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[1]Passed away on October 20, 2007
[2]Passed away on June 14, 2008

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INTRODUCTION

World Journal of Gastroenterology is an international, open-access, peer-reviewed, and multi-disciplinary weekly journal that serves gastroenterologists and hepatologists. The biggest advantage of the open access model is that it provides free, full-text articles in PDF and other formats for experts and the public without registration, which eliminates the obstacle that traditional journals possess and usually delays the speed of the propagation and communication of scientific research results. The open access model has been proven to be a true approach that may achieve the ultimate goal of the journals, i.e. the maximization of the values of the readers, the authors and the society.

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EDITORIAL

Hepatitis C comorbidities affecting the course and response to therapy

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Abstract

Several studies have demonstrated that the outcome of chronic hepatitis C (CHC) infection is profoundly influenced by a variety of comorbidities. Many of these comorbidities have a significant influence on the response to antiviral therapy. These comorbidities negatively affect the course and outcome of liver disease, often reducing the chance of achieving a sustained virological response with PEGylated interferon and ribavirin treatments. Comorbidities affecting response to antiviral therapy reduce compliance and adherence to inadequate doses of therapy. The most important comorbidities affecting the course of CHC include hepatitis B virus coinfection, metabolic syndrome, and intestinal bacterial overgrowth. Comorbidities affecting the course and response to therapy include schistosomiasis, iron overload, alcohol abuse, and excessive smoking. Comorbidities affecting response to antiviral therapy include depression, anemia, cardiovascular disease, and renal failure.

Key words: Comorbidities; Chronic hepatitis C; Response to therapy

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INTRODUCTION

The prevalence of hepatitis C virus (HCV) infection varies throughout the world, with the highest number of infections reported in Egypt. The use of parenteral antischistosomal therapy in Egypt is thought to have contributed to a prevalence of antibodies against HCV in various regions ranging from 6% to 28% (mean, 22%)[1]. An estimated 70% to 85% of infected patients are likely to develop chronic hepatitis, and up to 30% of these cases might progress to cirrhosis[2].

When treating chronic hepatitis C (CHC), many clinicians do not take into consideration the presence of other comorbid conditions that lead to more progressive liver disease, such as cirrhosis and hepatocellular carcinoma (HCC)[3-5]. In recent studies, it has been proved that such comorbidities might reduce the response rate to PEGylated interferon (PEG-IFN)/ribavirin (RBV) therapy in HCV patients[6,7].

Eventually amelioration of these comorbidities before embarking on IFN-based therapy would improve the sustained virological response (SVR) and impair progression to cirrhosis and HCC.

COMORBIDITIES AFFECTING THE COURSE OF CHC

Hepatitis B virus (HBV) co-infection

Coexistent HCV infection has been estimated to be present in 10% to 15% of patients with chronic hepatitis B, and is more common among injecting drug users[8]. Acute coinfection with HBV and HCV can shorten the duration of HBs antigenemia and lower the peak serum aminotransferase concentrations compared with acute HBV infection alone[9,10]. However, acute coinfections of HCV and HBV, or acute HCV on preexisting chronic HBV, have also been reported to increase the risk of severe hepatitis and fulminant hepatic failure[11].

However, combined chronic hepatitis B and C leads to more severe liver disease, an increased risk of hepatocellular carcinoma[3,4] and lower response to IFN[6]. Furthermore, co-infected patients represent a treatment challenge. No standard recommendations exist for treatment of viral hepatitis due to dual HBV/HCV infection, and therefore treatment must be individualized[4].

Management: Treatment decisions should be based upon

Figure 1 HCV-induced insulin resistance. Dotted lines represent inhibition, continuous lines represent activation. PI3K: Phosphatidyl-inositol 3 kinase.

Figure 2 HCV-induced insulin resistance[21-23]. SOC-3: Suppressor of cytokine-3; PPAR: Peroxisome proliferators activated receptor; HSCs: Hepatic stellate cells.

Figure 3 Core-protein induced insulin resistance[21-23].

the determination of the "dominant" hepatitis virus. The more active virus should be treated using IFN plus RBV for hepatitis C and IFN plus Lamivudine for hepatitis B[3,4]. Caution must be exercised in treating coinfected patients, by observing reactivation of untreated virus as flares of the latter might occur.

Metabolic syndrome (MS)

MS is a cluster of abnormalities, including obesity, insulin resistance, type 2 diabetes mellitus, dyslipidemia, and hypertension. Moreover, patients with chronic HCV infection have increased prevalence of insulin resistance and of type 2 diabetes compared with age-, sex-, and liver disease-matched controls[12].

