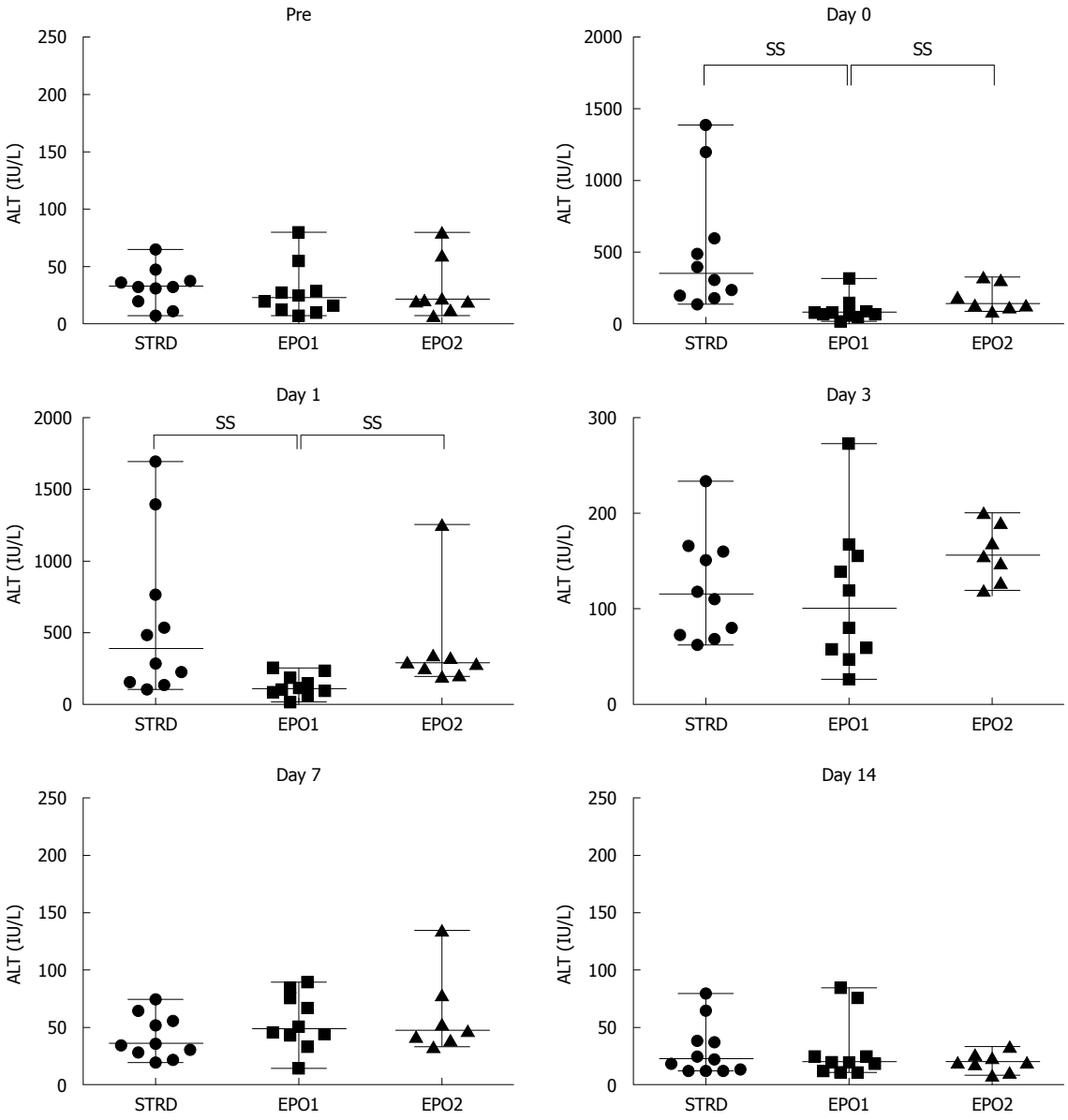

Figure 3 Changes in alanine transaminase. The alanine transaminase (ALT) level in the erythropoietin (EPO)1 group was significantly lower than that in the steroid (STRD) and EPO2 groups on Day 0 (P = 0.020, Kruskal-Wallis test) and Day 1 (P = 0.004, Kruskal-Wallis test). There were no significant differences in the ALT level after Day 3. Note that the scale of the Y-axis differs in each graph. SS: Statistically significant.

the EPO1 group was significantly lower than that in the STRD group on Day 0, and significantly lower than in the STRD and EPO2 groups on Day 1. The ALT level in the EPO1 group was significantly lower than in the STRD and EPO2 groups on both Day 0 and Day 1. There were no significant differences in AST and ALT levels between the three groups after Day 3. Our results demonstrated that the protective effect of Epo against IR/I was most pronounced at a very early stage after surgery, and furthermore, was stronger than that of steroids. During the very early post-surgical phase, there were no differences in LDH level, although the lactate level tended to be lower in the EPO1 group than in the STRD and EPO2 groups. The levels of proinflammatory cytokines, IL-6 and TNF-α, showed similar tendencies. The level of IL-6 in the EPO1 group was significantly lower than in the EPO2 and STRD groups. The level of TNF-α in the EPO1 and EPO2 groups was significantly lower than that in the STRD group.

The mechanism responsible for the protective effect of Epo against IR/I has been studied extensively by various groups, including our own[9-11]. As shown in this study, Epo protects organs and tissues from IR/I by inhibiting IR/I-induced cell apoptosis. Furthermore, Epo induces neovascularization and inhibits the secretion of acute inflammatory cytokines[4-6]. Epo binds to its specific cell-surface receptor (EpoR) and transduces signals to the nucleus[11]. The most important signaling pathways are the Jak2/STAT, Jak2/PI3K/AKT, and Jak2/MAPK/p38 pathways[12,13]. These signals activate anti-apoptotic genes, such as XIAP and BclxL, and suppress proapoptotic genes, such as BAD and GSK3β, resulting in inhibition of

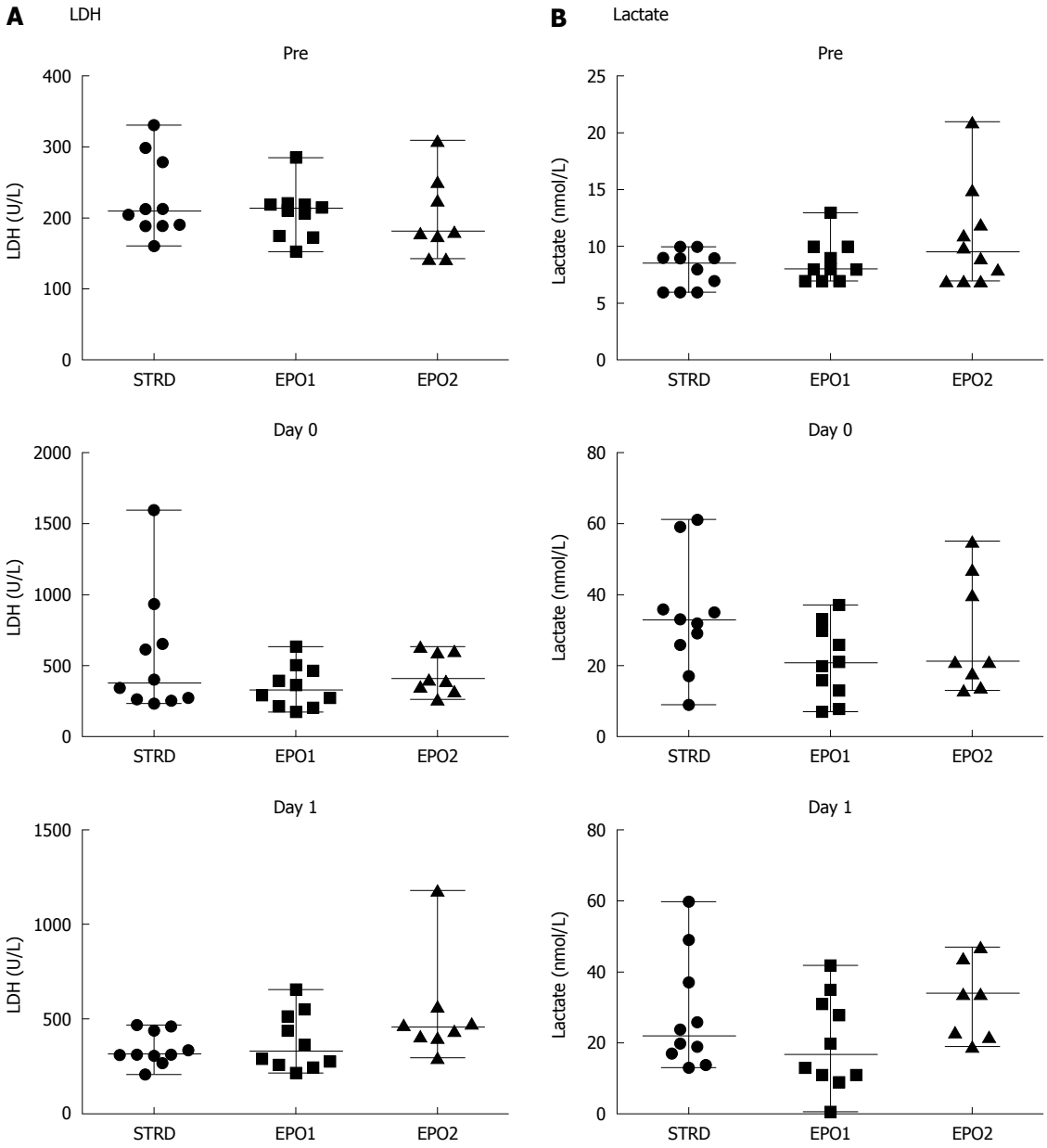

Figure 4 Lactate dehydrogenase (A) and lactate (B) levels at Pre, and on Day 0 and Day 1. There were no significant differences between the three groups in both lactate dehydrogenase (LDH) and lactate levels. Note that the scale of the Y-axis differs in each graph ($P > 0.05$ for hemoglobin and hematocrit, Kruskal-Wallis test). STRD: Steroid group; EPO: Erythropoietin.

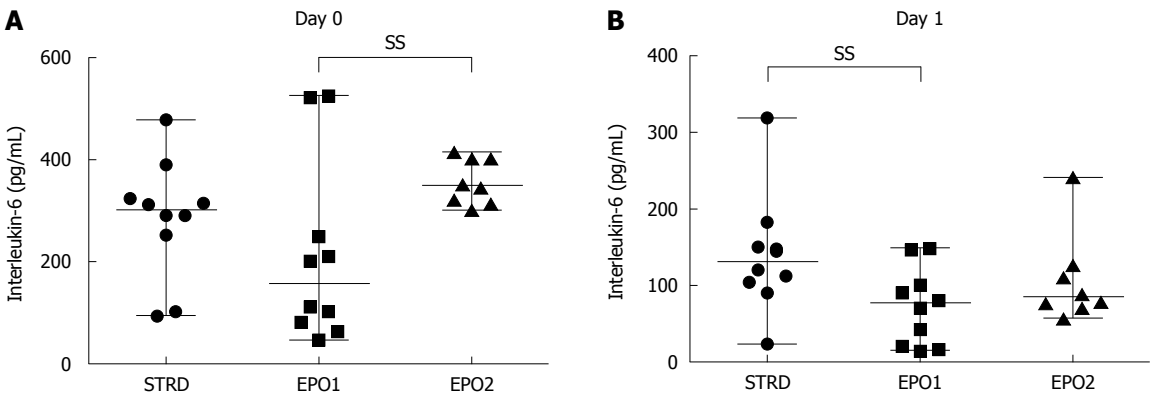

Figure 5 Interleukin-6 level on Day 0 (A) and Day 1 (B). The interleukin-6 level in the erythropoietin (EPO)1 group was significantly lower than that in the EPO2 group and the steroid (STRD) group on Day 0 ($P = 0.0036$, Kruskal-Wallis test). Note that the scale of the Y-axis differs in each graph. SS: Statistically significant.

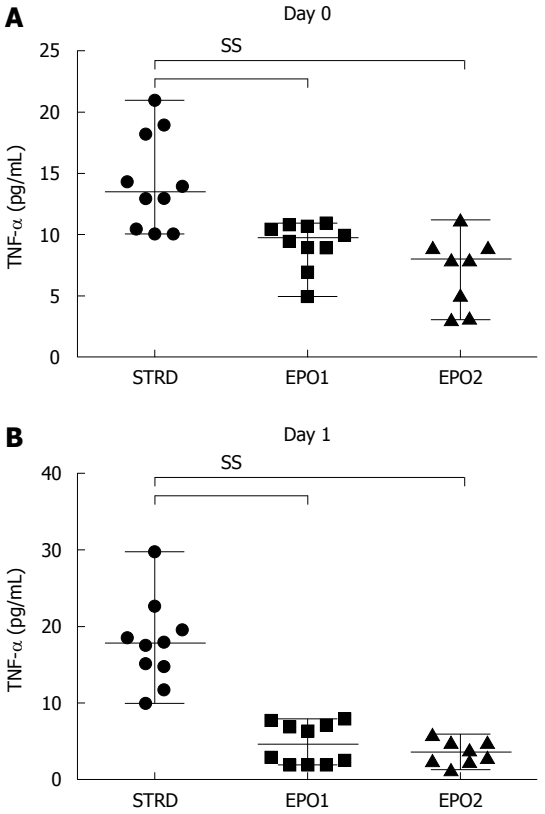

Figure 6 Tumor necrosis factor-α level on Day 0 (A) and Day 1 (B). Levels of tumor necrosis factor (TNF)-α in the erythropoietin (EPO)1 and EPO2 groups were significantly lower than that in the steroid (STRD) group on both Day 0 (P = 0.0006, Kruskal-Wallis test) and Day 1 (P < 0.0001, Kruskal-Wallis test). Note that the scale of the Y-axis differs in each graph. SS: Statistically significant.

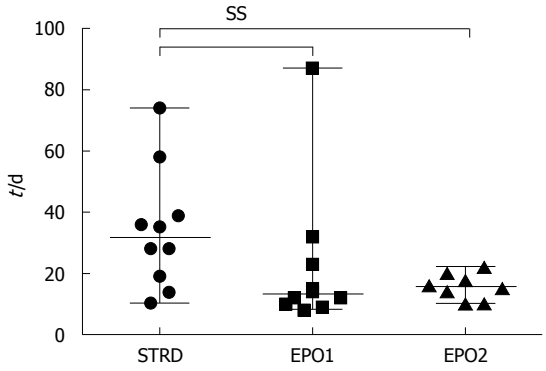

Figure 7 Length of hospitalization. Hospitalization in the steroid (STRD) group was significantly longer than that in the erythropoietin (EPO)1 and EPO2 groups (P = 0.0463, Kruskal-Wallis test). SS: Statistically significant.

apoptosis[10,14-17]. EpoR usually has a homodimeric structure, but Epo has been shown to bind to heterodimeric EpoR, consisting of EpoR and the β common receptor when exerting its extra-haematopoietic effect. This heterodimeric receptor is expressed in various organs, including the liver [18].

We employed two different dose regimes for Epo: EPO1 (30000 U × 2) and EPO2 (60000 U × 1). The results, in terms of AST, ALT, and IL-6 levels, indicated that EPO1 was more effective. In terms of the TNF-α level, the EPO2 regimen showed a stronger inhibitory effect than the EPO1 regimen, but not to a significant degree. Although the reason for this finding is unclear, previous studies have reported that Epo directly and indirectly inhibits the expression of IL-6 and TNF-α, and reduces their systemic levels[19-21]. The extra-hematopoietic effects of Epo have been studied in various animal models and clinical settings. These previous studies each employed different doses of Epo. The use of Epo in animal models has been reviewed by Sharples *et al*[13], Johnson *et al*[22], and Arcasoy[6]. In clinical studies, Ehrenreich *et al*[23] used Epo at a total dose of 100000 U for the first 3 d after acute cerebral stroke, noting that this was safe and well tolerated, and also used a dose of 40000 U for 3 mo to treat schizophrenia[9]. Lipsic *et al*[5] used a single bolus injection of Epo at a dose of 60000 U for patients with acute myocardial infarction, and did not observe any significant increase in Hb. Mocini *et al*[24] used a single injection of Epo at a dose of 40000 U before cardiac surgery, but did not observe any significant improvement or an increase in erythropoiesis. The results suggest that the optimal dose of Epo and the mode of administration may vary according to species and target organ. Our present findings indicate that 30000 U of Epo × 2, rather than 60000 U of Epo × 1, has a stronger inhibitory effect on IR/I following the PM, and that the doses and the mode of administration we employed did not affect haematopoiesis (Figure 1).

The period of hospitalization was significantly longer in the STRD group than in the EPO1 and EPO2 groups. This was a prospective study performed during a single period. Therefore, the patients who were treated with steroid stayed longer in hospital. The occurrence of complications in the STRD, EPO1, and EPO2 groups was 20%, 0% and 0%, respectively. In this study, we evaluated only grade Ⅱ complications by Clavien's classification[25]. Although the number of patients in each group was small and more studies are needed, the data suggest that Epo might reduce the rate of complications after hepatic resection.

We conclude that Epo has greater potential than steroids to ameliorate IR/I following the PM, and that Epo at a dose of 30000 U, given just before the PM and just after surgery, yields better results. This protective effect of Epo may be applicable not only in patients undergoing hepatic resection, but also for much more severe IR/I, such as that occurring in liver transplantation.

COMMENTS

Background

Ischemia-reperfusion injury (IR/I) is an obstacle encountered in various medical fields. Especially in liver surgery, IR/I after the Pringle maneuver (PM) hinders curability. Up to now, steroids have been used for amelioration of IR/I, but stronger and more effective drugs to prevent IR/I are needed.

Research frontiers

Erythropoietin (Epo) is a hematopoietic cytokine that has been used to treat anemia in patients with end-stage renal disease. Recently, extra-hematopoietic effects of Epo have been reported, including an organ-protective effect against IR/I. However, there have been no reports of the hepatoprotective effect of Epo in a clinical setting.

Innovations and breakthroughs

This study is the first prospective study to have confirmed the protective effect of Epo against IR/I after the PM, in comparison with steroid treatment. It was found that Epo strongly ameliorated IR/I after the PM, and that the effect was better than that of the steroid. Epo would therefore contribute to better preservation of liver function after liver surgery.

Applications

This study demonstrated that Epo shows promise for preventing IR/I after the PM. This finding extends its application not only to liver resection, but also liver transplantation, where amelioration of IR/I would improve survival.

Terminology

IR/I is a tissue injury occurring after ischemia-reperfusion. Organs are continuously perfused with blood, but once the blood supply is interrupted, organs sustain damage due to an anaerobic environment (ischemia). Restoration of the blood supply then causes further organ damage (reperfusion injury).

Peer review

This is an original study because it is the first prospective randomized study of its kind, although the number of patients in each group is small.

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BRIEF ARTICLE

mPGES-1 expression in non-cancerous liver tissue impacts on postoperative recurrence of HCC

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Abstract

AIM: To investigate whether microsomal prostaglandin E synthase-1 (mPGES-1) expression in hepatocellular carcinoma (HCC) and in non-cancerous liver affects HCC prognosis after hepatectomy.