It has been observed that overweight, insulin resistance, and liver steatosis have a negative impact on the course of CHC, being associated with more severe and progressive liver fibrosis[5]. HCV proteins are associated with the dysfunction of mitochondria and endoplasmic reticulum that promote oxidative stress. The latter mediates signals that activate the expression of proinflammatory cytokines: tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8, tumor growth factor-α, and the fas ligand..

Insulin resistance: HCV proteins activate the expression of TNF-α which inhibits the function of insulin receptor

substrates (IRS) and decreases the expression of glucose transporter-4 and lipoprotein lipase in peripheral tissues, which are responsible for the promotion of insulin resistance (Figure 1).

Furthermore, reduced adiponectin levels, loss of adiponectin receptors, and decreased anti-inflammatory peroxisome proliferator-activated receptor α (PPAR-α) in the liver of HCV patients might contribute to reduced fatty acid oxidation, inflammation, and eventually, lipotoxicity[12].

Insulin resistance has been clearly associated with steatosis, more severe and progressive fibrosis, and a reduced response to PEG-IFN and RBV therapy in HCV patients[13]. Several recent studies have confirmed that SVR is impaired in patients with a high homoeostasis model assessment index[14].

Obesity/Hepatic steatosis: In hepatitis C infection, hepatic steatosis might be either metabolic [overweight & obesity (BMI ≥ 25), diabetes mellitus] or cytopathic due to the effect of the virus, as in genotype 3. However, genotype-4, which is predominant in Egypt, has no direct relation with the development of steatosis[15]; however, it develops as a secondary metabolic effect as evidenced by HCV core protein promotes IR via TNF-α production[16] (Figures 2 and 3). In addition, HCV non-genotype-3 induces insulin resistance through downregulation of IRS[17-19]. HCV genotype-3 might induce a cytopathic effect and autoimmune aggression on β-cells of the pancreas[20]. Furthermore, HCV produces necroinflammation of hepatocyte membranes with consequent malfunction of insulin receptors.

Several studies have recently confirmed that the SVR[7] is impaired in patients with high body mass index and for those with hepatic steatosis. These data indicate that HCV carriers should avoid weight gain by diet and physical exercise before initiation of antiviral therapy, all efforts should be made to improve the metabolic steatosis of the patient.

Type 2 diabetes mellitus: Type 2 diabetes mellitus is frequently associated with hyperinsulinemia and fatty liver disease. The chronically elevated circulating insulin levels found in type 2 diabetic patients might be responsible for accumulation of fat in the liver by downregulating

Figure 4 Insulin-induced IFN resistance. Interaction between insulin and the PEG-interferon α-signaling pathway. PI3K activated by insulin seems to be responsible for the block of STAT1 translocation that avoids the antiviral effect of interferon. Dotted lines represent inhibition, continuous lines represent activation. IRS-2: Insulin substrate-2; 2′,5′-OAS: 2′,5′-oligoadenylate synthetase; PKR: Protein kinase.

mitochondrial β-oxidation and blocking secretion of triglycerides from the liver. Diabetic individuals are at higher risk to develop non-alcoholic steatohepatitis, which might progress to cirrhosis in up to 5% of cases[24,25].

Intestinal bacterial overgrowth (IBOG): Chronic liver disease is associated with slow, transient, altered gut permeability and translocation of intestinal bacteria and toxins to portal and systemic circulation[26,27]. These toxins contribute to liver cell injury by induction of proinflammatory cytokines (TNF-α, IL-6, and IL-8) in the liver. Concomitant IBOG with HCV infection will aggravate the risk of severe hepatitis and progressive liver disease as a result of the immune response against HCV infection.

Management: Intestinal decontamination by metronidazole and probiotics to prevent endotoxin formation, open bowel by synthetic disaccharides (lactulose), and trigger peristalsis by prokinetics.

COMORBIDITIES AFFECTING THE COURSE AND RESPONSE TO THERAPY OF CHC

Schistosomiasis co-infection
Schistosoma mansoni has been the major risk factor of liver diseases in Egypt, especially in rural areas. In many patients, HCV infection is associated with schistosomiasis because of iv anti-schistosomal therapy[28,29]. Schistosomiasis is an immunological disease, which suppress cellular immunity, triggering Th2 cytokine response favoring chronicity of hepatitis C infection.

Patients with CHC and concomitant schistosomiasis respond poorly to IFN therapy and have higher relapse rates compared to patients with HCV infection only. This might be due to HCV genotype 4 *per se*, or to the negative influence of schistosomiasis on the immune system,

leading to higher HCV RNA titers, and more severe liver damage with a higher incidence of cirrhosis. The latter is promoted by the longer duration of both infections[30].

Diagnosis of schistosomiasis was based on a history of Schistosoma infection, detection of S. mansoni ova in stool, or a rectal biopsy[31]. At baseline, patients with CHC and schistosomiasis had higher HCV RNA titers compared to patients without schistosomiasis[30].

Management: Praziquantel 40 mg/kg single dose (can be repeated up to three weekly consecutive doses) before initiation of antiviral therapy.

MS
Insulin resistance has been clearly associated with steatosis, a more severe and progressive fibrosis, and a reduced response to PEG-IFN and RBV therapy in HCV patients[13].

How does concomitant MS with HCV infection impair the response to interferon?

In obese patients, subcutaneous fat impairs absorption of interferon at the site of injection. In addition, hepatic steatosis decreases contact between interferon and hepatocytes receptors[16]. Steatosis interferes with the signaling cascade of interferon [Janus Kinase (JAK) activate signal transduction and activator of transcription (STAT) which express *IFN* genes][32]. On the other hand, the HCV core protein promotes insulin resistance *via* TNF-production and insulin resistance induces steatosis, fibrogenesis, and IFN-resistance[16,33,34]. In addition, HCV core protein and TNF-α upregulate suppressor of cytokines-3 which binds to JAK, inhibiting phosphorylation of STAT1[35] and eventually interfering with IFN signaling[33]. Furthermore, obesity in general is associated with a suppressed immune response[36,37] (Figures 3 and 4).

Management of MS: Amelioration of metabolic factors before starting interferon therapy favors a good response to interferon therapy. HCV patients should avoid weight gain by life style modification (hypocaloric diet/exercise), use of insulin sensitizers, in the form of Metformin, to reduce hepatic gluconeogenesis, and pioglitazone to sensitize insulin receptors and mobilize visceral fat to subcutaneous tissues. Antioxidants (vitamin E, betaine, silymarin, and β-carotine), hepatoprotective drugs (UDCA) might add therapeutic benefit to inhibit the toxic effects of free radicals. Gut decontamination with metronidazole and probiotics to prevent gut endotoxin formation should be considered; the later induce proinflammatory cytokines in the liver that promote steatosis and steatohepatitis.

Iron overload
In hepatitis C patients, increased iron absorption in cirrhosis and difficulty in excreting iron from the body contribute to development of iron overload[38]. There is growing evidence that iron overload enhances the amount of liver injury and progression to cirrhosis and HCC. In addition, it decreases the SVR to IFN/RBV treatment[39,40].

Management: Venesection, restrict diets rich in iron, give antioxidants such as silymarin, betaine and vitamin E to nullify the toxic effects of free radicals.

Alcohol abuse

Alcohol abuse favors the development of alcoholic steatosis, steatohepatitis, and subsequently cirrhosis. It plays a role in resistance to interferon therapy through immunosuppression of CD4+ and NK cells[41], by increased hepatic iron load, and by inhibiting the IFN-α-activated signals[42,43].

Most studies found that alcohol decreased the response to interferon-based therapy and this effect is alcohol dose-dependent[42-44]. Median daily alcohol use > 30 g/d is associated with failure to respond to PEG-IFN and RBV for treatment of hepatitis C. Past alcohol use should be evaluated when considering treatment for hepatitis C[42].

Management: Stop alcohol abuse, intestinal antibiotics and probiotics to prevent gut endotoxin formation; and antioxidants as vitamin E, betaine and silymarin to block lipid peroxidation. Pentoxifylline is an oral phosphodiesterase inhibitor which decreases expression of TNF-α (and other proinflammatory cytokines) and which may inhibit apoptosis. When the full course is completed, the SVR is similar, regardless of alcohol intake[45].

Excessive smoking

Heavy smoking increases the severity of hepatic inflammation and fibrosis when associated with hepatitis C infection[46]. Heavy smoking induces resistance to interferon therapy by suppression of CD4+ and NK activity[41] inducing apoptosis of T-cells[47], and increasing hepatic iron load[48].

Management: Stop smoking, venesection to reduce iron level, limit diet rich in iron and use of antioxidants as silymarin, vitamin E, betaine, β-carotine, lecithin and selenium.

COMORBIDITIES AFFECTING RESPONSE TO INTERFERON THERAPY

These comorbidities reduce compliance and adherence to inadequate PEG-IFN or RBV doses.

Depression

Depression is significantly more prevalent in chronically HCV-infected patients than in the general population[49], which negatively affect patients' functional health, ability to work, self-perceived health, health-related quality of life (HRQL) and well being[50].

The presence of mild/moderate depression at baseline is not considered an absolute contraindication to initiate antiviral therapy with PEG-IFN and RBV. However, this condition is certainly associated with a higher risk of developing severe depression during therapy that might lead to higher rates of treatment discontinuation in the absence of adequate antidepressant therapy[51].

It has been reported that IFN-α downregulated glucocorticoid receptor (GR) and serotonin receptor 1A (5-HTR1A) levels in cell lines. These levels of GR and 5-HTR1A, following IFN-α-induced downregulation, recovered after withdrawal of IFN-α or addition of desipramine or fluoxetine. These data provide insights regarding the pathogenesis of IFN-α-induced depression[52].