METHODS: The relationship between patient clinical profiles, tumor factors, surgical determinants, and mPGES-1 expression and the recurrence-free survival rate were examined in 64 patients who underwent curative hepatectomy between March 2003 and December 2006.

RESULTS: The scores for mPGES-1 expression were higher in well differentiated and moderately differentiated HCC tissues than in poorly differentiated HCC tissues (well differentiated, 5.1 ± 2.7; moderately differentiated, 5.1 ± 1.7; poorly differentiated, 3.0 ± 1.8). In non-cancerous liver tissues, the mPGES-1 levels were higher in injured liver tissues than in normal tissues. Cirrhotic livers had higher mPGES-1 levels than livers with chronic hepatitis (normal livers, 3.3 ± 0.7; chronic hepatitic livers, 5.4 ± 1.9; cirrhotic livers, 6.4 ± 1.6). A univariate analysis revealed that the recurrence-free survival rate was significantly lower in patients with vascular invasion, a higher mPGES-1 level in non-cancerous liver tissue, a larger tumor diameter (≥ 5 cm), and a lower serum albumin level (≤ 3.7 g/dL). The mPGES-1 expression in HCC tissues did not correlate well with postoperative recurrence. A multivariate analysis demonstrated that the presence of vascular invasion and higher mPGES-1 levels were statistically significant independent predictors for early postoperative recurrence of HCC.

CONCLUSION: Increased mPGES-1 expression in non-cancerous liver tissues is closely associated with the early recurrence of HCC after curative resection.

Key words: Curative resection; Hepatocellular carcinoma; Microsomal prostaglandin E synthase-1; Non-cancerous liver tissue; Recurrence-free survival

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a common cause of cancer death worldwide[1,2]. Hepatectomy is one of the best treatment modalities for HCC. Recent advances in surgical techniques and perioperative management have led to improved survival after curative resection. However, the rates of postoperative recurrence remain high (60%-80%)[3], and such recurrences can originate from intrahepatic metastases of the primary HCC and from the multicentric occurrence of new tumors[4]. With regard to the latter, many studies have reported a significant association between HCC development and underlying liver disease[5,6]. Therefore, HCC tumor factors as well as the underlying hepatic status should be carefully examined to predict tumor recurrence after curative resection and to choose optimal treatments.

A variety of malignant tumors in many visceral sites have appeared after chronic inflammation[7]. Clinical and biochemical evidence suggests that prostaglandin E_2 (PGE_2) produced at inflammation sites and its receptors play an important role in the development of malignant tumors, including HCC and other cancers[8,9]. The biosynthesis of PGE_2 from arachidonic acid requires two enzymatic activities that include cyclooxygenase (COX) and prostaglandin E synthase (PGES), which is the terminal enzyme for PGE_2 biosynthesis. Three PGES isoforms have been identified, including microsomal PGES-1 (mPGES-1), mPGES-2, and cytosolic PGES[10,11]. In particular, mPGES-1, an enzyme induced by pro-inflammatory stimuli, has received much attention[8,11]. Previous studies have indicated that mPGES-1 overexpression was associated with various types of cancer, including HCC[12,13]. Therefore, mPGES-1 may play an important role in HCC recurrence in the remnant liver tissue after curative resection for HCC.

The aim of the present study was to clarify whether mPGES-1 expression in HCC and non-cancerous liver tissues affects the clinical course of HCC patients undergoing curative resection.

MATERIALS AND METHODS

Patients and follow-up

Sixty-four consecutive patients (42 males and 22 females) underwent curative liver resection for HCC at the Division of Surgery, National Hospital Organization, Nagasaki Medical Center, between March 2003 and December 2006. In all cases, the diagnosis of HCC was confirmed by pathological examination of the resected specimens.

The inclusion criteria for the study were as follows: (1) the absence of extrahepatic metastasis; (2) curative resection defined as histological evidence of the complete removal of HCC tumors; and (3) no additional therapies or multi-modality treatment for HCC until the development of recurrence. Written informed consent was obtained from all the patients. They were regularly followed up at our outpatient clinic and were prospectively monitored for disease recurrence by serum levels of α-fetoprotein (AFP) and des-γ-carboxy prothrombin (DCP), and ultrasonography or computed tomography every 3 mo. Suspected intra-hepatic recurrence was confirmed by hepatic angiography, and if necessary, by percutaneous needle biopsy. The follow-up period was at least 12 mo or until death in patients who died within 12 mo of their operation. The study was conducted in accordance with the Helsinki Declaration and the guidelines issued by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the Ethics Committee at National Hospital Organization, Nagasaki Medical Center.

Tissue samples

HCC tissues and non-cancerous liver tissues from the opposite liver lobe in which HCC developed were obtained. The tissues were frozen in liquid nitrogen and stored at -80℃ until use. For immunohistochemical analysis, the tissues were formalin-fixed and paraffin-embedded.

Histologically "normal" livers (free of hepatitis B or C viral infections and without any significant pathological abnormalities) were obtained from 7 patients with liver metastases from colorectal cancer.

Immunohistochemistry

For immunohistochemical analysis of the mPGES-1 protein, formalin-fixed and paraffin-embedded tissue blocks were cut into 4 μm-thick sections. The sections were deparaffinized in xylene and subsequently rehydrated in sequential ethanol (100%-70%). After washing 3 times with 10 mmol/L phosphate-buffered saline (PBS) (pH 7.4), antigen retrieval was performed by first heating in a microwave at 95℃ for 20 min, then by washing twice in PBS for 10 min. The sections were treated with peroxidase-blocking solution (DAKO Japan, Kyoto, Japan) for 5 min, and incubated with the primary antibody for 60 min at room temperature. The primary antibody used was a 1:100 dilution of a mPGES-1 polyclonal antibody (Cayman Chemical, Ann Arbor, MI, USA). A standardized two-step method with ENVISION plus (DAKO) was used for detection. The reaction products were visualized using diaminobenzidine as a chromogen (DAKO), and counterstained with Mayer's hematoxylin (DAKO). The specificity of the antibody was checked by the adsorption with corresponding blocking peptides (Cayman Chemical) using a 1:1 ratio of primary antibody to blocking peptide.

Scoring criteria for mPGES-1 expression

Two blinded investigators (MI and KN) evaluated the immunostained sections. To assess the mPGES-1 protein staining results, the cytoplasmic immunoreactive intensity was scored as previously described[14]. In summary, the staining intensity for mPGES-1 was scored in each specimen on a scale of 0-3, with 0 = negative staining, 1 = weakly positive staining, 2 = moderately positive staining, and 3 = strongly positive staining (Figure 1). The staining intensity was evaluated for the maximum intensity among

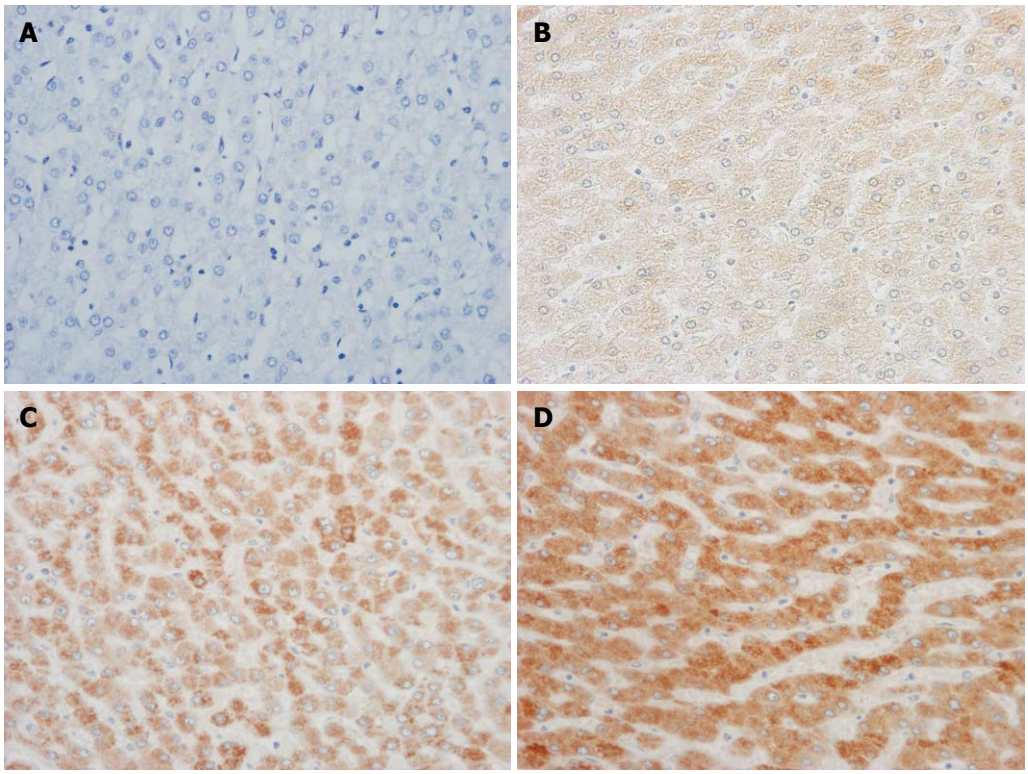

Figure 1 Grading of immunohistochemical staining for microsomal prostaglandin E synthase-1 protein in representative liver tissue (original magnification, × 200). A: No immunoreactivity for microsomal prostaglandin E synthase-1 (mPGES-1) (grade 0); B: Weakly positive for mPGES-1 (grade 1); C: Moderately positive for mPGES-1 (grade 2); D: Strongly positive for mPGES-1 (grade 3).

positive cells ("maximum intensity of staining", Ⅰ) and the intensity level observed in the largest number of positive cells ("most extensive intensity level", Ⅱ). The extent to which positive cells were observed in each specimen ("extent of distribution of positive cells", Ⅲ) was estimated and scored on a scale of 0-4, with 0 = negative, 1 = positive in 1%-25% of cells, 2 = positive in 26%-50% of cells, 3 = positive in 51%-75% of cells, and 4 = positive in 76%-100% of cells. Each section was evaluated for the sum of these three parameters (Ⅰ + Ⅱ + Ⅲ). Immunoreactivity for mPGES-1 protein was compared statistically using the average of the sum in each histological category. The patients in the present study were divided into two groups, including the higher expression (the sum of the categorical score, 6 to 10) and the lower expression groups (the sum of the categorical score; less than 6).

Western blotting analysis

We performed a Western blotting analysis on representative samples of HCC and non-cancerous liver tissues. The tissues were homogenized on ice in RIPA buffer [PBS, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] containing 100 ng/mL phenylmethylsulphonyl fluoride, 4 mg/mL aprotinin, 2 mg/mL leupeptin, 1 mg/mL pepstatin, 10 mg/mL antipain, 10 mg/mL soybean trypsin inhibitor, and 2 mmol/L ethylenediaminetetraacetic acid. The homogenates were clarified by centrifugation. Protein concentrations were measured using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). After boiling for 5 min in the presence of 2-mercaptoethanol, samples containing 50 mg of tissue lysates were separated on 12.5% SDS-polyacrylamide gels and then transferred onto equilibrated Hybond PVDF membranes (Amersham International, Buckinghamshire, UK). After skim milk blocking, the membranes were then incubated with the mPGES-1 polyclonal antibody (at a dilution of 1:500). Bound antibodies were detected with horseradish peroxidase-labeled rabbit anti-goat IgG (Southern Biotechnology Associates, Birmingham, AL, USA) using an enhanced chemiluminescence detection system (ECL kit; Amersham International, Buckinghamshire, UK).

Analysis of the risk factors for HCC recurrence after curative resection

The following clinicopathological factors were evaluated for their association with HCC recurrence: age, gender, presence of hepatitis B surface antigen (HBsAg) or anti-hepatitis C virus antibody (anti-HCV Ab), platelet count, preoperative blood chemistry (serum levels of total bilirubin, alanine aminotransferase and albumin), presence of liver cirrhosis, and mPGES-1 expression. The evaluated operative factors included the intraoperative blood loss and the hepatectomy method. The tumor factors were the greatest tumor diameter, the number of tumor nodules, the presence of vascular invasion, the presence of capsular formation, the histological grade, and the serum levels of AFP and DCP. The hepatectomy method was classified as anatomical or non-anatomical resection ac-

Table 1 Microsomal prostaglandin E synthase-1 expression in hepatocellular carcinoma and non-cancerous liver tissue *n* (%)

	No. of cases	Patients with higher scores	Patients with lower scores	Scores (mean ± SD)	*P*
Hepatocellular carcinoma tissues					
Well differentiated	18	7/18 (38.9)	11/18 (61.1)	5.1 ± 2.7	-
Moderately differentiated	40	14/40 (35.0)	26/40 (65.0)	5.1 ± 1.7	0.959[a]
Poorly differentiated	6	0	6/6 (100)	3.0 ± 1.8	0.009[a]
Non-cancerous liver tissues					
Normal	2	1/2 (50.0)	1/2 (50.0)	3.3 ± 0.7	-
Chronic hepatitis	31	19/31 (61.3)	12/31 (38.7)	5.4 ± 1.9	0.006[b]
Cirrhosis	31	15/31 (48.4)	16/31 (51.6)	6.4 ± 1.6	0.002[b], 0.039[c]
Normal livers from colorectal cancer	7	0	7/7 (100)	3.5 ± 0.5	-

[a]*vs* well differentiated hepatocellular carcinomas; [b]*vs* normal livers; [c]*vs* chronic hepatitis.

cording to the methods described by Makuuchi *et al*[15] and Takayama *et al*[16]. The anatomic resection consisted of the systematic removal of the hepatic segment which is confined by the tumor-bearing portal tributaries. In the non-anatomic resection, the liver was divided along a line so as to secure a surgical margin of at least 5 mm, if possible.

Statistical analysis

Statistical analyses were performed using either Student's *t*-test or the Mann-Whitney *U* test to compare variables between the groups. A recurrence-free survival curve was plotted using the Kaplan-Meier method. A statistical comparison of the recurrence-free survival was performed using the log-rank test. A multivariate analysis by the Cox proportional hazard model was used to identify the independent risk factors for tumor recurrence. A P value < 0.05 was considered statistically significant. Statistical analyses were performed using the StatView for Windows software program (version 5.0, SAS Institute Inc., Cary, NC, USA).

RESULTS

Characteristics of the patients

There were 42 male (65.6%) and 22 female (34.4%) patients. The mean age was 64 years (range, 38-86 years). Twenty-one patients were positive for HBsAg, 32 were positive for anti-HCV Ab, and 11 were negative for both. Thirty-one patients had a cirrhotic liver, while 33 did not. The maximum tumor size was 12 cm, and 57 patients (89.1%) had a solitary tumor. More than 90% of the patients enrolled in the study had a Child-Pugh classification of A for liver function. Fifty percent of the patients had a tumor size > 3 cm. In the pathological differentiation, HCC was well differentiated in 18 patients, moderately differentiated in 40, and poorly differentiated in 6. The median observation period was 49 mo (range, 3-74 mo).

Immunohistochemical analysis of mPGES-1 protein

The expression of mPGES-1 protein in the HCC and non-cancerous liver tissues was examined immunohistochemically. Various degrees of staining for mPGES-1 protein were observed. The scores for mPGES-1 expression in the HCC and non-cancerous liver tissues are summarized in Table 1. The marked expression of mPGES-1 was demonstrated in well differentiated as well as in moderately differentiated HCC tissues (scores; 5.1 ± 2.7 and 5.1 ± 1.7, respectively). Conversely, mPGES-1 expression was significantly weaker in poorly differentiated HCC tissues (score; 3.0 ± 1.8, $P < 0.05$). Seven of 18 cases (38.9%) with well differentiated HCC and 14 of 40 cases (35.0%) with moderately differentiated HCC had high expression scores, whereas none of the patients with poorly differentiated HCC had high expression scores.

The mPGES-1 levels increased significantly with fibrotic stage of the liver tissues (scores; normal liver 3.3 ± 0.7, chronic hepatitic livers 5.4 ± 1.9, cirrhotic livers 6.4 ± 1.6). High expression scores were observed in 1 of 2 normal livers (50%), 19 of 31 chronic hepatitic livers (61.3%), and 15 of 31 cirrhotic livers (48.4%). There was no significant correlation between tumor differentiation and non-cancerous liver tissue in the expression of mPGES-1 (data not shown). Additionally, mPGES-1 expression in normal livers obtained from 7 patients with liver metastasis was lower than that in damaged livers ($P < 0.05$).

Western blotting analysis of mPGES-1

To confirm the specificity of the mPGES-1 antibody and the presence of mPGES-1 protein in the specimen, Western blotting analysis was performed on representative samples of HCC and non-cancerous liver tissues. Both tissue types yielded a single band with a molecular weight of 16 kDa, indicating the presence of mPGES-1 protein (Figure 2).

Correlation between the levels of mPGES-1 expression and recurrence-free survival time

We evaluated the correlation between the levels of mPGES-1 expression in HCC and non-cancerous liver tissues and recurrence-free survival time. No statistically significant difference was observed in the recurrence-free survival time between the higher and lower expression groups in HCC tissues (Figure 3A). In contrast, a statistically significant difference in the recurrence-free survival time was observed between the higher and lower expression groups in non-cancerous liver tissues ($P = 0.006$, Figure 3B).

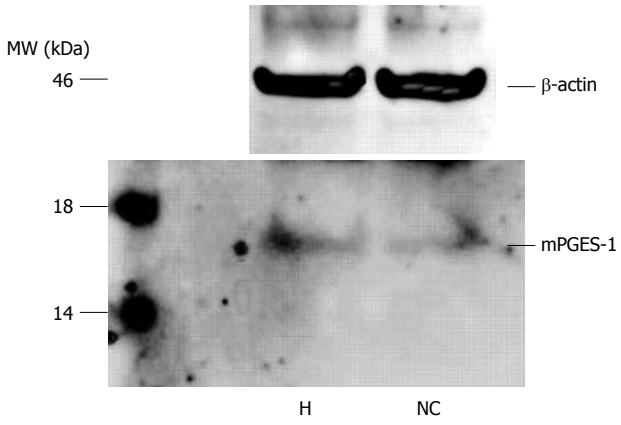

Figure 2 Western blotting analysis for microsomal prostaglandin E synthase-1. A band of 16 kDa in molecular weight, thus indicating the presence of microsomal prostaglandin E synthase-1 (mPGES-1) protein, is identified in both hepatocellular carcinoma tumors (H) and non-cancerous liver (NC) tissues. MW: Molecular weight.