The pharmacokinetics of PEGylated IFN are different from those of standard IFN[53] and the time of depression occurrence also differs between PEGylated and standard IFN[54]; therefore, it will be important to determine if the mechanisms described by Cai et al[52] on GR and 5-HTR1A receptors also apply to PEGylated IFN. In addition, anxiety and depression impair the level and activity of B cells, T cells, and NK cells[55]. No differences in depression rates were observed by Neri et al[56] comparing PEGylated-α 2a and PEGylated-α 2b, and this finding has recently been confirmed in the large randomized comparison trial 'IDEAL'[57].

Depression and anxiety of significant severity can adversely effect compliance and tolerance to medication. Although anemia and depression were associated with HRQL impairment, depression was the most consistent predictor[50]. Several studies concluded that individuals who experience significant worsening of depressive symptoms during IFN therapy are less likely to achieve a virological response during therapy and an SVR after therapy withdrawal[13].

Management: Therapeutic intervention has been shown to be effective in the management of IFN-induced depression in a controlled study[58]. The most effective and well-studied antidepressants in this setting are those of the selective serotonin reuptake inhibitor class, in particular citalopram 25 mg/d[59]. Importantly, all patients who received antidepressant treatment were able to complete the full course of IFN therapy; while discontinuation was necessitated in patients in the placebo arm[60].

Anemia

Anemia that develops in a patient receiving HCV therapy often has multiple potential contributing factors, including RBV, interferon or PEG-interferon, underlying liver disease caused by HCV infection, and co-morbid conditions, such as HIV infection or chronic renal failure[61]. The anemia associated with RBV most often occurs as dose-dependent hemolytic anemia, typically developing within the first 4 wk of therapy[62,63]. At higher doses of RBV (1000-1200 mg/d), hemoglobin levels frequently decline by 2-3 g/dL. In addition to causing hemolysis, RBV can also downregulate the number of erythropoietin receptors[64]. Interferon can also contribute to the development of anemia by suppressing bone marrow production of erythrocytes, but this process is generally slower and might account for the continued decline in hemoglobin concentration during the second and third months of treatment. Finally, patients developing anemia during HCV therapy often have inappropriately poor serum erythropoietin responses[65], probably related to their underlying liver disease. It is often not possible to

pinpoint one particular drug as the primary cause of the anemia, because of the mixed nature of HCV treatment-associated anemia.

Management of anemia associated with HCV Therapy: The conventional standard of care for managing anemia during HCV antiviral therapy has consisted of reducing the RBV dose by half if the hemoglobin level decreases to less than 10 g/dL, and to completely stop the RBV if the hemoglobin level drops below 8.5 g/dL. A decrease in RBV dose, especially in the first several months of treatment, can diminish response rates considerably. Furthermore, the strategy of decreasing the RBV dose will only partially correct the anemia. On average, the hemoglobin level will increase 1 g/dL with RBV dose reduction[66]. Finally, some patients who have co-morbid conditions, such as diabetes mellitus, coronary artery disease, and chronic obstructive pulmonary disease, might poorly tolerate even mild levels of anemia.

The recombinant erythropoietin hormone, epoetin α, has emerged as an excellent option for improving HCV treatment-related anemia while supporting optimal treatment doses of RBV and interferon. Recombinant erythropoietin hormone acts by increasing the number of erythroid progenitor cells, and has demonstrated efficacy and safety in patients with chronic renal disease, those with malignancies receiving chemotherapy. Patients receiving once weekly epoetin α (4000 unites) had significantly higher hemoglobin levels at week 16[67]. The major drawbacks of using any of the recombinant erythropoietin medications are high cost and slightly increased risk of thrombotic events.

Cardiovascular disease

HCV RNAs were found in the hearts of patients with cardiomyopathies, and negative strands of HCV RNA were also detected in the hearts, suggesting that HCV replicates in myocardial tissues[68].

A major subset of CHC patients currently considered ineligible for PEG-IFN/RBV is represented by those with co-existing clinically significant heart disease. Durante-Mangoni et al[69] prospectively evaluated safety and efficacy of PEG-IFN/RBV treatment in CHC patients with heart disease. They concluded that treatment with PEG-IFN/RBV might be safely offered to CHC patients with co-existing, clinically significant, heart disease. In qualified centers, CHC patients with overt hear disease should not be denied treatment, whenever indicated[69].

However, some patients with coronary artery disease poorly tolerate even mild levels of anemia. Hence, doses should be reduced in more than 25% of patients for both PEG-IFN/RBV to avoid the serious adverse events on the sick heart.