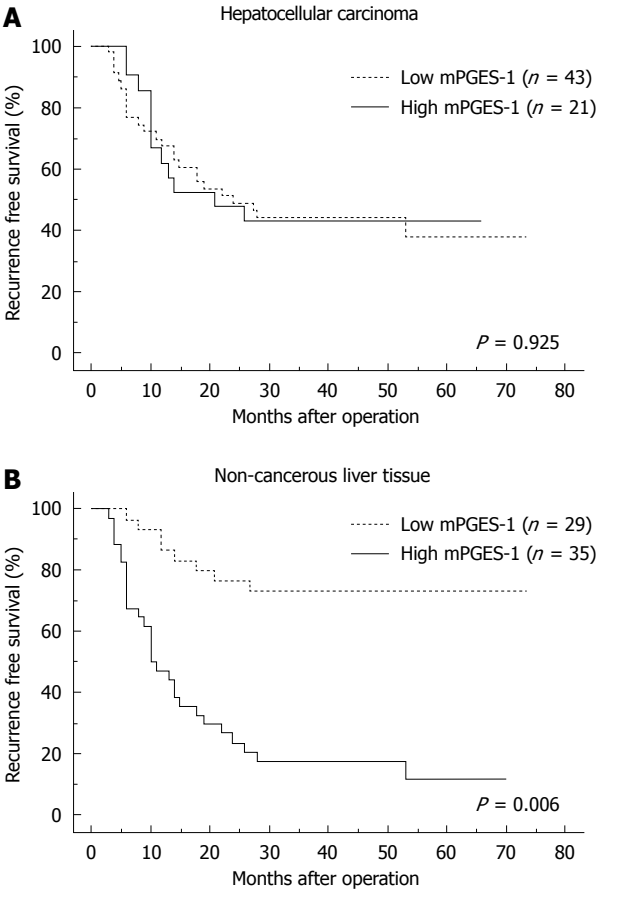

Figure 3 Recurrence-free survival time based on microsomal prostaglandin E synthase-1 expression in hepatocellular carcinoma tissues (A) and non-cancerous liver tissues (B). The recurrence-free survival time is significantly shorter in patients with an increased expression of microsomal prostaglandin E synthase-1 (mPGES-1) in non-cancerous liver tissues.

Correlation between various clinicopathological parameters and recurrence-free survival time

Various clinicopathological parameters were evaluated for their association with HCC recurrence (Table 2). A univariate analysis revealed that recurrence-free survival time was

Table 2 Univariate analysis of clinicopathological features related to postoperative recurrence

	No. of patients	Postoperative recurrence		*P*
		Yes	No	
Age (yr)				
≥ 60	45	23	22	0.733
< 60	19	9	10	
Gender				
Male	43	20	23	0.481
Female	21	12	9	
Hepatitis B surface antigen				
Positive	21	13	8	0.147
Negative	43	19	24	
Hepatitis C virus antibody				
Positive	32	12	20	0.151
Negative	32	20	12	
Total bilirubin (mg/dL)				
≥ 1.0	29	7	22	0.987
< 1.0	35	13	22	
Alanine aminotransferase (IU/L)				
≥ 50	26	11	15	0.635
< 50	38	21	17	
Albumin (g/dL)				
≥ 3.7	49	24	25	0.022
< 3.7	15	13	2	
Platelets (10^4/μL)				
≥ 10	46	21	25	0.465
< 10	18	11	7	
Liver cirrhosis				
Present	31	16	15	0.772
Absent	33	16	17	
T mPGES-1				
High	21	12	9	0.925
Low	43	25	18	
NC mPGES-1				
High	35	23	11	0.006
Low	29	9	21	
Hepatectomy				
Anatomic	31	21	10	0.149
Non-anatomic	33	16	17	
Operative blood loss (mL)				
≥ 500	15	12	3	0.091
< 500	49	25	24	
α-fetoprotein (ng/mL)				
≥ 100	15	10	5	0.347
< 100	49	27	22	
DCP (mAU/mL)				
≥ 400	22	15	7	0.081
< 400	42	19	23	
Tumor diameter (cm)				
≥ 5	16	12	3	0.018
< 5	48	20	29	
Tumor number				
Multiple	7	3	4	0.789
Solitary	57	29	28	
Histological grade				
Well	18	8	10	0.495
Moderate	40	21	19	
Poor	6	3	3	
Capsular formation				
Present	49	25	24	0.320
Absent	15	7	8	
Vascular invasion				
Present	16	11	5	< 0.001
Absent	48	9	39	

DCP: Des-γ-carboxy prothrombin; mPGES-1: Microsomal prostaglandin E synthase-1; T mPGES-1: mPGES-1 expression in hepatocellular carcinoma tumor tissue; NC mPGES-1: mPGES-1 expression in non-cancerous liver tissue.

Table 3 Multivariate analysis of the risk factors for postoperative recurrence

Variables	Hazard ratio	95% CI	*P*
Vascular invasion (present)	4.116	1.813-9.344	< 0.001
NC mPGES-1 expression (high)	4.074	1.760-9.428	0.001
Tumor diameter (≥ 5 cm)	2.060	0.860-4.935	0.105
Albumin (< 3.7 g/dL)	1.745	0.589-3.165	0.315

NC mPGES-1: Microsomal prostaglandin E synthase-1 expression in non-cancerous liver.

shorter in cases with vascular invasion, higher mPGES-1 levels in non-cancerous liver tissue, a larger tumor diameter (≥ 5 cm), and lower levels of serum albumin (< 37 g/L). The operative factors were not significantly correlated with recurrence-free survival time. A multivariate analysis demonstrated that the presence of vascular invasion and higher mPGES-1 levels in the non-cancerous liver tissue were significant independent predictors for the early recurrence of HCC after curative hepatectomy (Table 3).

DISCUSSION

The present study demonstrated that the rate of HCC recurrence was high after curative resection. This finding was consistent with those described in other recent reports[1-6]. Tumor recurrence is caused by metastatic lesions, residual microscopic lesions that remain after curative resection, or multicentric occurrence in the setting of hepatitis or cirrhosis[17,18]. The prevention of tumor recurrence is key to the improvement of prognosis for HCC patients after a hepatectomy[19]. In the present study, a multivariate analysis indicated that the two independent predictors for HCC recurrence after curative resection were the presence of vascular invasion and increased mPGES-1 expression in the non-cancerous liver tissue.

Vascular invasion is a well-known risk factor for a poor prognosis after curative resection. The presence of vascular invasion is considered one of the strongest predictors of intrahepatic metastasis caused by the spread of cancer cells *via* the portal venous system[17-19]. Although several reports have demonstrated that postoperative adjuvant therapy prevented postoperative HCC recurrence[20,21], its efficacy has yet to be determined. Other therapeutic modalities for treating postoperative recurrence are urgently needed.

The most interesting finding in the present study was that increased mPGES-1 expression in the non-cancerous liver tissue was an independent predictor for early HCC recurrence after curative resection. Increased mPGES-1 levels induce PGE_2 synthesis, which may create a suitable environment for occult intrahepatic metastases to survive and spread after hepatectomy. This hypothesis is supported by several studies showing that PGE_2 was implicated in migration, secretion of various types of matrix metallo-proteinases, and cell adhesion in HCC cells[22-24]. Additionally, increased PGE_2 levels in the non-cancerous liver tissue leads to prolonged acceleration of necroinflammation and regeneration in the remnant liver[25]. The inflamed liver may also provide a good environment for occult intrahepatic metastases to grow in response to different growth factors[26]. In the present study, active hepatitic and/or cirrhotic livers had increased mPGES-1 expression compared to normal livers. The repeated cycles of necroinflammation, degeneration, and regeneration increase hepatocyte turnover, which facilitates spontaneous mutation and may hinder DNA repair[26]. The release of reactive oxygen species including superoxide and H_2O_2 in this situation may also contribute to uncontrolled cell growth, apoptosis, and senescence[27]. Another possible mechanism is that mPGES-1 itself may act as a landscaping tumor promoter. mPGES-1 lies downstream of the PGE_2-biosynthetic pathway of COX-2. Recent studies reported that mPGES-1 was expressed in several cancers and was linked to carcinogenesis[12,28]. mPGES-1 derived from the stromal component may promote tumor growth by producing bioactive PGE_2, which acts angiogenetically or immunosuppressively, and affects carcinoma cells in a paracrine fashion[12,28]. Therefore, the increased expression of mPGES-1 in the non-cancerous liver tissue may create conditions suitable for HCC recurrence from metastasis or multicentric occurrence. However, the precise mechanisms remain to be elucidated.

The mPGES-1 expression in HCC tissues did not correlate well with postoperative recurrence. This finding suggested that the mPGES-1 in HCC tissues *per se* did not determine the malignant potential of HCC tissues, although overexpression of mPGES-1 was associated with various types of cancer[12,13].

The data indicate that COX-2 inhibitors are chemopreventive for several kinds of cancers[29], however, there have been no reports on HCC patients. Although the COX-2 inhibitors have a reduced gastrointestinal toxicity in comparison to traditional non-steroidal anti-inflammatory drugs, some adverse effects have been reported[30]. From this standpoint, more selective inhibition of the prostanoid pathway to PGE_2 is thus highly desirable. mPGES-1 is the terminal enzyme for PGE_2 biosynthesis, and thus it is considered the most selective agent for that pathway. Although there have been several reports concerning the selective inhibitors of mPGES-1[31,32], further studies are still needed in clinical settings.

In conclusion, increased mPGES-1 expression in non-cancerous liver tissue is closely associated with the early recurrence of HCC after curative resection. The present study also indicates that an inhibitor of mPGES-1 may be a new therapeutic option to improve the survival rate of HCC patients after curative resection.

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COMMENTS

Background

Microsomal prostaglandin E synthase-1 (mPGES-1) is the terminal enzyme in the formation of prostaglandin E_2 from prostaglandin H_2. Data indicate that increased expression of mPGES-1 is associated with various types of cancers. However, the impact of mPGES-1 expression on the clinical course of hepatocellular carcinoma (HCC) has not yet been elucidated.

Research frontiers

In HCC, tumor recurrence is caused by metastatic lesions, residual microscopic lesions that remain even after curative resection, and multicentric occurrence in the setting of hepatitis or cirrhosis. The research was performed to clarify the risk factors for the recurrence of HCC after curative resection in Nagasaki Medical Center.

Innovations and breakthroughs

The present study demonstrates that various degrees of mPGES-1 expression occur in HCC and non-cancerous liver tissues. This is the first report to demonstrate that increased expression of mPGES-1 in non-cancerous liver tissue is an independent predictor for HCC recurrence after curative resection.

Applications

mPGES-1 expression in non-cancerous liver tissue could be a useful biomarker for screening high risk groups of patients with HCC after curative resection. In the near future, a selective mPGES-1 inhibitor may prevent postoperative recurrence of HCC and improve the prognosis of HCC patients.

Terminology

mPGES-1 is a protein belonging to the membrane-associated proteins involved in eicosanoid and glutathione metabolism super family. mPGES-1 is induced by pro-inflammatory stimuli, down-regulated by anti-inflammatory glucocorticoids, and functionally coupled with cyclooxygenase-2. Thus, mPGES-1 plays a central role in the biosynthesis of prostaglandin E_2.

Peer review

The paper reported that increase in mPGES-1 in non-cancerous liver was an independent prognostic factor in patients received surgical therapy to HCC. Although the results might be of importance, several questions are addressed, and several points to be improved are suggested.

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BRIEF ARTICLE

Suspected uncomplicated cecal diverticulitis diagnosed by imaging: Initial antibiotics *vs* laparoscopic treatment

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Abstract

AIM: To compare the recurrence rate following initial antibiotic management to that following laparoscopic treatment for suspected uncomplicated cecal diverticulitis.

METHODS: We examined the records of 132 patients who were diagnosed with uncomplicated cecal diverticulitis and a first attack during an 8-year period. The diagnosis of uncomplicated diverticulitis was made based on imaging findings, such as inflamed diverticulum or a phlegmon with cecal wall thickening. Concurrent appendiceal dilatation from 8 to 12 mm was observed in 36 patients (27%). One hundred and two patients were treated initially with antibiotics only, whereas 30 underwent laparoscopic treatment, including partial cecectomy (n = 8) or appendectomy with diverticulectomy (n = 9) or appendectomy alone (n = 13). We compared clinical outcomes in both groups over a median follow-up period of 46 mo.

RESULTS: All patients were successfully treated with initial therapy. Of the 102 patients who initially received only antibiotic treatment, 6 (6%) had a recurrence (3 in the cecum and 3 in the ascending colon or transverse colon) during the follow-up period. Five of these patients were managed with repeated antibiotic treatment and 1 underwent ileocolic resection for perforation. Of the 30 patients treated by the laparoscopic approach, 2 (7%) had a recurrence (ascending colon) which was treated with antibiotics.

CONCLUSION: Initial antibiotic management for suspected uncomplicated cecal diverticulitis showed comparable efficacy to laparoscopic treatment in the prevention of recurrence.

Key words: Antibiotics; Cecal diverticulitis; Laparoscopy; Radiological imaging

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INTRODUCTION

Right colonic diverticulitis is a common complication of right-sided diverticular disease and has a higher prevalence in Oriental countries than in Western countries[1]. Cecal diverticulitis is uncommon and is rarely detected preoperatively because it is usually misdiagnosed as appendicitis[2].

There has been some controversy regarding the optimal management of cecal diverticulitis. Some surgeons recommend surgical treatment, claiming that cecal diverticulitis

does not usually resolve with medical therapy and has a high rate of recurrence with complications[3-5]. In contrast, other authors favor conservative treatment, stating that it is a safe and effective treatment regimen with a low recurrence rate[6-8]. Differences due to ethnicity or pathophysiological mechanisms including disease state may account for this variation in outcome.

With the increasing use of radiologic evaluation for right lower quadrant (RLQ) pain, the diagnosis of cecal diverticulitis is possible and may be assessed in both uncomplicated and complicated cases[9].

However, the appropriate treatment of suspected uncomplicated cecal diverticulitis diagnosed by radiologic evaluation is not definite and there have been few reports comparing the long-term recurrence rate following antibiotic only management and laparoscopic treatment for suspected uncomplicated cecal diverticulitis in an Asian population.

The aim of this study was to evaluate the treatment outcomes of suspected uncomplicated cecal diverticulitis diagnosed by radiologic imaging. To this end, we reviewed the long-term recurrence in a series of Asian patients who received initial antibiotic management, and compared this to laparoscopic treatment.

MATERIALS AND METHODS

During an 8-year period (2001 to 2008), 8814 patients admitted with RLQ pain were assessed (Table 1). We performed routine computed tomography (CT) in these patients, and in some indeterminate cases, adjuvant specific appendiceal ultrasonography was performed.

Clinical information was reviewed retrospectively using an existing database which revealed 164 patients with suspected cecal diverticulitis following radiologic evaluations. All patients were of Asian descent with a first documented attack. Twenty one patients who underwent surgery for suspected perforation or generalized peritonitis and 11 patients who were lost to follow-up were excluded, thus, 132 patients with suspected uncomplicated cecal diverticulitis were included in the present study. Uncomplicated diverticulitis was diagnosed as inflamed diverticulum or a phlegmon with cecal wall thickening, using radiological imaging (Figure 1). None of the patients had symptoms of peritonitis or the formation of an inflammatory mass. Diverticulitis accompanied by appendiceal dilatation from 8 to 12 mm was observed in 36 patients (27%).

The treatment method was determined at the discretion of the doctor who first examined the patients or patient preference. Therefore, 102 patients who received initial antibiotic management were classified as group 1 and 30 patients who underwent laparoscopic treatment were classified as group 2.

The antibiotic regimen consisted of a second generation cephalosporin and metronidazole which was administered for 4-7 d or until the abdominal pain subsided. Most patients received intravenous antibiotics, however, 8 received oral antibiotics. The diagnosis was confirmed by CT (3-D colon) or colonoscopic examination in all patients at least once during the follow-up period.

Table 1 Clinical and radiological diagnosis in consecutive patients with right lower quadrant pain (n = 8814)

Diagnosis	n (%)
Appendicitis	4718 (53.6)
Cecal diverticulitis	164 (1.8)
Ascending colonic diverticulitis	202 (2.3)
Terminal ileum diverticulitis	10 (0.1)
Mesenteric lymphadenitis	392 (4.4)
Gynecologic disease	501 (5.7)
Urologic disease	93 (1.1)
Uncommon findings (malignancy, IBD, *etc.*)	121 (1.4)
Nonspecific ileocolitis	806 (9.1)
No remarkable findings	1807 (20.5)

IBD: Inflammatory bowel disease.

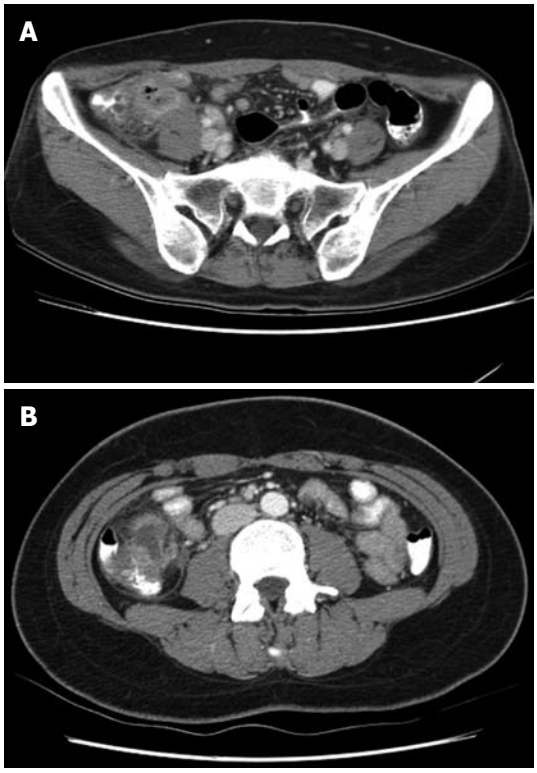

Figure 1 Radiologic evaluation of acute cecal diverticulitis (computed tomography). A: Inflamed diverticulum; B: Diverticulitis with phlegmon.

Laparoscopic treatment consisted of partial cecectomy including appendix and diverticulum in 8 patients, using one or two endoGIA (Covidien, Mansfield, MA, USA). Diverticulectomy was performed in 9 patients where technically feasible, especially in the case of diverticula arising from the anterior aspect of the cecum. Concurrent appendectomy was performed to prevent future diagnostic confusion. The remaining 13 patients underwent appendectomy alone, followed by direct visualization of the inflamed diverticulum located in the lateral or posterior side of the cecum.

Patients were reviewed using their medical records and were interviewed on the telephone to identify any recurring symptoms and surgical interventions. The median

Table 2 Clinical characteristics and outcomes in both groups (mean ± SD) *n* (%)

	Group 1 (*n* = 102)	Group 2 (*n* = 30)	*P*
Age (yr)	37.5 ± 11	39.0 ± 13	0.512
Sex (M/F)	50/52	15/15	0.925
WBC (× 10^3/L)	10.6 ± 3.6	12.3 ± 5.1	0.034
Mean hospital stay (d)	5.8 ± 2	7.3 ± 2	0.003
Mean medical costs ($)	1253 ± 168	1657 ± 157	0.001
Follow-up (mo)	46	45	0.725
Readmission rate	8 (8)	3 (10)	0.710
Recurrence	6 (6)	2 (7)	0.875
Treatment at recurrence	5 antibiotics 1 ileocolic resection	2 antibiotics	-

WBC: White blood cell.

follow-up time was 46 mo (range, 10-112 mo). Using this information, we evaluated the outcomes including readmission and recurrence rate between the two groups using the χ^2 and *t*-tests.

RESULTS

Of the 102 patients in group 1, 7 had undergone a previous appendectomy. Eight patients were suspected of having a pericolic abscess and 31 patients had a visible fecalith on diagnostic imaging. Twenty five patients had concurrent appendiceal dilatation. All patients were successfully treated without complications.

Of the 30 patients in group 2, 2 had a pericolic abscess and 11 had concurrent appendiceal dilatation. The operative findings revealed an inflamed diverticulum and phlegmon with adjacent bowel wall thickening. All patients with partial cecectomy or diverticulectomy were confirmed as having diverticulitis on pathologic examination. Appendix examination revealed that most patients (21/30) had secondary appendiceal serositis and the remaining patients had a normal appendix. The postoperative period was uneventful in most patients with the exception of 2 who developed wound infections. Group 2 patients had a higher white blood cell count at admission, a longer hospital stay, and higher medical costs.

During the follow-up period, 2 patients died of unrelated causes (liver cirrhosis, pancreas cancer). To date, all other patients are alive. Recurrence was defined as the development of the same symptoms and radiological evidence of diverticulitis.

Of the 102 patients in group 1, 6 had recurrence at a median of 15 mo (range, 5-25 mo) after treatment. Three recurrences were in the cecum and 3 were in the ascending colon or proximal transverse colon. Of these 6 patients, 5 were successfully treated with antibiotics and 1 underwent laparoscopic ileocolic resection for perforated diverticulitis. Two patients were readmitted due to RLQ pain, however, these patients were treated medically and did not undergo surgery for appendicitis.

In group 2, 2 patients (7%) had recurrence 21 mo and 24 mo after treatment and required further antibiotic treatment. Of these patients, 1 had undergone partial cecectomy and the other had undergone diverticulectomy. The location of recurrent diverticulitis was the distal ascending colon and hepatic flexure colon in each. One patient readmitted with RLQ pain, was treated medically (Table 2).

DISCUSSION

Although cecal diverticulitis is an uncommon condition and is preoperatively almost indistinguishable from appendicitis, it has a high prevalence in the Oriental population[10,11].

In the same 8-year period, we performed 4871 appendectomies, and the frequency of cecal diverticulitis was high (1 in 30 appendectomies). The high number of appendectomies and a specialized radiologist for RLQ diseases may have contributed to the higher diagnostic rate of cecal diverticulitis in our institution.

Other studies also demonstrated that right colonic diverticulitis can be correctly diagnosed using radiologic evaluation[12-15]. Since diverticula which develop in the right colon are generally of a limited number and are frequently solitary, the evaluation is not difficult. However, in our experience, the differential diagnosis using imaging studies between appendicitis and appendiceal diverticulitis or between perforated appendicitis and perforated diverticulitis is still problematic.

The treatment of suspected uncomplicated cecal diverticulitis diagnosed by radiological imaging has not been uniform. Uncomplicated cecal diverticulitis can usually be treated with antibiotics. However, if the disease is not fully differentiated from acute appendicitis the patient treated with initial antibiotics may be readmitted due to RLQ pain. Moreover, the clinical course of uncomplicated cecal diverticulitis may not be easily determined because most patients are young, and are only followed up for a short period.

Laparoscopic minimal surgery, such as diverticulectomy or partial cecectomy, is a good therapeutic option, however, the procedure is not always easy. Simple diverticulectomy using one or two staplers may be performed in some cases, but concerns regarding conversion or extended dissection of inflammatory tissue have been raised. The location of a diverticulum may be associated with technical difficulties. Diverticulitis that originates from the anterior aspect of the cecum may be more easily managed. However, diverticulitis found in the lateral or posterior aspect of the cecum may result in a difficult laparoscopic procedure. A phlegmon with adjacent inflammation also complicates the situation for surgeons.

To our knowledge, laparoscopic diverticulectomy, particularly in cases of cecal diverticulitis, has been rarely reported[16,17]. A possible explanation for this is that the disease is uncommon and therefore, this procedure may be difficult and risky, if technically infeasible or performed by an inexperienced surgeon.

Treatment with both antibiotics and laparoscopic surgery carry a risk of recurrence. Extensive surgery was often performed during re-operation in order to decrease the

risk of leaving an inflamed diverticulum. However, we presume that the clinical features of patients with cecal diverticulitis might differ between the Asian and non-Asian population. In most Asian patients, a low recurrence rate may be expected and non-operative management could be performed, even in recurrent cases[18-21].

Moreover, diverticulitis does not always recur in the same place. Multiple diverticula are often found in Asian patients with cecal diverticulitis. We also demonstrated right-sided colonic recurrent diverticulitis at a different site.

Many clinicians prefer non-operative management if right-sided uncomplicated diverticulitis is recognized preoperatively and may achieve long-term remission and control of the disease. We also believe that the natural course of cecal diverticulitis has mostly benign features. Many patients were successfully treated with initial antibiotic management at the time of the first attack and had a low readmission and recurrence rate. Moreover, patients with recurrence may be retreated non-operatively. These findings suggest that cecal diverticulitis, if not combined with definite complications, seems to have a benign nature and may be treated non-operatively.

The treatment of complicated cecal diverticulitis or a suspected mass is less controversial due to high morbidity and unexpected pathologies[22]. Surgical treatment is well accepted in these cases.

In conclusion, we suggest that initial antibiotic management is an effective treatment option for suspected uncomplicated cecal diverticulitis diagnosed by radiological evaluation and shows comparable long-term results in the prevention of recurrence, to that of laparoscopic treatment in Asian patients.

COMMENTS

Background

Although the optimal treatment of suspected uncomplicated cecal diverticulitis remains controversial, non-operative management of this disease is increasing. The authors reviewed the long-term recurrence rate following initial antibiotic management for uncomplicated cecal diverticulitis diagnosed by radiological imaging, compared with laparoscopic treatment in Asian patients.

Research frontiers

The efficacy of initial antibiotic management for suspected uncomplicated cecal diverticulitis diagnosed by radiological imaging has not been fully addressed.

Innovations and breakthroughs

Recent studies have reported good outcomes following antibiotic management of uncomplicated diverticulitis. However, there have been few reports on cecal diverticulitis which is common in Asian patients. Cecal diverticulitis is not easily differentiated from appendicitis. Therefore, many clinicians are concerned about the long-term recurrence rate following initial non-operative management, compared with surgical treatment.

Applications

Laparoscopic surgery may be unnecessary in localized or uncomplicated diverticular disease. The surgical treatment options for cecal diverticulitis range from diverticulectomy to right hemicolectomy and are reserved only for definite cases of complicated diverticulitis.

Terminology

Uncomplicated diverticulitis was defined as inflamed diverticulum or a phlegmon with cecal wall thickening, and was not associated with complications, such as perforation, obstruction, or visible abscess.

Peer review

Over all, the study demonstrates success of antibiotic alone therapy in a large number of patients suffering from cecal diverticulitis - a rather uncommon entity.

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BRIEF ARTICLE

Comparative analysis of dideoxy sequencing, the KRAS StripAssay and pyrosequencing for detection of KRAS mutation

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Abstract

AIM: To compare the differences between dideoxy sequencing/KRAS StripAssay/pyrosequencing for detection of KRAS mutation in Chinese colorectal cancer (CRC) patients.

METHODS: Formalin-fixed, paraffin-embedded (FFPE) samples with tumor cells ⩾ 50% were collected from 100 Chinese CRC patients at Beijing Cancer Hospital. After the extraction of genome DNA from FFPE samples, fragments contained codons 12 and 13 of KRAS exon 2 were amplified by polymerase chain reaction and analyzed by dideoxy sequencing, the KRAS StripAssay and pyrosequencing. In addition, the sensitivities of the 3 methods were compared on serial dilutions (contents of mutant DNA: 100%, 50%, 20%, 15%, 10%, 5%, 1%, 0%) of A549 cell line DNA (carrying the codon 12 Gly>Ser mutation) into wild-type DNA (human normal intestinal mucosa). The results of dideoxy sequencing, the KRAS StripAssay and pyrosequencing were analyzed by Chromas Software, Collector for KRAS StripAssay and the pyrosequencing PyroMarkTM Q24 system, respectively.

RESULTS: Among 100 patients, KRAS mutations were identified in 34%, 37% and 37% of patients by dideoxy sequencing, the KRAS StripAssay and pyrosequencing, respectively. The sensitivity was highest with the KRAS StripAssay (1%), followed by pyrosequencing (5%), and dideoxy sequencing was lowest (15%). Six different mutation types were found in this study with 3 main mutations Gly12Asp (GGT>GAT), Gly12Val (GGT>GTT) and Gly13Asp (GGC>GAC). Thirty-three patients were identified to have KRAS mutations by the 3 methods, and a total of 8 patients had conflicting results between 3 methods: 4 mutations not detected by dideoxy sequencing and the KRAS StripAssay were identified by pyrosequencing; 3 mutations not detected by dideoxy sequencing and pyrosequencing were identified by the KRAS StripAssay; and 1 mutation not detected by pyrosequencing was confirmed by dideoxy sequencing and the KRAS StripAssay. Among these discordant results, the results identified by dideoxy sequencing were consistent either with the KRAS StripAssay or with pyrosequencing, which indicated that the accuracy of dideoxy sequencing was high.

CONCLUSION: Taking a worldwide view of reports and our results, dideoxy sequencing remains the most popular method because of its low cost and high accuracy.

Key words: DNA mutational analysis; KRAS; Mutation; Dideoxy sequencing; KRAS StripAssay; Pyrosequencing

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INTRODUCTION

Colorectal cancer (CRC) is a common malignant tumor of the gastrointestinal tract, with yearly increasing morbidity and mortality in China. Recently, the development of targeted drugs (for example, cetuximab, panitumumab) has brought advancement in CRC therapy. Cetuximab, which targets the epidermal growth factor receptor (EGFR), shows activity in refractory CRC patients expressing EGFR[1], and in CRC patients with tumors that do not express EGFR immunohistochemically[2]. Thus, there is not an association between EGFR expression and cetuximab efficacy[2,3]. The activating mutations in exon 2 of KRAS play an important role in the progression of CRC, which can induce unlimited proliferation of tumor cells[4,5]. One study reported that KRAS mutations could become an independent prognostic factor in advanced CRC patients treated with cetuximab[6] and there was a significant negative association between KRAS mutations and cetuximab efficacy[7]. Some clinical trials, such as CRYSTAL, OPUS and EVEREST, demonstrated that CRC patients with wild-type KRAS could benefit from the addition of cetuximab to the standard chemotherapy regimen, but patients with mutated KRAS could not[8-10]. Thus, detection of KRAS mutations is strongly recommended before administration of cetuximab.

The mutation rate of KRAS is slightly different among different trials and different areas; for example, the prevalence of KRAS mutation was 35.6% in the CRYSTAL trial[8], but 42.3% in the CO.17 trial[11]; the frequency of KRAS mutation is about 40% in the United States, 34% in the Netherlands, 49% in France and 26.5% in Taiwan, China[12]. The KRAS mutation rate in Chinese CRC patients is about 37% according to our previous study (about 600 patients were studied) using dideoxy sequencing. Most mutations occur in codons 12 and 13 (about 95%), and only a few in codon 61 (about 5%)[13-15]. Up to now, many methods have been used to detect KRAS mutations, including dideoxy sequencing, polymerase chain reaction (PCR)-single-strand conformation polymorphism, PCR-restriction fragment length polymorphism (RFLP), pyrosequencing, denaturing high performance liquid chromatograph, and so on[16]. Along with the advancement of technology, many new kits have been developed, such as the DxS K-RAS Mutation Test Kit and the KRAS StripAssay, which brought new choices for researchers.

As a new method, the KRAS StripAssay has been used in Europe and America, but is not available in China. In order to determine the sensitivity of the KRAS StripAssay, and confirm the feasibility of dideoxy sequencing, we compared the differences between dideoxy sequencing, the KRAS StripAssay and pyrosequencing for mutation detection in codons 12 and 13 of KRAS. Codons 12 and 13 of KRAS were detected by the 3 methods in 100 CRC patients proposed for treatment with cetuximab.

MATERIALS AND METHODS

Patient samples and control samples

A total of 100 patients in Beijing Cancer Hospital with CRC confirmed by histopathology between October 2008 and August 2009 were investigated for KRAS mutations in our laboratory. Formalin-fixed, paraffin-embedded (FFPE) samples with ≥ 50% tumor cells were collected. An A549 cell line was preserved in our laboratory and normal intestinal mucosa was provided by the tissue bank of our hospital.

Genomic DNA extraction

Genomic DNA of FFPE sections was extracted using E.Z.N.A.FFPE DNA Kit (Lot. D3399-01, OMEGA, USA) according to the manufacturer's instructions. Genomic DNAs of A549 and normal intestinal mucosa were extracted using EasyPure Genomic DNA Extraction Kit (Lot. D60916, TransGen Biotech, China) according to the manufacturer's instructions. All genomic DNAs were stored at -20℃ until further research.

Preparation of serial dilutions

The concentrations of DNA from the A549 cell line and normal intestinal mucosa were determined by fluorometry. Serial dilutions were prepared by putting A549 cell DNA into wild-type DNA to produce dilutions with the following contents of mutant DNA: 100%, 50%, 20%, 15%, 10%, 5%, 1%, 0%.

Dideoxy sequencing

A DNA fragment including exon 2 of the KRAS gene was amplified by PCR using primers (KRAS-F: 5'-GGTACTGGTGGAGTATTTGATAG-3', KRAS-R: 5'-TGGTCCTGCACCAGTAATATG-3') with a product size of 248 bp. Each PCR reaction consisted of 10 × LA PCR buffer Ⅱ 2 μL, 10 mmol/L dNTPs 2 μL, LA *Taq* 0.2 μL (DRR200A, TAKARA), genomic DNA 2 μL, 10 μmol/L forward primer 0.5 μL, 10 μmol/L reverse primer 0.5 μL in a final volume of 20 μL. The cycling conditions were 94℃ for 5 min, 45 cycles of 94℃ for 30 s, 56℃ for 30 s and 72℃ for 20 s, final extension at 72℃ for 10 min, and ended at 4℃. The PCR products were determined by 3% agarose gel electrophoresis and then sequenced using the same forward primer by Invitrogen 3730XL genetic analyzer. The sequencing results were analyzed with Chromas software under the condition of signal/noise > 98%.

KRAS StripAssay

The KRAS StripAssay kit (Lot. 5-590) was kindly pro-

vided by ViennaLab of Austria. All procedures were conducted according to the manufacturer's instructions. Briefly, PCR products were amplified in a tube containing 15 μL amplification mix, 5 μL diluted *Taq* DNA polymerase (1 U), 5 μL DNA template (50 ng genome DNA). The cycling conditions were 94℃ for 2 min, 35 cycles of 94℃ for 60 s, 70℃ for 50 s, 56℃ for 50 s and 60℃ for 60 s, final extension at 6℃ for 3 min. PCR products with fragment lengths 151 bp and 204 bp were determined by 3% agarose gel electrophoresis after PCR amplification. Following hybridization (45℃, shaking waterbath), stringent washing (45℃, shaking waterbath) and color development (room temperature), the results were interpreted using the enclosed Collector sheet.

Pyrosequencing technique

A DNA fragment including codons 12 and 13 of the KRAS gene was amplified by PCR using primers (forward: 5'-biotin-TGACTGAATATAAACTTGTGGTAGTTG-3', reverse: 5'-TCGTCCACAAAATGATTCTGAA-3') with a product size of 91 bp. Each PCR reaction consisted of 10 × PCR buffer 5 μL, 10 mmol/L dNTPs 4 μL, Hotstart *Taq* 0.4 μL [Gene Tech (Shanghai) Company Limited], genomic DNA 4 μL, 10 mmol/L forward primer 0.5 μL, 10 mmol/L reverse primer 0.5 μL in a final volume of 50 μL. The cycling conditions were 95℃ for 3 min, 45 cycles of 95℃ for 10 s, 56℃ for 20 s and 72℃ for 30 s, final extension at 72℃ for 5 min. The PCR products were determined by 3% agarose gel electrophoresis and ssDNA was prepared as described[16]. Mutation detection of KRAS codons 12 and 13 by the Pyrosequencing PyroMark™ Q24 system was done following the manufacturer's instructions (see http://www.pyrosequencing.com/ for more information).

RESULTS

Patient demographics and spectrum of KRAS mutations

The study included 54 males and 46 females with a median age of 59 years (range 22-82 years). The primary locations of tumors were the colon (n = 58) and rectum (n = 42). The mutation rate in females (about 43%) was slightly higher than that in males (about 30%), and the mutation rate in colon and rectal cancers was similar. All patients had a single mutation site. A total of 6 mutation types were detected in this study: GGT>GAT, GGT>GTT, GTT>GCT, GTT>TGT, GTT>AGT, GGC>GAC (wild-type codon 12: GGT; wild-type codon 13: GGC) (Figure 1). Three main mutations Gly12Asp (GGT>GAT), Gly12Val (GGT>GTT) and Gly13Asp (GGC>GAC) accounted for about 80.0% (28/34) of all mutations.

Sensitivity of the 3 methods

Serial dilutions with different contents of mutant DNA were detected by the 3 methods. All 3 methods could correctly identify the Gly12Ser mutation in dilutions containing 15% or more mutant DNA. Dideoxy sequencing failed to detect the mutation in dilutions containing 10% or less mutant DNA. Pyrosequencing failed to detect the mutation in dilutions containing 1% mutant DNA, and only the KRAS StripAssay could unambiguously identify 1% mutant DNA in the dilutions. Thus the sensitivity was highest in the KRAS StripAssay (1%), followed by pyrosequencing (5%), while dideoxy sequencing was lowest (15%) (Table 1).

Table 1 Sensitivity of the 3 methods in mutation detection

Methods	Mutant DNA/total DNA (%)							
	100	50	20	15	10	5	1	0
Dideoxy sequencing	Yes	Yes	Yes	Yes	No	No	No	No
Pyrosequencing	Yes	Yes	Yes	Yes	Yes	Yes	No	No
KRAS StripAssay	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No

Dideoxy sequencing could identify mutations in dilutions containing ≥ 15% mutant DNA, pyrosequencing could identify mutation in dilutions containing ≥ 5% mutant DNA, the KRAS StripAssay could identify mutant DNA as low as 1%.

KRAS mutations of 100 CRC patients using the 3 methods

The KRAS mutation was detected in 34/100 (34%) of patients by dideoxy sequencing with 15/54 (27.8%) of males and 19/46 (41.3%) of females, 37/100 (37%) of patients by KRAS StripAssay with 16/54 (29.6%) of males and 21/46 (45.7%) of females, and in 37/100 (37%) of patients by pyrosequencing with 18/54 (33.3%) of males and 19/46 (41.3%) of females. Three main mutations Gly12Asp, Gly12Val and Gly13Asp accounted for 82.4% (28/34), 78.4% (29/37) and 83.8% (31/37) of all mutations by dideoxy sequencing, KRAS StripAssay and pyrosequencing, respectively. The overall results of the 3 methods were similar, with a few discrepancies.