Renal failure

CHC and chronic renal failure might occur together because of the association of HCV infection with cryoglobulinemia and membranoproliferative glomerulonephritis, or by infection of chronic renal failure patients from exposure to HCV-contaminated blood or hemodialysis equipment.

Those with renal failure and chronic HCV infection might have significant liver disease. Although patients on dialysis tend to have milder liver disease and normal liver enzymes compared with patients with normal renal function[70], patients with end-stage renal disease (ESRD) and CHC might have severe chronic hepatitis on liver biopsy[71].

A liver biopsy should be performed in patients with CHC who are receiving hemodialysis[72] and do not have major comorbidities. Among dialysis patients who are not candidates for renal transplantation, antiviral therapy is recommended in those with fibrotic disease for viral eradication and potential reduction of the stage of fibrosis.

In candidates for renal transplantation, cirrhosis is a contraindication to renal transplantation[73]; a combined liver and kidney transplantation might be indicated in patients who progress to decompensated cirrhosis. In candidates for renal transplantation, treatment is appropriate in the pre-transplant setting[73]. Interferon therapy is ineffective and has an unacceptably high risk of precipitating rejection after transplantation. Even in patients with mild liver disease, antiviral treatment is recommended to obtain a SVR before transplantation[73], which will avoid the risk of progressive liver disease after transplantation.

Management of HCV infection associated with renal failure: RBV is cleared by the kidneys and thus contraindicated in patients with renal failure[74,75]. There is a risk of enhancement of the RBV-related hemolytic anemia, with a marked fall in hemoglobin levels. Interferon monotherapy at the standard dosing schedule of 3 million units subcutaneously three times weekly, or PEG-interferon monotherapy (α-2a or α-2b) injected once weekly, is used in patients with renal disease. The pharmacokinetics are similar to patients with renal disease down to a creatinine clearance of 20 mL/min. Trials of PEG-interferon in patients with ESRD have used either 135 mcg of PEG-interferon α-2a or 0.5-1.0 mcg/kg of PEG-interferon α-2b. In dialysis patients, the sustained virologic response achieved with interferon monotherapy is at least as good as in the general population[76]. Adverse events leading to drug discontinuation, in decreasing order of frequency, include flu-like symptoms, neutropenia, depression, and neurological symptoms. Drop-out rates seen with interferon given at 3 million units three times weekly are between 20% and 30%. PEG-interferon monotherapy trials in patients with ESRD are currently under way.

CONCLUSION

In chronic hepatitis C, control or amelioration of comorbidities before embarking on antiviral therapy represents the milestone for higher post-antiviral therapy response.

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EDITORIAL

Achalasia: A review of Western and Iranian experiences

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Abstract

Achalasia is a primary motor disorder of the esophagus, in which esophageal emptying is impaired. Diagnosis of achalasia is based on clinical findings. The diagnosis is confirmed by radiographic, endoscopic, and manometric evaluations. Several treatments for achalasia have been introduced. We searched the PubMed Database for original articles and meta-analyses about achalasia to summarize the current knowledge regarding this disease, with particular focus on different procedures that are used for treatment of achalasia. We also report the Iranian experience of treatment of this disease, since it could be considered as a model for medium-resource countries. Myotomy, particularly laparoscopic myotomy with fundoplication, is the most effective treatment for achalasia. Compared to other treatments, however, the initial cost of myotomy is usually higher and the recovery period is longer. When performing myotomy is not indicated or not possible, graded pneumatic dilation with slow rate of balloon inflation seems to be an effective and safe initial alternative. Injection of botulinum toxin into the lower esophageal sphincter before pneumatic dilation may increase remission rates. However, this needs to be confirmed in further studies. Due to lack of adequate information regarding the role of expandable stents in the treatment of achalasia, insertion of stents does not currently seem to be a recommended treatment. In summary, laparoscopic myotomy can be considered as the procedure of choice for treatment of achalasia. Graded pneumatic dilation is an effective alternative when the performance of myotomy is not possible for any reason.

INTRODUCTION

Achalasia is the most recognized primary motor disorder of the esophagus. In idiopathic achalasia, inhibitory ganglion cells in the myenteric plexuses of the esophagus undergo inflammatory degeneration. The term achalasia means "failure to relax"; the loss of ganglion cells leads to a defect in lower esophageal sphincter (LES) relaxation, which is the principal feature of idiopathic achalasia and causes functional obstruction of the esophagus, and reduced peristalsis in the esophageal body, which further impairs esophageal emptying. The functional obstruction in the LES is overcome only when the hydrostatic pressure of the retained material in the esophagus exceeds the LES pressure[1]. Secondary achalasia may occur following several conditions, which are listed in Table 1.