Thirty-three of the 100 patients were identified to have KRAS mutations by all 3 methods, and 8 patients (sample No. 5, 11, 14, 29, 44, 46, 48, 71) showed conflicting results between the 3 methods: 4 mutations (sample No. 5, 44, 46, 48) which were not detected by dideoxy sequencing and the KRAS StripAssay, were identified by pyrosequencing; 3 mutations (sample No. 11, 14, 71) which were not detected by dideoxy sequencing and pyrosequencing were identified by the KRAS StripAssay; one mutation (sample No. 29) not detected by pyrosequencing was idenified by dideoxy sequencing and the KRAS StripAssay (Figure 2). In addition, among these discordant results, the mutations identified by dideoxy sequencing were consistent either with the KRAS StripAssay or with pyrosequencing (Table 2). This indicated that although the sensitivity of dideoxy sequencing was low, its accuracy was high.

DISCUSSION

Along with national development and improvements in standard of living, morbidity and mortality of CRC has increased rapidly in China. The outcomes of the same

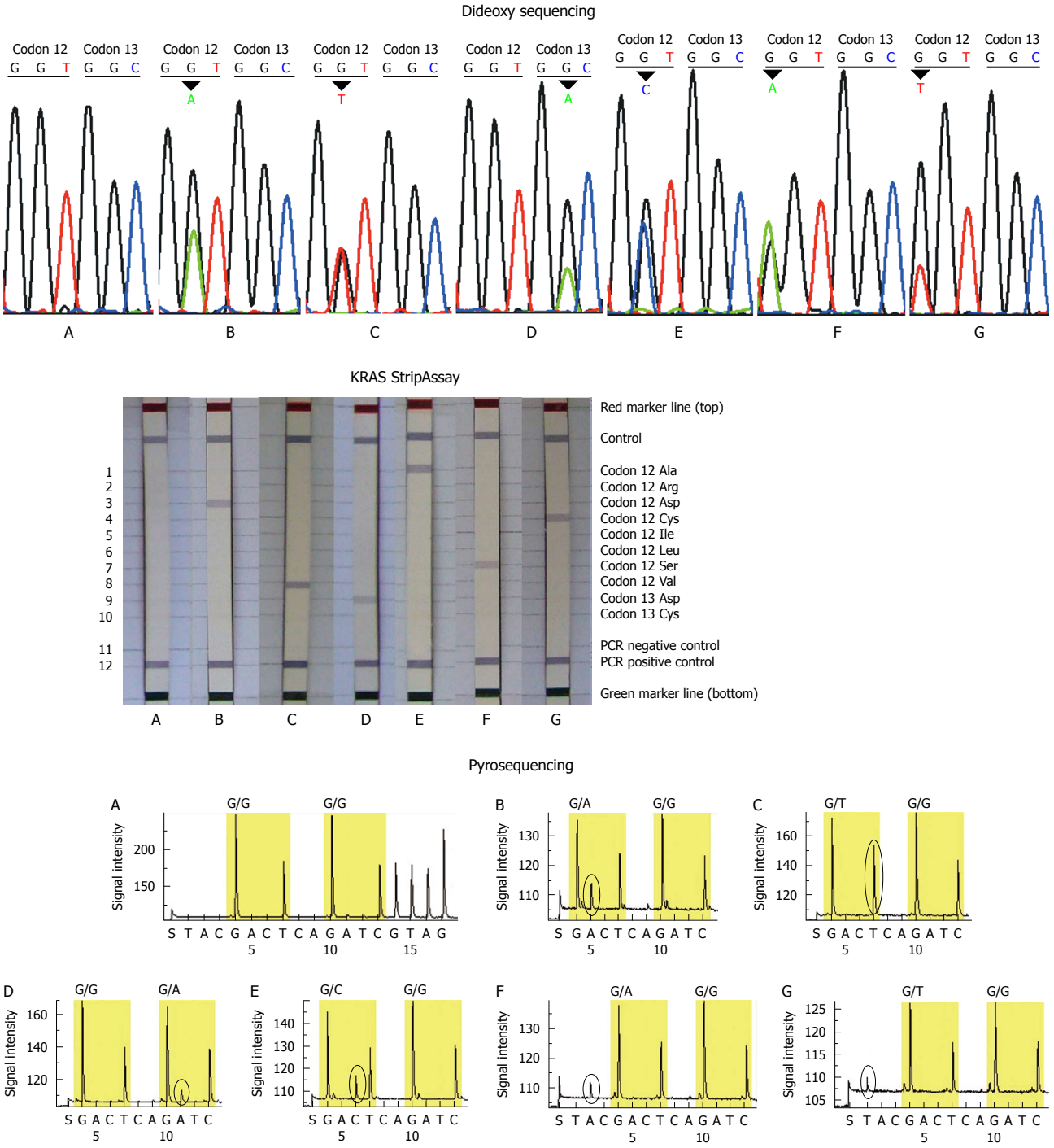

Figure 1 Mutation patterns of KRAS codons 12 and 13 by the 3 methods in this study. A: Wild-type codons 12 and 13 with sequence GGT(12)GGC(13); B: Mutant codon 12 with GGT>GAT; C: Mutant codon 12 with GGT>GTT; D: Mutant codon 13 with GGC>GAC; E: Mutant codon 12 with GGT>GCT; F: Mutant codon 12 with GGT>AGT; G: Mutant codon 12 with GGT>TGT.

treatment regimen in CRC patients were frequently found to differ, and thus it was important to develop individualized treatments. Because the effect of cetuximab was tightly associated with KRAS mutation status, the US Food and Drug Administration recommended that patients who were proposed for cetuximab treatment should undergo KRAS mutation analysis. As a result, besides the conventional methods, more and more techniques have been developed to detect KRAS mutation.

Recent reports have highlighted the advantages of the new methods, and we first compared dideoxy sequencing, the KRAS StripAssay and pyrosequencing for mutation detection in codons 12 and 13 of KRAS in Chinese CRC patients. The mutation rate of KRAS in our study was about 37%, and the mutation rate in females (about 43%) was higher than in males (about 30%) which was different from a report that KRAS mutation in males was higher than in females in Brazil[16].

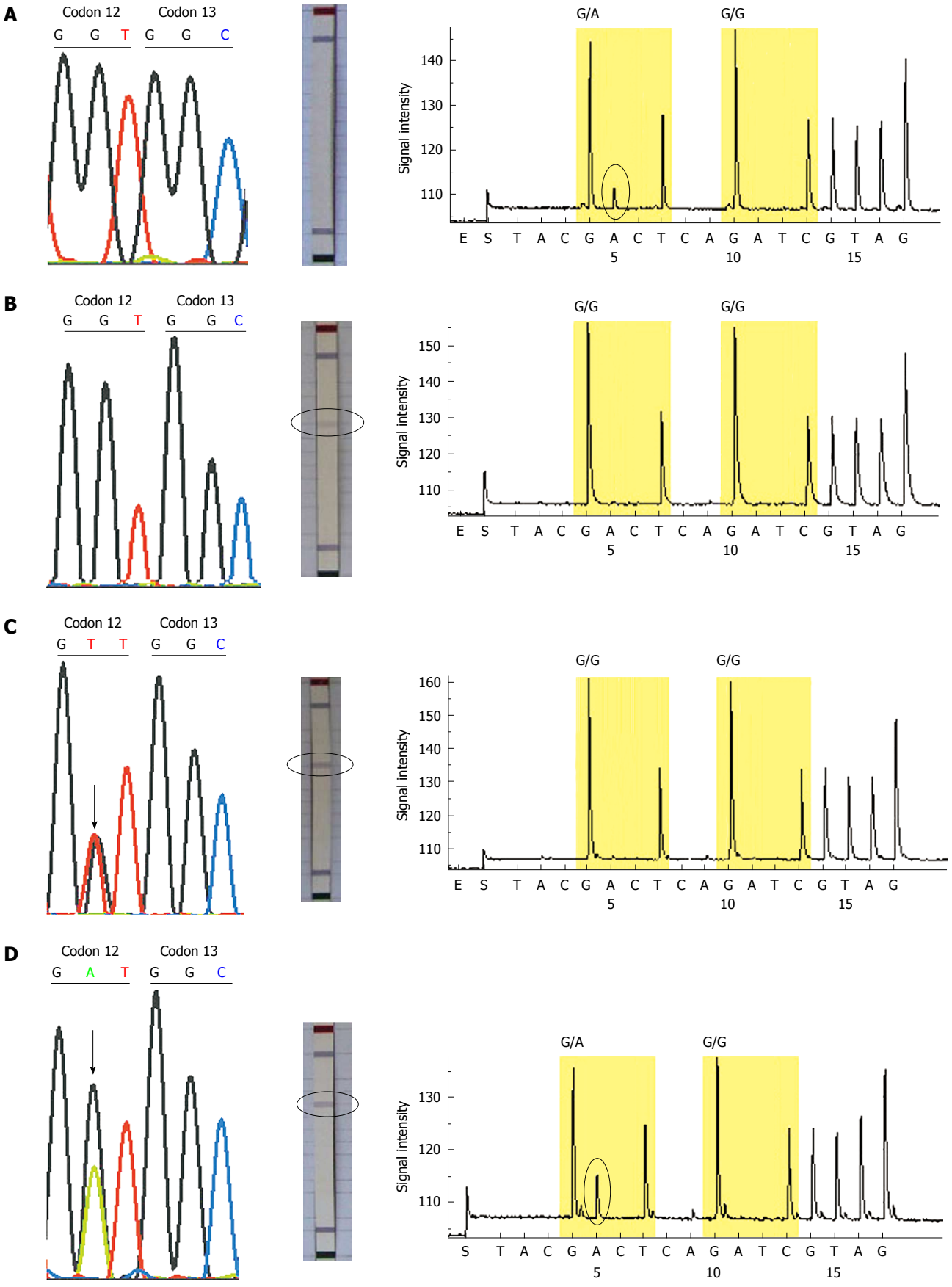

Figure 2 Representative discrepant samples and concordant samples between the 3 methods. A: Mutation of sample 5 was not detected by dideoxy sequencing and the KRAS StripAssay but identified by pyrosequencing; B: Mutation of sample 11 was not detected by dideoxy sequencing and pyrosequencing but identified by the KRAS StripAssay; C: Mutation of sample 29 was not detected by pyrosequencing but identified by dideoxy sequencing and the KRAS StripAssay; D: Mutation of sample 82 was detected by all 3 methods.

Table 2 Discrepant results detected by the 3 methods

Sample No.	Mutation type		
	Dideoxy sequencing	KRAS StripAssay	Pyrosequencing
5	Wild-type	Wild-type	GGT>GAT
11	Wild-type	GGT>TGT	Wild-type
14	Wild-type	GGT>GTT	Wild-type
29	GGT>GTT	GGT>GTT	Wild-type
44	Wild-type	Wild-type	GGT>GAT
46	Wild-type	Wild-type	GGT>GAT
48	Wild-type	Wild-type	GGT>GAT
71	Wild-type	GGT>GCT	Wild-type

These results were repeated at least twice.

The results from the 3 methods were similar, but a total of 8 patients had conflicting results between the 3 methods. We repeated these discrepant samples at least twice, the results being consistent. Because the results by dideoxy sequencing were consistent either with the KRAS StripAssay or with pyrosequencing, the results by dideoxy sequencing were likely to be more accurate. To support our hypothesis, we retrospectively analyzed the patients who were treated with cetuximab. Case 44 with KRAS mutation identified by pyrosequencing was treated with cetuximab and achieved a partial response after 6 weeks' treatment. Because patients with KRAS mutations could not benefit from cetuximab, the result of case 44 by pyrosequencing may be a false positive.

Our results showed that the sensitivities of the KRAS StripAssay and pyrosequencing were higher than that of dideoxy sequencing, but according to our large-scale sampling by dideoxy sequencing, the KRAS mutation rate was stable at 37-39% which was consistent with other reports. From the result of case 44, a false positive could occur in sensitive methods. We can analyze the 3 methods from the aspect of medical economics. At present, the cost is about 100-150 RMB/test for dideoxy sequencing, 1000 RMB/test for the KRAS StripAssay and 200-300 RMB/test for pyrosequencing. Payments in China are limited to the cheapest methods. Up to now, dideoxy sequencing and pyrosequencing have already been widely used to detect KRAS mutations[17,18], but the KRAS StripAssay has not been widely used all over the world.

Studies reported that the KRAS mutation could be used as a prognostic marker in non-small cell lung cancer and as an independent prognostic factor for CRC patients treated with cetuximab[6,19]. Whether there is a relationship between KRAS mutations and prognosis in Chinese CRC patients needs to be studied further.

In conclusion, we compared the differences between 3 methods in the detection of KRAS mutations, and used the KRAS StripAssay to detect KRAS mutations. Although new methods have been developed for detection of KRAS mutation, traditional methods are still in an invincible position and are used widely. In our following large-scale study, dideoxy sequencing will be chosen preferentially because of its ease of use.

ACKNOWLEDGMENTS

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COMMENTS

Background

At present, more and more colorectal cancer (CRC) patients are treated with cetuximab [a monoclonal antibody of epidermal growth factor receptor (EGFR)]. It had been confirmed that KRAS mutated patients could not benefit from cetuximab, so the US Food and Drug Administration recommended that patients proposed for cetuximab treatment should undergo KRAS mutation analysis.

Research frontiers

Besides the traditional methods (e.g. dideoxy sequencing), more and more methods (various kinds of kits) have been developed to detect KRAS mutation. The authors compared methods and demonstrated differences between dideoxy sequencing, the KRAS StripAssay and pyrosequencing, with indications that although dideoxy sequencing is a traditional method, it is still in an invincible position because of its low cost and high accuracy.

Innovations and breakthroughs

Recently, many reports have highlighted the advantages of new methods, and disregarded the traditional methods in the detection of KRAS mutation. This was the first study to compare the differences between dideoxy sequencing, the KRAS StripAssay and pyrosequencing in KRAS detection. The study indicated that although the sensitivity of dideoxy sequencing was lower than the other 2 methods, it was still widely used by many researchers because of its superior accuracy.

Applications

The study could help researchers to choose a suitable method for detection of KRAS mutation according to the sample size, equipment platform and economic status, *etc*. In their opinion, dideoxy sequencing could be easily carried out in any laboratory.

Terminology

In normal physiological conditions, KRAS is regulated by its upstream protein EGFR and plays an important role in the development and progression of tumors. Cetuximab could inhibit the tumors through blocking EGFR and the downstream signal pathway. If KRAS was mutated, KRAS protein was activated without the regulation of EGFR, so cetuximab could not have an effect.

Peer review

The paper deals with the comparison of 3 DNA sequencing methods in order to detect KRAS mutations. The authors postulate that dideoxy sequencing and pyrosequencing techniques have already been widely used to detect KRAS mutations but the KRAS StripAssay has not been widely used all over the world. The paper is well written and well documented.

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BRIEF ARTICLE

Possible key residues that determine left gastric artery blood flow response to PACAP in dogs

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Abstract

AIM: To determine the effect of pituitary adenylate cyclase-activating polypeptide (PACAP) on left gastric artery (LGA) flow and to unveil the structural or functional important sites that may be critical for discrimination of different receptor subtypes.

METHODS: Peptides, including PACAP-27, PACAP-38, amino acid substituted PACAP-27 and C-terminus truncated analogues PACAP (27-38), were synthesized by a simultaneous multiple solid-phase peptide synthesizer. Flow probes of an ultrasound transit-time blood flowmeter were placed around the LGA of beagle dogs. When peptides were infused intravenously, the blood flow was measured.

RESULTS: [Ala4, Val5]-PACAP-27 caused a concentration-dependent vasodepressor action which was similar to that caused by PACAP-27. The LGA blood flow response to [Ala4, Val5]-PACAP-27 was significantly higher than that to PACAP-27, which was similar to that to vasoactive intestinal polypeptide (VIP) at the same dose. [Ala6]-PACAP-27 did not increase the peak LGA flow. [Gly8]-PACAP-27 showed a similar activity to VIP. [Asn24, Ser25, Ile26]-PACAP-27 did not change the activity of peptides at all doses.

CONCLUSION: NH2 terminus is more important to biological activity of peptides and specific receptor recognition than COOH-terminus.

Key words: Pituitary adenylate cyclase-activating polypeptide; Pituitary adenylate cyclase-activating polypeptide 27; Pituitary adenylate cyclase-activating polypeptide P38; Left gastric artery; Blood flow

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INTRODUCTION

Pituitary adenylate cyclase-activating polypeptide (PACAP)

was originally isolated because of its similarity with vasoactive intestinal polypeptide (VIP). PACAP stimulates adenylyl cyclase activity in anterior pituitary cells of rats. So far, two forms of PACAP (PACAP-38 and PACAP-27) have been described. Considering that the 27-residue polypeptide (PACAP-27) corresponds to the N-terminus 27-amino acid sequence of PACAP-38 and shows a 68% identity with VIP[1] (Table 1), PACAP showing a 68% of identity with VIP has been considered a member of VIP-glucagon-growth hormone releasing factor-secretin superfamily.

Both PACAP and VIP exhibit an ability to stimulate adenylate cyclase in pituitary cells and in neural, pancreatic, and liver membrane[2]. However, PACAP is much more potent than VIP in pituitary cells and liver membrane. In the cardiovascular system, PACAP acts as a vasodepressor like VIP. Similarity would demand the validation of effective dose, the duration of response, and the latent period, *etc.*[3].

PACAP and VIP are co-expressed in nerve fibers and neurons in ganglia of guinea pig gallbladder[4]. Our previous study showed that their actions on the gallbladder are opposite, namely VIP relaxes the gallbladder whereas PACAP induces its contraction[5].

It has been shown that PACAP-38 and PACAP-27 are potent VIP-like vasodilators of the femoral arterial bed of dogs, while PACAP-38 differs from PACAP-27 and VIP in its prolonged effects on femoral blood flow[6]. Small arteries and arterioles in the gastrointestinal tract and pancreas are innervated by VIP- or PACAP-positive fibers. Both peptides are very potent vasodilators of gastrointestinal blood vessels in conscious dogs. These findings suggest that PACAP may participate in regulation of the gastrointestinal circulation. However, its effect on gastric blood flow is unknown[7,8].

Three receptor subtypes that mediate PACAP and VIP have been identified[9], including PACAP-specific receptor (PAC1) with a high affinity for PACAP and a much lower affinity for VIP, and PACAP/VIP receptors (VPAC1 and VPAC2) with a similar affinity for PACAP and VIP. All of them belong to the group of 7 transmembrane G protein-coupled receptors. PACAP and VIP act primarily as an inhibitory transmitter on most gastrointestinal and vascular smooth muscle cells, suggesting that PACAP may participate in regulation of the gastrointestinal circulation.

In the present investigation, gastrointestinal blood flow response to PACAP38, PACAP27 and their analogues with amino acid substitutions of corresponding VIP residues as well as substituted analogues at putative functional/structural important sites and C-terminal truncated analogues were studied in conscious beagle dogs to unveil the dose-response and structure-response relationships of these peptides in the left gastric artery (LGA).