The reported annual incidence of achalasia is approximately 1 per 100 000[2]. Men and women are affected with equal frequency[3]. Although achalasia is diagnosed mainly in people aged between 25 and 60 years, it may occur at any age[2-4]. In general, achalasia has a subtle onset, and symptoms progress gradually. Therefore, patients experience symptoms for months or years before their disease is diagnosed. In a series of 87 patients with newly-diagnosed achalasia, the mean duration of symptoms was 4.7 years. The delay in diagnosis was due to misinterpretation of symptoms by physicians and atypical clinical manifestations[5]. Many patients are treated for other disorders such as gastro-esophageal reflux disease (GERD) before the diagnosis of achalasia is made[3]. The main symptom of achalasia is dysphagia[3]. To relieve dysphagia and other symptoms of achalasia, several treatments have been introduced,

including medical therapy, myotomy, pneumatic dilation of the LES, and injection of botulinum toxin into the LES. Early treatment of achalasia may also reduce the reported increased risk of esophageal cancer among patients with achalasia[6,7].

In this article, we report results of our review of medical literature on the subject of achalasia. Our aim is to summarize the current knowledge as regards this disease, with particular focus on different procedures that are used for treatment. Furthermore, several articles about achalasia have been published from Iran, mainly from the Digestive Diseases Research Center, Shariati Hospital of Tehran University of Medical Sciences, a nationwide referral center for the disease, where we provide follow-up care for 675 patients with achalasia. We present results of the Iranian studies, since our experience could be considered as a model for medium-resource countries.

PATHOPHYSIOLOGY

In achalasia, inhibitory ganglion cells in the myenteric plexuses of the esophagus, which produce nitric oxide, undergo inflammatory degeneration[8-10]. The remaining ganglion cells are often surrounded by lymphocytes and, to a lesser extent, by eosinophils[8,11]. Loss of inhibitory ganglion cells has two consequences: (i) basal LES pressure rises, which impairs normal relaxation of the sphincter and esophageal emptying, and (ii) the smooth muscle layer in the esophageal body loses normal peristalsis.

Several studies have suggested that a viral infection or some other environmental factor may initiate inflammation in the myenteric plexus. For example, a recent study suggested that the inflammatory reaction might be triggered by human herpes virus type 1[12]. In certain individuals, the inflammation may lead to an autoimmune response against ganglion cells. Genetic susceptibility may play a role in this process[13]. There are some reports of an association between some human leukocyte antigens and the presence of circulating antibodies to enteric neurons and the loss of ganglion cells[14]. An hereditary association between achalasia and some other conditions such as adrenal glucocorticoid deficiency and alacrima has been reported[15]. In our study on 25 children with achalasia, 2 cases (8%) had Achalasia, Alacrima, and Adrenal insufficiency (triple A syndrome or Allgrove syndrome) and 3 cases (12%) had Achalasia and Alacrima (double A syndrome). All of the 5 cases were siblings from two families[16].

CLINICAL FEATURES

The prevalence of the main symptoms of achalasia is shown in Table 2. The most common symptom of achalasia is dysphagia to solid foods (over 99%), followed by dysphagia to liquids (90% to 95%)[17]. Although dysphagia to liquids can occur in patients with other esophageal motility disorders (e.g. progressive systemic sclerosis), this symptom strongly suggests achalasia[3].

Regurgitation, active or passive, occurs in 70% to

Table 1 Causes of secondary achalasia

Malignancy, especially carcinoma
Chagas' disease
Amyloidosis
Sarcoidosis
Neurofibromatosis
Eosinophilic gastroenteritis
Multiple Endocrine Neoplasia, type 2B
Juvenile Sjögren's syndrome with achalasia and gastric hypersecretion
Chronic idiopathic intestinal pseudo-obstruction
Anderson-Fabry disease

Table 2 Prevalence of symptoms in achalasia

Symptom	Percentage (%)
Dysphagia to solids	99
Dysphagia to liquids	93
Active regurgitation	84
Passive regurgitation	68
Weight loss	61
Chest pain	59
Nocturnal cough	45
Heartburn	35
Nocturnal dyspnea	20
Hiccup	8

80% of cases. Regurgitation can be troublesome; this may lead to aspiration when patients lie down[2,3]. Weight loss, chest pain, and heartburn occur in approximately 40% to 60% of patients. Although severe weight loss can happen, the usual weight loss is 5 to 10 kg.

Occasionally, chest pain is the presenting symptom of achalasia. This is more common in younger patients, and tends to diminish with advancing age[17]. In our study of 213 achalasia patients, chest pain was the only symptom whose prevalence between the sexes was significantly different, being more common among women than men (70.9% *vs* 54.5%, $P = 0.03$, respectively)[18]. In both sexes, chest pain did not relate to the duration of symptom and the LES pressure. Chest pain was less frequently reported by patients over 56 years of age compared to those younger than 56 years ($P < 0.05$)[18]. It seems that chest pain is a distinctive symptom of achalasia which is affected by sex as well as age. Although chest pain was not improved following pneumatic dilation in some studies[17], others reported significant improvement after the procedure[18].