MATERIALS AND METHODS

Peptide synthesis

On the basis of sequence homology of PACAP and VIP as well as the structural results by NMR, positions 4, 5, 6, 8, 24, 25, and 26 of PACAP-27 and VIP were selected as substitution sites in the present study. Peptides, including PACAP-27, PACAP-38, amino acid substituted PACAP-27 and C-terminal truncated analogues PACAP 27-38 were synthesized by a simultaneous multiple solid-phase peptide synthesizer (PSSM-8; Shimadzu, Kyoto, Japan), using the 9-fluorenylmethoxycarbonyl strategy. After cleavage, all peptides were purified with the SynProPep System[10] and characterized by sequencing, amino acid analysis, and fast atom bombardment mass spectrometry to confirm the high homogeneity with the desired structure. VIP was purchased from Peptide Institute (Osaka, Japan).

Table 1 Amino acid sequence of pituitary adenylate cyclase-activating polypeptide-27, pituitary adenylate cyclase-activating polypeptide-38, vasoactive intestinal polypeptide and their analogues

Peptides and their analogues	Amino acid sequence
PACAP-27	HSDG I, FTD S Y, SRYRK, QMAVK, KYLAA, VL-NH2
VIP	HSDAV, FTDNY, TRLRK, QMAVK, KYLN S, ILN-NH2
[Ala4]-PACAP-27	HSDA I, FTDSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Val5]-PACAP-27	HSDGV, FTDSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Ala4, Val5]-PACAP-27	HSDAV, FTDSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Ala6]-PACAP-27	HSDG I, ATDSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Gly8]-PACAP-27	HSDG I, FTGSY, SRYRK, QMAVK, KYLAA, VL-NH2
[Asn24, Ser25, IIe26]-PACAP-27	HSDG I, FTDSY, SRYRK, QMAVK, KYLN S, IL-NH2
PACAP 1-33	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQ
PACAP 1-34	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQR
PACAP 1-35	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQRV
PACAP 1-36	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQRV, K
PACAP 1-37	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQRV, KN
PACAP 1-38	HSDGI, FTDSY, SRYRK, QMAVK, KYLAA, VLGKR YKQRV, KNK-NH2

PACAP: Pituitary adenylate cyclase-activating polypeptide; VIP: Vasoactive intestinal polypeptide.

Methods

The study was approved by the Ethical Committee of the National Institute for Physiological Sciences (Okazaki, Japan) on Animal Use for Experiment. Five beagle dogs of either sex weighing 8-14 kg were used. After fasted for 18 h, the animals were anesthetized with thiamylal (20 mg/kg) and atropine (0.5 mg) and maintained with N_2O-O_2-ether throughout the procedure. Flow probes of an ultrasound transit-time blood flowmeter (Transonic Systems, New York) were placed around the LGA. Connectors of the probes were pulled out of the abdominal cavity through a subcutaneous tunnel and fixed at the chest. After a 4-wk

recovery period, the animals were restrained in Pavlov stands and experiments were conducted in the conscious state. PACAP27, PACAP38, VIP, and PACAP-27 analogues (2.5, 5, 10, 25, 50 and 100 pmol/kg in 1 min) were infused intravenously. The blood flow was measured. Blood flow response to oral ingestion of 300 mL milk served as a control.

Statistical analysis

All data were presented as mean ± SE. Statistical analysis was carried out by one-way analysis of variance using least-significant difference when equal variances or Tamhane's T2 was assumed or when equal variances were not assumed for multiple comparisons. Independent sample *t* test was used for comparison between two independent data. $P < 0.05$ was considered statistically significant with n = the number of animals.

RESULTS

Effects of PACAP-27 and VIP

Different concentrations of PACAP-27, PACAP-38 and VIP were employed. The LGA blood flow responses to PACAP-27 at the doses of 2.5, 5, 10, 25, 50 and 100 pmol/kg were 30.07 ± 8.52, 66.62 ± 16.04, 100, 195.29 ± 35.07, 276.45 ± 47.33, 322.76 ± 60.36, respectively, while those to PACAP-38 at the same doses were 37.35 ± 5.11, 91.69 ± 11.15, 137.60 ± 13.81, 186.91 ± 25.66, 214.12 ± 31.42, 229.73 ± 42.11, respectively. The blood flow responses to VIP at the doses of 10, 25, 50 and 100 pmol/kg were 25.56 ± 8.32, 56.88 ± 9.56, 87.41 ± 1.72, 148.60 ± 17.17, respectively (Figure 1).

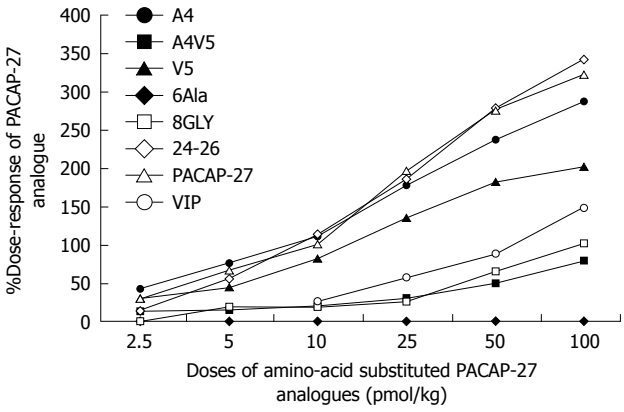

Figure 1 Left artery blood flow response to different doses of amino-acid substituted pituitary adenylate cyclase-activating polypeptide-27 analogues. PACAP: Pituitary adenylate cyclase-activating polypeptide; VIP: Vasoactive intestinal polypeptide.

Effects of N-termini (1-8) substituted PACAP-27 analogues

Effect of N-termini 4 and 5 substituted PACAP-27 analogues with corresponding VIP residues: Intravenous infusion of substituted PACAP27 analogues increased the peak LGA blood flow in a dose-dependent manner. [Ala4, Val5]-PACAP-27 caused a concentration- dependent vasodepressor action similar to that caused by PACAP-27. Both showed a comparable activity to PACAP-27 at the doses of 2.5-100 pmol/kg), demonstrating that a single amino-acid residue substitution at position 4 or 5 of PACAP-27 does not significantly change its biological function. Interestingly, analogues with a substitution at positions 4 and 5, [Ala4, Val5]-PACAP-27 (12.68 ± 4.88, 42.18% of PACAP27) showed a similar activity to PACAP-27 (30.07 ± 8.52) at the dose of 2.5 pmol/kg. However, the responses to [Ala4, Val5]-PACAP-27 (14.58 ± 6.73, 20.63 ± 6.08, 29.99 ± 9.77, 48.53 ± 10.79, 79.20 ± 4.66) at the doses of 5, 10, 25, 50 and 100 pmol/kg were significantly lower than those to PACAP27 (21.88%, 20.63%, 15.36%, 17.55% and 24.54%, $P < 0.05$) and those to PACAP27 at positions 4 and 5 (66.62 ± 16.04, 100, 195.28 ± 35.07, 276.45 ± 47.33, 322.76 ± 60.36), while exhibited a similar activity to VIP (25.56 ± 8.32, 56.88 ± 9.56, 87.41 ± 1.72, 148.60 ± 17.17) at the dose of 10-100 pmol/kg, suggesting that positions 4 and 5 of PACAP-27 are the key NH2-terminal residues of PACAP-27 that discriminate interactions of PACAP with specific receptor subtypes in the LGA (Figure 2).

Effect of N-terminus 6, 8 substituted PACAP-27 analogue: Intravenous infusion of [Ala6]-PACAP-27 did not increase the peak LGA flow, indicating that amino-acid residue replacement at position 6 of PACAP-27 results in loss of its biological function. Phenylalanine, an amino acid residue at position 6 of PACAP -27, was critical for PACAP-27 to exert its action on LGA flow.

The responses to [Gly8]-PACAP-27 were significantly lower at the doses of 5-100 pmol/kg (17.97 ± 4.21, 18.07 ± 4.13, 26.21 ± 7.22, 64.06 ± 15.82, 101.51 ± 17.40) than those to PACAP-27 (26.98%, 18.07%, 13.42%, 23.17%, 31.45%) ($P < 0.05$). [Gly8]-PACAP-27 at the dose of 10-100 pmol/kg showed a similar activity to VIP. Changes in amino-acid residue at position 8 made the biological function of PACAP-27 less potent, suggesting that position 8 of PACAP-27 plays a key role in conformation of PACAP-27 (Figure 2).

Effects of C-termini (24-26) substituted PACAP-27 analogues with corresponding VIP residues

The replacement of C-terminal residues of PACAP-27, [Asn24, Ser25, Ile26]-PACAP-27 (14.75 ± 6.97, 55.43 ± 21.31, 112.66 ± 32.25, 185.23 ± 38.60, 279.30 ± 59.33, 341.83 ± 72.38) did not significantly change the responses at the doses of 2.5-100 pmol/kg, while the responses were 83.21%, 112.66% and 101.03% to PACAP-27 at 5, 10 and 50 pmol/kg, indicating that the three C-terminal residues are not critical for the difference between PACAP-27 and VIP (Figure 2).

Effects of C-terminal deletion in PACAP-38 on peak LGA flow

The effects of C-terminal deletion in PACAP-38 on the peak LGA flow were monitored and compared with the response to PACAP-27, showing that almost all C-terminal deletions in PACAP-38 had no significant effect on the peak LGA flow (Figure 3).

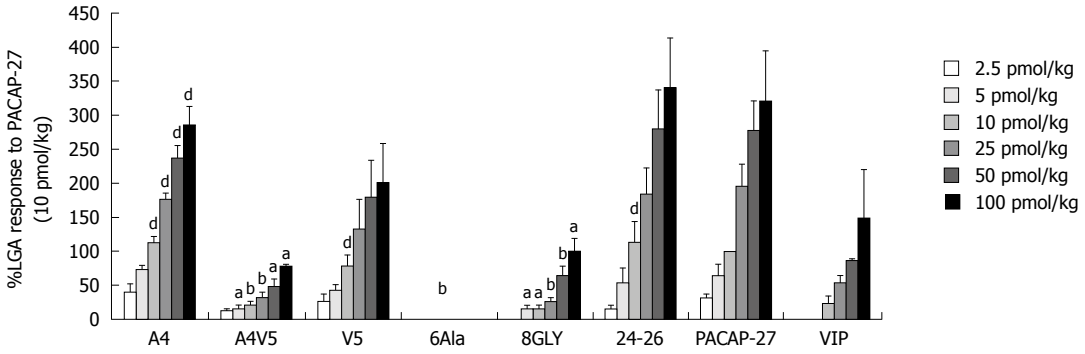

Figure 2 Effects of different doses of amino-acid substituted pituitary adenylate cyclase-activating polypeptide-27 analogues, pituitary adenylate cyclase-activating polypeptide-27 and vasoactive intestinal polypeptide on left artery blood flow. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* pituitary adenylate cyclase-activating polypeptide (PACAP)-27; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* vasoactive intestinal polypeptide (VIP). LGA: Left gastric artery.

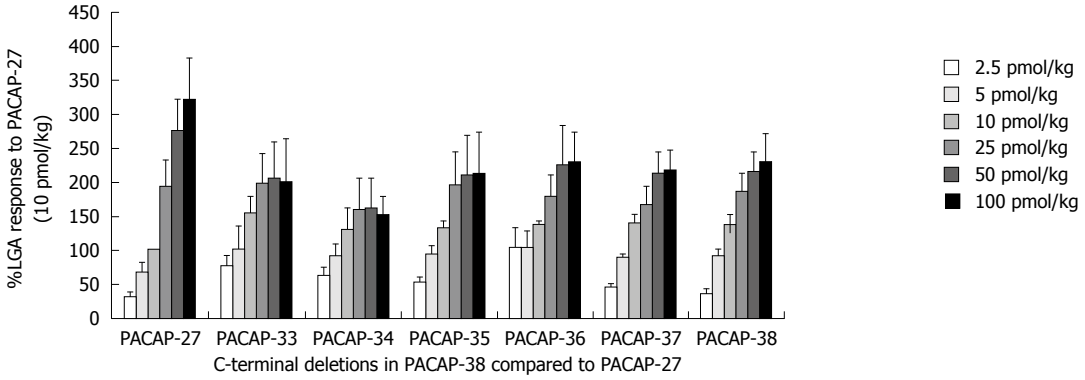

Figure 3 Effects of C-terminal deletions in pituitary adenylate cyclase-activating polypeptide-38 and pituitary adenylate cyclase-activating polypeptide-27 on left artery blood flow (mean ± SE). PACAP: Pituitary adenylate cyclase-activating polypeptide; LGA: Left gastric artery.

DISCUSSION

It has been shown that PACAP has potent gastrointestinal effects[11]. The present study demonstrated that both PACAP and VIP were potent vasodilators of the left gastric arterial bed in dogs, and PACAP was more potent than VIP.

Similarly, it has been reported that although PACAP-27 and N-terminus 27 amino acids in PACAP-38 show a high homology with VIP[12], PACAP is more potent than VIP in stimulating adenylate cyclase in pituitary cells[13].

Autoradiography can clearly identify two PACAP binding sites: one is PACAP preferring, the other has an identical affinity to VIP and PACAP[14].

The physiological actions of these widely distributed peptides, including PACAP, VIP and their analogues, are produced by activating the three common G-protein coupled receptors (VPAC1, VPAC2 and PAC1) which preferentially stimulate adenylate cyclase and increase intracellular cAMP, although stimulation of other intracellular messengers, including calcium[15] and phospholipase D[16] has also been reported.

The three receptor subtypes have been classified into "VPAC receptors" for VIP and PACAP, and "PAC1 receptors" which are the PACAP-preferring subtype[17]. The VPAC receptors can be further divided into VPAC1R and VPAC2R subtypes, based upon helodermin binding/potency (VPAC2R is helodermin preferring).

The actions of PACAP and VIP on gallbladder are opposite, namely VIP relaxes the gallbladder, whereas PACAP induces its contraction both *in vivo* and *in vitro*. The three receptor subtypes that recognize PACAP and VIP as the gallbladder have been found in blood vessels of the gastrointestinal system. Intravenous infusion of PACAP analogues increases the intestinal system blood flow in a dose-dependent manner. The PACAP becomes more potent as the amino chain of PACAP extends.

The aim of this study was to determine the effect of PACAP on LGA flow and to unveil the structural or functional important sites that may be critical for discrimination of different receptor subtypes. PACAP-27 analogues with amino acid substitutions or deletions at selected sites were synthesized.

In the present study, deletion of amino-acid residues from C-terminus of PACAP-38 did not significantly change the effect of PACAP on LGA, which is not consistent with the reported findings in gallbladder[5], demonstrating that the COOH-terminus of PACAP-38 has no key residues for the activity of PACAP-27 in LGA, and that PACAP-27 and PACAP-38 may act on the same receptor subtype.

The C-terminal deletions in PACAP-38 had no signifi-

cant influence on the peak LGA flow in this study, showing that no particular amino acid residue is responsible for the decreased potency of PACAP-27 and PACAP-38, which is consistent with the prolonged response of PACAP-38 to the femoral blood flow in dogs[18].

The change in single amino-acid residue at position 4 or 5 of the amino chain of PACAP-27 did not significantly change the biological function of PACAP-27. However, substituting the amino-acid residues at positions 4 and 5 with corresponding VIP residues significantly changed the biological function of PACAP-27. The responses of LGA flow to [Ala4, Val5]-PACAP-27 and VIP were similar, demonstrating that positions 4 and 5 are the key NH2-terminal residues of PACAP-27 that distinguish interactions with PAC1 receptors from those with VPAC1 and VPAC2 receptors in the LGA.

In our previous study on VIP and PACAP in guinea pig gallbladder[5], VIP induced relaxation while PACAP27 induced contraction of gallbladder. [Ala4, Val5]-PACAP27 were more potent than PACAP27 ($P < 0.01$) in stimulating the gallbladder. It has also been identified in a previous study[5] that [Ala6] PACAP-27 has no significant activity and [Gly8] PACAP-27 is significantly ($P < 0.05$) less potent (25%) than PACAP-27, which are consistent with the findings in the present study, demonstrating that position 4 and 5 are the key residues of PACAP-27 and substitutions at both sites with VIP residues may influence on specific receptor recognition. In this case, positions 4 and 5 substituted PACAP-27 may choose VPAC receptors instead of PAC receptors. Positions 6 and 8 are also important for the effect of PACAP27. It has been shown that a hydrophobic β-coil may form in the N-terminal region and that this structure may be important in receptor-binding affinity[19].

There is evidence that both N- and C-terminal regions are important for the biological activity of peptides and recognition of specific receptors[20,21]. It was reported that replacement of the COOH-terminal of PACAP-27 with VIP has no effect on the relaxation of LGA[22].

In conclusion, NH2 terminus plays an more important role in the recognition of specific receptors than the COOH-terminal. No particular amino acid residue is responsible for the decreased potency of PACAP-27 and PACAP-38. Further study is needed to determine the sites important to the structure and functions of PACAP.

COMMENTS

Background

Pituitary adenylate cyclase-activating polypeptide (PACAP) was originally isolated because of its similarity with vasoactive intestinal polypeptide (VIP). PACAP-27 corresponds to the N-terminal 27-amino acid sequence of PACAP-38 and shows a 68% identity with VIP. The effects of PACAP and VIP on the cardiovascular system and gallbladder have extensively studied. The effects of PACAP on excretion and motility of the gastrointestinal tract have also been investigated. The findings suggest that PACAP may participate in regulation of the gastrointestinal circulation. The influence of PACAP on left gastric blood flow was observed in the present study.

Research frontiers

Small arteries and arterioles in the gastrointestinal tract and pancreas are innervated by VIP- or PACAP-positive fibers. Their actions on the gallbladder are opposite: VIP relaxes the gallbladder whereas PACAP induces its contraction. Both peptides were found to be very potent vasodilators of the gastrointestinal blood vessels in conscious dogs, suggesting that PACAP may participate in regulation of the gastrointestinal circulation.

Innovations and breakthroughs

Flow probes of an ultrasound transit-time blood flowmeter were placed around the left gastric artery (LGA). Connectors of the probes were pulled out of the abdominal cavity through a subcutaneous tunnel and fixed at the chest. After a recovery period, the animals were restrained in Pavlov stands and the experiments were conducted in the conscious state. This method can also be used in other experiments on gastrointestinal blood flow.

Applications

The motility and secretion function of gastrointestinal tract are closely related with blood flow. Based on the mechanisms of motility and secretion, the effects of brain-gut peptides on blood flow help understand the physiology of the digestive system and treatment of digestive diseases. The methods can also be used in other experiments on the effects of peptides on gastrointestinal blood.

Peer review

In this study, peptides including PACAP-27, PACAP-38, amino acid substituted PACAP-27 and C-terminal truncated analogues PACAP (27-38) were synthesized and blood flow from the LGA of dogs was measured in response to these peptides infused at various concentrations. The results indicate that amino acid substituted PACAP can cause a concentration dependent vasodepressor action similar to that caused by PACAP-27. The study is interesting, but the data should be further clarified.

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BRIEF ARTICLE

Clinical significance of C-reactive protein values in antibiotic treatment for pyogenic liver abscess

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Abstract

AIM: To investigate the clinical significance of C-reactive protein (CRP) values in determining the endpoint of antibiotic treatment for liver abscess after drainage.

METHODS: The endpoints of antibiotic treatment in 46 patients with pyogenic liver abscess after complete percutaneous drainage were assessed by performing a retrospective study. After complete percutaneous drainage, normal CRP values were considered as the endpoint in 18 patients (experimental group), and normal body temperature for at least 2 wk were considered as the endpoints in the other 28 patients (control group).

RESULTS: The duration of antibiotic treatment after complete percutaneous drainage was 15.83 ± 6.45 d and 24.25 ± 8.18 d for the experimental and the control groups, respectively (P = 0.001), being significantly shorter in the experimental group than in the control group. The recurrence rate was 0% for both groups. However, we could not obtain the follow-up data about 3 patients in the control group.

CONCLUSION: CRP values can be considered as an independent factor to determine the duration of the antibiotic treatment for pyogenic liver abscess after complete percutaneous drainage.

Key words: Liver abscess; C-reactive protein; Antibiotic treatment; Drainage; Retrospective studies

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INTRODUCTION

Over the past two decades, complete percutaneous drainage combined with antibiotics has been considered as the routine treatment for liver abscess. This combined approach is clinically effective and has significantly reduced the death rate associated with liver abscess[1]. However, the duration and the protocol for the antibiotic treatment after percutaneous drainage is a matter of debate. Conventionally, the duration of antibiotic treatment is determined by the overall health condition after percutaneous abscess drainage[2-4] or by the white blood cell count[5]. The duration of antibiotic treatment was usually prolonged as much as possible[6]. However, assessments performed by considering the overall health condition as the end-point

of antibiotic treatment tend to be subjective. Moreover, standardized criteria for different patients cannot be developed easily. The white blood cell count can be affected by various factors such as physiological situations that may not truly reflect the clinical condition[7]. Prolonged antibiotic treatment has various disadvantages such as pyogenic antibiotic resistance and double infection. To decrease the incidence of antibiotic-resistant bacteria, reduce medical expenses, and increase patient compliance, a more sensitive parameter is required to determine the optimum duration of antibiotic treatment after percutaneous abscess drainage. C-reactive protein (CRP) is an acute-phase protein that is synthesized by liver endothelial cells, which is considered as the most valuable indicator of inflammation, and is a useful marker to determine the usage of antibiotics and assess the efficacy of the antibiotics[8-11]. However, there is no clinical report on the validity of using CRP values to determine the endpoint of liver abscess treatment. We analyzed the CRP values of the patients who were admitted to our hospital between June 2007 and February 2010. The investigation report is as follows.

MATERIALS AND METHODS

Subjects

We performed a retrospective study on 46 patients with liver abscess who were admitted to our hospital between June 2007 and February 2010. The diagnosis was based on typical clinical symptoms such as fever and upper abdominal pain along with the results of liver examination by ultrasonic and computed tomography. There were 32 male and 14 female patients; 8 patients had an abscess on their left liver lobe and 38 had an abscess on their right liver lobe. Ten patients had diabetes and 9 had cholecystitis. Among the 46 cases, 11 had positive culture results, including 4 from blood culture and 6 from abscess culture, and 1 from both; 8 (73%) of 11 patients showed *K. pneumoniae* infection. All the patients received effective antibiotic treatment and underwent percutaneous drainage of the liver abscess when the fluid was identified during ultrasonography.

Study methods

Inclusion criteria: The patients fulfilling the following criteria were included: (1) those undergoing effective antibiotic treatment; and (2) those who had undergone percutaneous abscess drainage when the fluid was identified during ultrasonography.

Exclusion criteria: The patients fulfilling any of the following criteria were excluded: (1) those who had undergone surgery; (2) those who had discontinued hospitalization before the treatment ended or continued medication out of the hospital. The duration of antibiotic treatment could not be determined for these patients; (3) those who had not chosen the right antibiotics and had prolonged hospitalizations; and (4) those who had not undergone percutaneous drainage.

Endpoints of the treatment: (1) Normal CRP values or (2) normal body temperature for at least 2 wk.

Experimental group: Normal CRP values were considered as the endpoint of antibiotic treatment in those patients.

Control group: Normal body temperature for at least 2 wk was considered as the endpoint of antibiotic treatment in those patients.

Mode and duration of follow-up: Visits were continued until March 2010 after hospitalization was ended.

Major endpoints: Duration of antibiotic treatment and recurrence rate.

CRP and white blood cell counts: CRP and white blood cell counts were determined every 3-5 d for the patients with normal CRP values were considered as the endpoint. Antibiotic treatment was stopped when the CRP values returned to normal. CRP and blood tests were not regularly performed for the control group, and the major criteria for discontinuing antibiotic treatment was normalization of body temperature for at least 2 wk.

Statistical analysis

The mean ± SD values were used for quantification. SPSS13.0 software was used for statistical analysis. *T*-test was used to compare the quantitative data. Chi-square analysis was used to compare the measurement data. P values < 0.05 were considered to be statistically significant.

RESULTS

Comparison of the known criteria

Normal CRP values were considered as the endpoint of antibiotic treatment in 18 patients (experimental group), and normal body temperature for at least 2 wk were considered as the endpoint in other 28 patients (control group). The gender, age, size of liver abscess before and after percutaneous drainage, duration of antibiotic treatment before the percutaneous drainage and the time of follow-up were comparable between the two groups (P > 0.05). In both groups, none of the patients showed recurrence of pyogenic liver abscess. We could not obtain follow-up information of 3 patients in the control group, in the intention-to-treat analysis, these patients would be considered as treatment failure.

Comparison of antibiotic treatment

The duration of antibiotic treatment after complete percutaneous drainage for the experimental and the control groups were 15.83 ± 6.45 and 24.25 ± 8.18 d, respectively (P = 0.001). The total duration of antibiotic treatment was 23.06 ± 7.36 d for the experimental group and 31.11 ± 7.30 d for the control group (P = 0.001) (Table 1).

Table 1 Data of liver abscess patients and duration of antibiotic treatment (mean ± SD)

	Experimental group	Control group	*P* value
Age (yr)	57.72 ± 10.39	58.68 ± 11.73	0.779
Women, *n* (%)	5 (28)	9 (32)	0.754
Size of abscess before therapy (diameter)	7.22 ± 1.44	8.01 ± 2.63	0.291
Size of abscess after therapy (diameter)	3.64 ± 0.89	4.02 ± 1.77	0.484
Antibiotic treatment before percutaneous drainage (d)	7.22 ± 5.39	6.82 ± 4.59	0.788
Antibiotic treatment after percutaneous drainage (d)	15.83 ± 6.45	24.25 ± 8.18	0.001[1]
Duration of follow-up (mo)	9.25 ± 5.67	9.24 ± 4.94	0.995
Recurrence rate	0%	11% (follow-up information for 3 individuals was not obtained)	0.151
Total duration of antibiotic treatment (d)	23.06 ± 7.36	31.11 ± 7.30	0.001[1]

[1]Statistical difference between the two groups of data.

Comparison of CRP values and white blood cell counts

The overall average time required for the white blood cell count and the percentage of neutrophils to normalize after the initiation of antibiotic treatment was 17.95 ± 8.00 d. The average time taken for the CRP values to normalize was found to be 21.44 ± 7.06 d for these 46 patients. There was significant difference ($P = 0.045$) between the two durations. The white blood cell count and the percentage of neutrophils had normalized before the CRP values returned to normal. In 5 patients, it was normal even before the percutaneous liver abscess drainage.

DISCUSSION

Liver abscess is a rare disease, with an incidence of 1.0-17.59 cases per 1 000 000 people[12,13]. The death rate for untreated cases is 100%. Currently, the major therapies for liver abscess are antibiotic treatment, percutaneous drainage combined with antibiotics, and surgery. Usually, antibiotics can be used alone for single liver abscess smaller than 3 cm in size. Percutaneous drainage combined with antibiotics is performed for liver abscesses larger than 3 cm. Surgery has to be performed in the cases of multiple abscesses[14]. Due to the effectiveness of these treatments, the death rate of the patients with liver abscess has decreased gradually over the last 20 years; the current death rate is only 6%-14%[1,15].

However, the optimum duration of antibiotic treatment is still a matter of debate. The current treatment protocols are all based on clinical experience, and there is no medical evidence to validate these protocols. Two representative treatment procedures have been suggested: (1) administration of antibiotics alone, for at least 6 wk; after successful percutaneous drainage, antibiotics are continued for another 7 d until all the symptoms disappear[2,3]; and (2) after complete percutaneous drainage, antibiotics are intravenously administered for at least 3 wk, which is followed by oral administration for 1 or 2 mo to prevent recurrence[4]. Furthermore, a study in the United States recommended prolonged antibiotic treatment in the cases of liver abscess[4]. Therefore, antibiotic treatment for liver abscess is considered to be a prolonged procedure. The referred endpoint indicators are non-specific. There are no exact markers to determine the endpoint of antibiotic treatment for liver abscess.

In China, there is no established guideline for the duration of antibiotic treatment after percutaneous drainage. In 45 diabetic patients with pyogenic liver abscess, treatment was not stopped after normalization of body temperature and physical condition; instead, the treatment was continued for 12 wk even after recovery from the abscess[16]. Another study reported that combined use of antibiotic treatment and percutaneous drainage for 4-6 wk was an extremely effective approach[17]. However, the appropriate treatment procedures and the endpoint of antibiotic treatment recommended in these studies required further investigations.

In recent years, Rahimian *et al*[18] reported that antibiotic treatment for liver abscess should not be prolonged. They also reported that short-term antibiotic treatment did not increase the death rate. They had treated 73 patients with liver abscess with intravenous antibiotic administration for 17.5 d, and the associated death rate was 2.5%. However, they did not mention the process of determination of the endpoint of antibiotic treatment. In 2009, the continuing education website of John Hopkins University reported that that after effective percutaneous drainage, the duration of the antibiotic treatment should be determined on the basis of the normalization of the white blood cell count and body temperature, and the treatment should be continued for 14-42 d[5] (unpublished data).

The total white blood cell count and the percentage of neutrophils have been used as standard indicators for the detection of infection, since the measurement methods for these parameters are simple, cheap, and of great clinical utility. These indicators are widely employed in most hospitals, especially in general hospitals. However, the use of the white blood cell count and the percentage of neutrophils as the endpoint of the treatment do not completely reflect the clinical condition. White blood cell count can vary due to various pathological conditions, and they may be influenced by physiological and various other factors such as postprandial intense exercise, cold temperature, pain, and fear. In a retrospective study in China, among 28 patients with thoracic abscess, 4 had normal white blood cell count and significantly elevated

CRP levels[19]. This finding indicates that some patients, especially some elderly patients had lower response to infection. Moreover, among children with lower respiratory system infection, there is no significant difference between the white blood cell counts of patients with pyogenic infection and those with virus infection[20]. Elevated white blood cell count has been traditionally considered as a diagnostic criterion even for patients with appendicitis; however, a prospective study indicated that the sensitivity and specificity of the elevated white blood cell count for diagnosing appendicitis were only 76% and 52%, respectively. The receiver operating characteristic (ROC) curve also indicated that elevated white blood cell count was not clinically relevant for diagnosing appendicitis[7]. ROC curve, is a graphical plot of the sensitivity, or true positives, or false positives, also known as a relative operating characteristic curve, because it is a comparison of two operating characteristics as the criterion changes. So ROC analysis provides tools to select possibly optimal models and to discard suboptimal ones independently. In our data, the white blood cell count and the percentage of neutrophils of some patients were normal even in the initial stage of the disease or before percutaneous drainage. Therefore, white blood cell count did not completely reflect their condition; consequently, it cannot be used as a criterion for medication.

After the onset of inflammation, CRP synthesis increases within 4-6 h, doubling every 8 h. The CRP level reaches the peak value (around 150-350 mg/L) within 36-50 h after infection. The high levels persist through the inflammation period. Therefore, when the infection is controlled, the CRP levels decrease quickly, and the decrease is strongly correlated with the relief from symptoms and with the duration of the treatment. However, the CRP level is not affected by factors such as gender, age, anemia, hyperglobulinemia, and pregnancy[21]. Clinically, the CRP levels have been used to determine whether antibiotic treatment should be started and to judge the effectiveness of the antibiotics. However, very few studies have considered the CRP value as a criterion for determining the endpoint of antibiotic treatment. In 1995, a report suggested that when both CRP and white blood cell counts are normal, antibiotic treatment for the abscess should be discontinued. The accuracy of using CRP values for the assessment of the abscess was as high as 99%, and there were no reports of negative results from the blood culture[9]. However, there have been no further studies on these findings. We realized that CRP value could be considered as an endpoint criterion for liver abscess treatment. In our data, the duration of the shortest treatment was only 11 d, the longest treatment period was not more than 4 wk, and the recurrence rate had not increased.

In summary, the CRP level could be used as an independent factor for determining the duration of the antibiotic treatment in the management of pyogenic liver abscess after complete percutaneous abscess drainage. It can be widely used in clinics. However, for further evaluation of the viability of CRP assessments, studies using more samples and random control trials should be performed.

COMMENTS

Background

Complete percutaneous drainage combined with antibiotics has significantly reduced the death rate associated with pyogenic liver abscess. However, the duration and the protocol for the antibiotic treatment after percutaneous drainage is a matter of debate in those patients. The referred endpoint indicators are non-specific. There are no exact markers to determine the endpoint of antibiotic treatment for liver abscess.

Research frontiers

Antibiotic treatment for liver abscess was considered to be a prolonged procedure. In recent years, it has been reported that antibiotic therapy for treating liver abscess should not be prolonged as short-term antibiotic treatment did not increase the death rate of the patients. However, the process of determination of the endpoint of antibiotic treatment was not mentioned. In 2009, the continuing education website of John Hopkins University reported that that after effective percutaneous drainage, the duration of the antibiotic treatment should be determined on the basis of the normalization of the white blood cell count and body temperature of the patients.

Innovations and breakthroughs

There are no exact markers to determine the endpoint of antibiotic treatment for pyogenic liver abscess at present. C-reactive protein (CRP) is considered as a useful marker to determine the usage of antibiotics and assess the efficacy of the antibiotic. However, there is no clinical report on the validity of using CRP values to determine the endpoint of liver abscess therapy. The authors proposed that the CRP value can be considered as an independent factor to determine the duration of the antibiotic treatment for pyogenic liver abscess after complete percutaneous drainage. In this study, normal CRP values were considered as the endpoint of antibiotic treatment.

Applications

Using CRP value as the endpoint of antibiotic treatment can decrease the duration of antibiotic treatment, thus decreasing the incidence of antibiotic-resistant bacteria, reducing medical expenses, and increasing patient compliance.

Terminology

CRP is an acute-phase protein that is synthesized by liver endothelial cells, which is considered as the most valuable indicator of inflammation. Percutaneous abscess drainage is a procedure performed to remove or drain a contained collection of infected fluid (abscess) from an area of the body such as the chest, abdomen, or pelvis.

Peer review

This manuscript is about a retrospective study to investigate the possibility of using CRP to determine the endpoint of antibiotic treatment along with percutaneous drainage for pyogenic liver abscess patients, and the data indicate that CRP value can be considered as an independent factor to determine the duration of the combination of antibiotic administration and percutaneous drainage. The manuscript is innovative and of putative interest for the readers.

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CASE REPORT

Treatment of portal vein tumor thrombus using ^{125}Iodine seed implantation brachytherapy

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Abstract

We reported two cases of liver metastasis with portal vein tumor thrombus that developed after liver transplantation for hepatocellular carcinoma (HCC). Both the patients were women aged 43 and 55 years, who had liver metastasis and portal vein tumor thrombus formation after liver transplantations for HCC. For the treatment of portal vein tumor thrombus, ^{125}I seeds were implanted into the hepatic tissue under the guidance of preoperative computed tomography (CT) images with a total radiation dose of 130 Gy. Enhanced spiral CT scan was performed for evaluation of the liver at 12 and 16 wk after treatment. Thereafter, upper abdominal CT examination was performed every 2-3 mo. No severe complications associated with the ^{125}I seeds were seen in these two patients. The upper abdominal CT images (obtained after 3 and 4 mo of treatment) showed that the thrombosis reactions were complete reaction and restoration of the patency of the partially obstructed portal vein with partial obstruction. In the case with complete obstruction of the portal vein, the thrombosis was resolved completely, but blood flow could not be restored. After this treatment, one of the patients is still alive, while the other died within 6 mo after the treatment due to lung metastasis complicated with lung infection, leading to respiratory failure.

Key words: Computed tomography-guided ^{125}Iodine seed implantation brachytherapy; Hepatocellular carcinoma; ^{125}I radioisotopes; Brachytherapy; Portal vein tumor thrombus

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a serious disease which is mainly treated with two approaches: surgical resection and orthotopic liver transplantation. However, these approaches are associated with a high risk of postoperative recurrence and metastasis. Intrahepatic metastasis of HCC after liver transplantation is similar to that after HCC resection in some respects; for instance, both conditions frequently invade the portal vein system. Portal vein tumor thrombosis (PVTT) is very common in liver metastasis after liver transplantation for HCC. To date, no effective treatment for PVTT has been recognized[1].

Three-dimensional (3-D) conformal radiotherapy (CRT) is widely used in treatment of HCC with portal

vein thrombosis. However, this therapy has the following shortcomings: (1) inaccuracy in locating the lesions due to interference by the respiratory movement; (2) a relatively long treatment duration; and (3) damage to the surrounding tissues[2].

^{125}I seed implantation is a type of brachytherapy. Its safety in the treatment of tumors has been recognized over the world. The emergence of computer-aided 3-D treatment planning system (TPS) and computed tomography (CT)-guided precision positioning system has enhanced the accuracy of particle implantation and reduced the extent of damage to the surrounding normal tissues. Therefore, this therapeutic method has been widely used. However, to date, there has been no published report on ^{125}I seed implantation for the treatment of HCC recurrence with PVTT after liver transplantation.

We herein report two cases in which CT-guided ^{125}I seed implantation was used for the treatment of recurrent liver metastasis with PVTT developed after liver transplantation for HCC. Satisfactory results were found during the follow-up in 1 case after 6 mo and in the other up to the present.

CASE REPORT

Case 1

The patient was a 43-year-old Chinese woman who underwent allogenic liver transplantation in the First Affiliated Hospital of China People's Liberation Army Third Military Medical University on February 17, 2006. On July 5, 2007, the abdominal CT examination revealed the left intrahepatic metastasis of HCC. She was treated with ultrasound-guided radiofrequency twice: one in August and the other in October 2007. On December 18, 2007, upper abdominal contrast-enhanced CT scan showed left intrahepatic metastasis, portal vein occlusion of the left extrahepatic segment, and intrahepatic segment-filling defect (2-cm long) in the portal trunk. α-fetoprotein was 43010 μg/L. After informed consent was obtained from the patients for the surgery, CT-guided ^{125}I seed implantation was performed on January 4, 2008. An enhanced abdominal CT scan was performed before seed implantation. The 3-D CT images were transferred to the TPS. On the basis of the obtained CT images of lesions, the spatial distribution of implanted seeds was simulated. A combination of preplanning and real-time technique was adopted, and the number and activity of the implanted seeds were calculated. Spiral CT-guided percutaneous manual implantation of the particles was performed (Figure 1). Since the thrombus originated from the left branch of the portal vein and spread to the trunk, there was no significant extension of the thrombus in the left branch of the portal vein. The ^{125}I seed implantation was done in the portal area around the capsules. We implanted 22 seeds (0.8 mCi/seed) with a total radiation dose of 130 Gy (110-140 Gy) to the thrombus. The liver function tests showed elevated levels of serum transaminase for about 1 wk after the treatment,

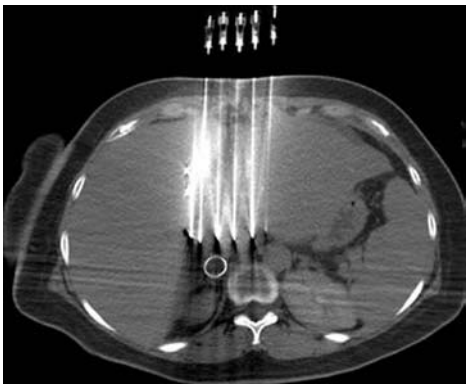

Figure 1 ^{125}I seed implantation process.

which returned to the level of pre-brachytherapy after liver recovery treatment. Abdominal CT after 3 mo showed the disappearance of the portal vein tumor thrombus and the obvious restoration of the portal vein. Thrombosis reaction was complete reaction (Figure 2). This patient died within 6 mo of treatment because of respiratory failure caused by bilateral pulmonary metastasis complicated with pulmonary infection.