Heartburn occurs frequently in achalasia. Patients with heartburn have lower LES pressures than those without this symptom[19]. Heartburn may occur as a result of gastro-esophageal reflux or other causes, such as direct irritation of the esophageal mucosa by foods, pills, and the lactate produced by bacterial fermentation of retained carbohydrates[19,20]. Hiccup is also a frequent symptom in achalasia, partly because of the obstruction of the distal esophagus[21]. Functional and structural abnormalities of the lung, such as tracheo-bronchial compression and abnormalities on high-resolution CT-scan, may occur in half of the patients[22]. The frequency

Table 3 Frequency of cardinal symptoms in achalasia			
Symptom	**Frequency (%)**		
	Each meal	Daily	Weekly	None
Dysphagia to solids	84	13	2	1
Dysphagia to liquids	60	25	8	7
Active regurgitation	33	36	15	16
	Daily	Weekly	Monthly	None
Passive regurgitation	20	44	4	32
Chest pain	12	39	8	41

of cardinal symptoms in achalasia is shown in Table 3.

Since patients with achalasia may experience retrosternal fullness following a meal, they may eat more slowly and induce regurgitation to relieve the feeling. They may perform some maneuvers to augment esophageal emptying, such as lifting the neck or throwing the shoulders back[3].

Compared to the general population, patients with achalasia are at substantially increased risk, even as high as 33-fold, of developing esophageal cancer. The cancer typically is of squamous cell type[6,7]. However, some series did not find any increase in the risk, particularly with early treatment of achalasia. We followed up 365 patients with achalasia for a mean duration of 43 mo; no case of esophageal cancer was identified. This may be related to the fact that our study participants were fairly young (mean age, 38 years) and the duration of follow-up was not very long[23].

DIAGNOSIS

Diagnosis of achalasia is based on clinical findings. The diagnosis is confirmed by radiographic, endoscopic, and manometric evaluations.

Radiography

The usual findings on a plain chest X-ray are widening of the mediastinum, due to esophageal dilation, and absence of the normal gastric air. When achalasia is suspected, barium swallow is the primary screening test. Barium swallow typically shows a dilated esophagus that terminates in a beak-like narrowing as a result of contraction in the LES. When dilation is very severe, the esophagus may have a sigmoid shape[3]. The overall sensitivity of barium swallow for diagnosis of achalasia is approximately 95%[24], but in early stages of the disease it may be reported as normal. For example, in a prospective study achalasia was suggested by barium examination in only 21 out of 33 patients who eventually were diagnosed with achalasia[2].

The timed barium esophagogram, which assesses esophageal emptying at 1, 3 and 5 min after swallowing of barium, can be more helpful than usual barium swallow. Vaezi et al[25] reported that assessing both symptom improvement and objective improvement in esophageal emptying can better identify the response rate to pneumatic dilation and need for repeated dilations in the future. In their study of 37 patients, there was a significant

association ($P < 0.001$) between improvement in patient symptoms and barium height. In 38 out of 53 (72%) pneumatic dilations, the degree of symptom and barium height improvement was comparable. In 8 out of 26 (31%) patients, however, there was < 50% improvement in barium height despite near complete symptom resolution. Age was the only difference between the groups and patients with improvement in both symptoms and barium height, i.e. the first group, were significantly older than the second. They concluded that the timed barium esophagogram before and after dilation may identify a subset of patients with poor esophageal emptying but with good improvement in symptoms who may benefit from early repeated pneumatic dilation[25]. Similarly, Chuah et al[26] found in their study of 32 patients with achalasia who received pneumatic dilation that the timed barium esophagograms correlated with symptomatic improvement in up to 71% of patients, although seven patients who noted complete symptom resolution showed less than 50% improvement in barium column height and esophageal diameter. In a study of 52 patients, we also found that the volume of barium retention at 5 min could predict the LES pressure before and after balloon dilation in achalasia[27], and in a study of 43 patients, surface area of barium retention at 5 min appeared to be an even better predictor for resting LES pressure[28]. In a randomized clinical trial of 51 patients who underwent surgery or pneumatic dilation, results of the timed barium esophagogram also correlated well with outcome. Poor improvement in barium height following the treatments was associated with an increased risk of treatment failure[29].

Manometry

Manometry is the most sensitive tool for diagnosis of achalasia. Elevated resting LES pressure (usually > 45 mmHg), incomplete LES relaxation, and aperistalsis in the smooth muscle portion of the body of the esophagus are three characteristic manometric features of achalasia[30]. Swallows may be followed by either no esophageal contraction or simultaneous contractions. Simultaneous contractions may also occur spontaneously. Another common feature is that resting pressure in the body of the esophagus is slightly higher than in the stomach[3].