Case 2

The patient was a 55-year-old Chinese woman, who underwent allogenic liver transplantation for HCC under general anesthesia on May 18, 2006 in the same hospital. On December 26, 2007, dual-modality positron emission tomography/CT examination showed that the upper posterior part of the right liver had abnormal uptake, thereby suggesting HCC metastasis. On January 12, 2008, ultrasound and CT images showed that the posterior branch of the right portal vein tumor thrombus had invaded the portal vein trunk, the length of the tumor thrombus was about 1.5 cm, and the left main trunk had a small embolus. On January 16, 2008, ^{125}I seed implantation was performed. For the thrombus in the right posterior portal branch, the seeds were implanted directly inside; for the left main trunk, they were implanted in the periportal zone about 1 cm away from the portal area. The treatment protocol was the same as that described in the 1st case, and 27 seeds were implanted at a dose of 0.5-0.8 mCi/seed (Figure 3). No postoperative complications associated with the implantation were observed in this patient. Four months after the treatment, upper abdominal CT showed the following findings: (1) the tumor thrombus in the right branch of the portal vein had resolved with only implanted seeds remaining; (2) the patency of the posterior branch of the right portal vein was not completely restored; and (3) the filling defect in the left branch of the portal vein had disappeared (Figure 4). To date, she is still alive. Transient increase in transaminase occurred in the two patients, which decreased to the preoperative levels after 1 wk. Serious complications, such as acute bleeding during seed implantation, hematoma, infection, abdominal pain, bilirubin, seed migration to the lung, were not observed.

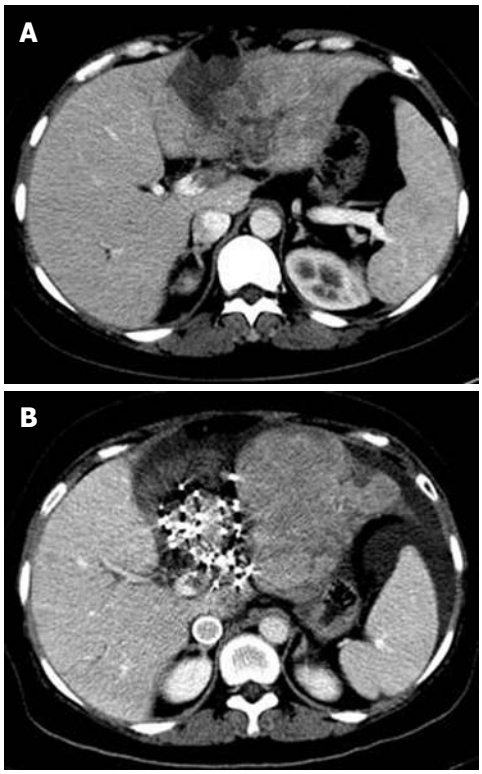

Figure 2 Enhanced computed tomography shows thrombus before ^{125}I seed implantation (A) and absence of tumor thrombi 3 mo after ^{125}I seed implantation (B).

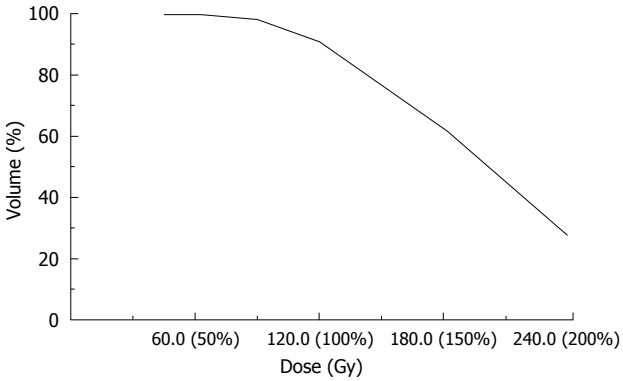

Figure 3 A histogram of therapeutic dose of normal and tumor tissues. target volume = 10.0 cm^3, planning target volume = 13.7 cm^3, target volume ratio = 137.4%, volume fraction for prescription, 100% = 91.2%, volume fraction for prescription, 150% = 63.5%, dose homogeneity index = 30.4%, dose non-uniformity ratio = 69.6%.

DISCUSSION

PVTT is a common complication in HCC patients with liver metastasis after liver transplantation. PVTT not only leads to the development of metastatic tumors in the liver, but also is a sign of liver function deterioration. Conventional radiotherapy can be used in the treatment of PVTT. However, the use of this therapy is limited because the liver has a low radiation tolerance. The tolerance dose for the whole liver is 30-35 Gy[3], but this dose is not sufficient to control the growth of HCC. Lawrence *et al*[4] reported

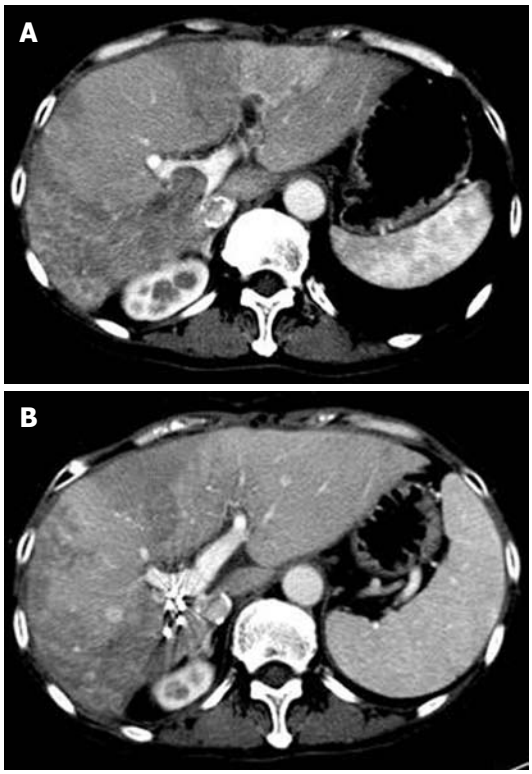

Figure 4 Enhanced computed tomography shows thrombus before ^{125}I seed implantation (A) and absence of tumor thrombi 4 mo after ^{125}I seed implantation (B).

that for lesions in 70%, 50% and 20% of the liver, tolerance doses were 42, 52 and 70 Gy, respectively.

The use of ^{125}I seed implantation in the treatment of PVTT has the following advantages over 3D-CRT: (1) the dose distribution is more conformal to the shape and size of the tumor with the former than with the latter; (2) since irradiation of the lesion is maintained during the decay of the isotopes, the duration of exposure of the tumor is extended; therefore, the tumors receive a higher dose of irradiation, while causing minimal injury to the surrounding normal tissues; and (3) tumor positioning is accurate; the tumor is accurately located without interference from respiratory movements. The application of ^{125}I seed implantation in the treatment of liver cancer has been reported previously. Rafael *et al*[5] performed ^{125}I seed implantations in 56 cases of such intrahepatic metastasis of colorectal cancer where the implantations were either undertaken because the surgeries were contraindicated or performed intraoperatively for residual tumors. In these patients, the actual tumor control rates in the first, third and fifth years were 41%, 23% and 23%, respectively, and the actual survival rates in the first, third and fifth years were 71%, 25%, and 8% (the median survival time was 20 mo), respectively. Subir *et al*[6] reported 64 cases of liver cancers in which intraoperative ^{125}I seed implantations (total dose of 160 Gy) were performed for any of the following reasons: incomplete removal of tumor by surgery, residual primary tumors after surgical resection, and metastasis. In the 64 cases, the actual tumor control rates in the first, third and fifth years were 44%, 22%, and 22%,

respectively, and the actual survival rates in the first, third and fifth years were 73%, 23%, and 5%, respectively.

The complication occurrence of ^{125}I seed implantation in liver tumors has been reported to be low. Nag *et al*[6] reported that in their study of 64 patients treated with ^{125}I seed implantation, only 2 had liver abscesses, but without other complications. Ricke *et al*[7] reported that among 37 patients with liver cancers and some patients with other cancers treated with CT-guided interstitial radiotherapy, 2 patients had severe complications after the treatment (< 5%). A high dose rate (HDR) was only used in temporary implantation because of its high energy of rays and severe damage to the surrounding tissues. There were serious adverse reactions induced by prolongation of seed implantation with HDR, but complications of permanent implantation with low-active seeds were very light. In PVTT treatment, the postoperative liver function abnormalities were transient and treatment-responsive, with only mild liver dysfunction in most cases. There was no seeds migration to the lung. Seeds were put inside the tumor and outside the vessels rather than inside the vessels.

In conclusion, a combination of ^{125}I seed implantation and other interventional means is a new method to control the HCC metastasis. However, analyses of more cases and further researches on brachytherapy are still required to assess the safety and efficacy of this new method.

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Science of weight loss supplements: Compromised by conflicts of interest?

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Abstract

Weight loss supplements often contain powerful pharmacoactive ingredients with the potential to cause harm. Trials used to determine product safety and effectiveness, meanwhile, tend to be small, of short duration, and frequently lack financial conflict of interest disclosures. These factors could conspire to place consumers at risk, especially when published research cited in advertising cloaks products with the suggestion that their safety and effectiveness have been proven by science. Examples of current and former weight loss products backed by potentially conflicted or low quality research include Metabolife-356, Hydroxycut, Xenadrine and LeptiCore. Published research, especially in the field of weight loss supplements, needs better conflict of interest disclosure, and regulators should consider how research findings are used in marketing claims.

Key words: Weight loss supplements; Cissus quadrangularis; Hydroxycut; Xenadrine; Metabolife; Lepticore; Garcinia cambogia; Conflict of interest

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TO THE EDITOR

Hasani-Ranjbar *et al*[1] recently reviewed evidence behind dietary supplements used for weight loss. While this review provides data suggestive of methodological weaknesses, such as sample sizes and trial duration, and provides a sentence suggesting that safety concerns may not be fully addressed in the reviewed studies, the paper appears to underemphasize inherent flaws in the publications it reviews. Weaknesses that deserve special attention are the small size and short duration of the trials, and links to industry sponsorship, which are frequently not disclosed or inadequately disclosed in the studies themselves. These factors are important for both methodological reasons, and also reasons specific to the weight loss products being reviewed.

Substances tested for weight loss often have potent pharmacological effects[2], may be used by millions of consumers without medical supervision, and can be marketed without meeting the regulatory standards imposed on traditional pharmaceuticals[3]. These factors underscore the importance of assessing product safety as well as efficacy[3,4]. Safety in particular is difficult to establish when studies are of small size and short duration. Of the 19 human studies reviewed by Hasani-Ranjbar *et al*[1], the average number of participants was 64.4 (range 24-153), and the average study duration was 15 wk (range 2-36 wk). Such methodologically weak studies, combined with lax dietary supplement regulation and oversight, have several potential public health implications, including: (1) infrequent or rare side effects may not be detected

Table 1 Selected studies supporting popular weight loss supplements

Xenadrine
Advertisements for this popular and widely advertised weight loss supplement cite a published study supportive of its effectiveness[11]. In addition to being small (n = 47) and of short duration (6 wk), the study lacks financial disclosure, or any mention of a funding source
LeptiCore
Marketing text for this weight loss product cites a published study of 62 participants followed for 6 wk who reportedly experienced significant reductions in weight, body fat, and other metrics associated with chronic disease, such as cholesterol and waist size[12]. The study does not disclose a funding source, beyond the name of the company that provided the tested substance. The paper states that the authors have no competing interests, though one author appears to be a chief scientific officer of a dietary supplement company[1], and appears on US Patent Office[2] filings as the inventor of a weight loss supplement whose patent is held by the same supplement company the author appears to be employed by (The patent was granted in 2010, and originally filed in 2000)
Hydroxycut Advanced
Hydroxycut was the top selling weight loss supplement in the US, then withdrawn from the market after being linked to 23 cases of liver toxicity and one death[3]. Marketing materials for Hydroxycut cited two published studies asserting product effectiveness that were small, of short duration, reported no serious side effects[13,14], and did not disclose relationships between authors and the product manufacturer[15] or that funding was received from the product manufacturer[16]
Hydroxycut has been renamed Hydroxycut Advanced, reformulated and returned to market, distributed by IHS. An active ingredient suspected of causing liver toxicity in the original formulation, Garcinia cambogia[2,3], has been removed and replaced with other ingredients, including CQ. At least 3 recently published studies support the safety and effectiveness of CQ for weight loss but lack financial disclosures or funding sources, beyond mentioning that the CQ being tested was provided by GHA[17-19]. The studies all share an author who is listed as a chief scientific officer for GHA[1] on internet sites, but not in the publications in question, and appears on US Patent Office[2] filings as the inventor of a weight loss supplement whose patent is held by GHA. IHS and GHA have collaborated in the past, though it is unclear whether the CQ currently used in IHS's product is provided by GHA
Concerns
1 Small, short term studies, and those funded by industry[7] may over-state product safety and effectiveness
2 A lack of funding source declaration reduces validity of findings, since readers are unable to assess the potential for this type of conflict of interest
3 Being a patent holder for a weight loss supplement should be considered a financial conflict of interest in these cases, since a patent holder may stand to gain financially from scientific reports of supplement effectiveness
4 The undeclared, potential financial or professional relationships between the patent holder/author and the manufacturer of the substance being studied also appears to be a conflict of interest, since the author would have a personal financial interest in the financial success of the product being studied

[1]http://www.clinicaltrials.gov, and professional networking websites, accessed May 7, 2010; [2]http://www.uspto.gov, accessed May 7, 2010. IHS: Iovate Health Sciences; CQ: Cissus quadrangularis; GHA: Gateway Health Alliances.

by studies of only a few dozen subjects, resulting in a body of evidence that over-states product safety; (2) findings from short-term studies may not reflect actual usage patterns among consumers, who may use products for longer-term weight loss maintenance; (3) sustaining weight loss long-term studies are difficult, so short-term studies may be more likely to garner positive findings, and since publication bias has been reported in favor of positive findings[5], the resulting body of evidence may over-state product effectiveness; (4) to gain market approval under current regulations in the United States, dietary supplement manufacturers only need to submit a relatively low-level of evidence suggestive of product safety[4], (5) products may then go to market backed by findings that over-state their safety and effectiveness; and (6) post market surveillance may only detect adverse events (AEs) once the number of product users blossoms into the tens of thousands, and only after harms have occurred. Unlike in research settings, it is rarely possible to conclusively link harm to product usage in daily life, and in the United States post market surveillance only detects an estimated 1% of such AEs[6].

In pharmaceutical research, industry-funded studies may be more likely to report positive findings[7], and the same is likely for weight loss supplements[8]. Readers can account for this if papers provide clear conflict of interest disclosures. However, the standard financial conflict of interest and funding disclosures regularly seen in rigorous pharmaceutical research may not appear as frequently in dietary supplement research[8]. In some cases, this is further obfuscated when a lack of conflict declaration is used to denote a lack of conflict instead of a declarative statement that there is no conflict. As such it is much more difficult to assess which studies are potentially biased by industry support.

These weaknesses are illustrated by a study, reviewed by Hasani-Ranjbar *et al*[1], of an ephedrine-containing product named Metabolife-356[9]. This manufacturer-funded study reported significant reductions in body-weight and body-fat, with only minor side effects such as dry mouth, insomnia, nervousness, palpitation and headache[9]. However, once the number of users rose into the millions, Metabolife-356 was linked to 92 serious adverse cardiovascular events, including 5 deaths[10], and withdrawn from the US market. These harms were not, and statistically could not be, detected by studies as small (n = 67) as the one in question. The potential for similarly misleading conclusions to be reached about the safety or effectiveness of three currently available products is detailed in Table 1, with the sometimes-opaque relationships between study authors, commercial interests and products delineated in Figure 1. The similarities in potentially flawed conflict-of-interest disclosures suggest a possible trend extending to multiple publications about multiple products in multiple journals.

Industry funding does not necessarily denigrate the

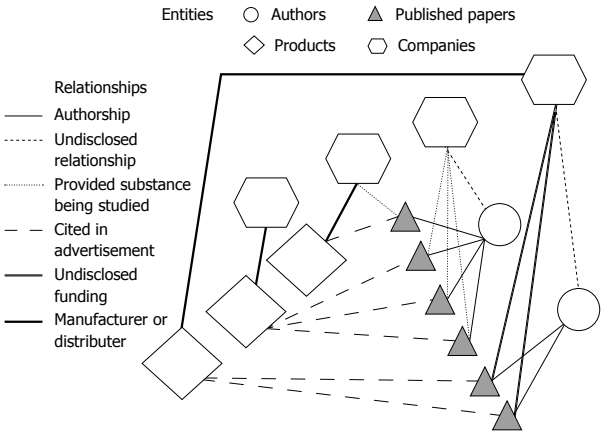

Figure 1 Relationships between select authors, evidence, products and manufacturers (detailed in Table 1).

quality of research, and in many cases is financially necessary to advance scientific understanding. However, undeclared financial conflicts of interest at best reduce face-validity of findings, and at worst represent deception. Financial disclosures that are generally the norm in scientific publications need to be in place in dietary supplement research as well, and should be provided by authors, required by journals, and insisted upon by peer-reviewers. A lack of financial conflicts should be noted by confirmatory statements rather than by omission of conflict declarations. Funding sources should also be noted by reviews of published papers, especially when the reviewed literature contains methodological weaknesses such as small sample size and short duration. When used for marketing, small, short duration pilot studies may have disproportionately large impact, providing false assurance to consumers with low science-literacy that products are "clinically tested" and thus safe and effective. It may also be prudent for regulatory authorities such as the US Federal Trade Commission or Food and Drug Administration to consider how published research is currently used in the marketing of consumer products, especially those with a track record of causing harm.

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Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
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12th World Congress of Endoscopic Surgery

April 14-18
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Digestive Disease Week Annual Meeting

May 06-08
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The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
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June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
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ILTS: International Liver Transplantation Society ILTS Annual International Congress

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Montreal, Canada
International Liver Association's Fourth Annual Conference

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September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

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The 7th Biannual International Symposium of Society of Coloproctology

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3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

Organization as author

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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Books

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Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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