In most patients the amplitude of esophageal contractions is low. On the other hand, in vigorous achalasia the simultaneous esophageal contractions have high amplitudes (e.g. > 60 mmHg). Some studies have suggested that vigorous achalasia may represent an early form of achalasia in which some inhibitory ganglion cells may not yet be destroyed[8], and that patients with vigorous achalasia may benefit more from botulinum toxin injection than those with classic achalasia[31]. At present, however, the distinction between vigorous and classic achalasia seems to have little clinical significance.

Endoscopy

Endoscopy in achalasia typically reveals a dilated

esophagus that often contains retained material. The esophageal mucosa usually appears normal, although inflammation and ulceration may result from chronic inflammation caused by retained food or pills. Endoscopy may be reported as normal if it is carried out in the early stages of the disease or it is not performed by experienced endoscopists[2]. Achalasia at early stages may also be misdiagnosed as GERD. Food stasis and GERD are main factors contributing to esophageal mucosal inflammation in achalasia. The association between endoscopic food stasis and histological inflammation is significant, but endoscopic signs of esophagitis and histological inflammation are poorly associated. Because of low sensitivity of endoscopy to detect inflammation, surveillance endoscopy with biopsy sampling and assessment of stasis is warranted to detect early neoplastic changes[32]. The stasis may predispose the esophagus to *Candida* infection.

Although the LES in achalasia is contracted, the endoscope can usually be traversed easily into the stomach aided by gentle pressure on the scope[3].

Endoscopic ultrasonography (EUS) may show widening of the mean longitudinal and circular smooth muscle layers of the LES; however, this finding is not specific for achalasia[33]. We compared the esophageal muscularis propria thickness in achalasia patients with a control group using EUS and assessed the relationship between EUS findings and demographic features in both groups. The esophageal muscular layer was significantly thicker in patients with achalasia compared to control group ($P < 0.05$). Among patients with achalasia, the thickness at 5 and 10 cm above the gastro-esophageal junction appeared to be correlated with age, being higher among older people[34].

Clinical and endoscopic features of some other conditions, such as neoplasms, may be similar to those of achalasia. Since gastric adenocarcinoma is the most common neoplasm associated with pseudo-achalasia, the esophago-gastric junction and the gastric fundus should be carefully examined for any evidence of neoplasm. With certain features, malignancy is more likely: duration of symptoms less than 6 mo; presentation after the age of 60 years; excessive weight loss in spite of short duration of symptoms; difficult passage of the endoscope through the gastro-esophageal junction[35]. In these cases, repeated evaluations and biopsies are recommended.

Symptomatic scoring

Several scoring system have been proposed to evaluate the severity of the symptoms in achalasia. One of the scoring systems is shown in Table 4. In this scoring system, scores for the following five symptoms; dysphagia to solids, dysphagia to liquids, passive regurgitation, active regurgitation, and chest pain are summed up to calculate the total score. In a study of 116 patients with achalasia, we found a good correlation between this score and LES pressure ($r = 0.29$, $P < 0.01$)[36]. Among the main symptoms, active and passive regurgitation and dysphagia to liquids were significantly correlated to the

Table 4 Scoring system for evaluation of clinical symptoms

Symptom	Score by frequency of symptoms			
	Each meal	Daily	Weekly	None
Dysphagia to solids	3	2	1	0
Dysphagia to liquids	3	2	1	0
Active regurgitation	3	2	1	0
	Daily	Weekly	Monthly	None
Passive regurgitation	3	2	1	0
Chest pain	3	2	1	0

LES relaxation pressure ($P = 0.001$, 0.002, and 0.046, respectively)[36].

TREATMENT

The mainstay of therapy is to reduce LES pressure in order to improve esophageal emptying by gravity. Several therapeutic modalities have been introduced to achieve this goal, including medical therapy, surgical myotomy (open or laparoscopic), pneumatic dilation of the LES, injection of botulinum toxin into the LES, and insertion of self-expanding stents. There are 3 recent meta-analyses of publications which have investigated different treatment approaches. One of these included 105 articles involving 7855 subjects, and investigated symptom relief, prevalence of gastro-esophageal reflux, and complications following treatments[37]. Pneumatic dilation was more successful in symptom relief than botulinum toxin injection (68% vs 41%, $P = 0.02$, respectively). Symptom relief with laparoscopic myotomy plus an anti-reflux procedure was better (90%) than with all other treatments. Furthermore, complication rate was low with this method (6.3%)[37]. Likewise, another meta-analysis of randomized and controlled treatment trials, which included 17 articles with 761 participants and investigated remission and relapse rates and complications, found a better remission rate and lower relapse rate for laparoscopic myotomy compared to other treatments. There was no difference between open and laparoscopic myotomy, in the only trial that compared these two methods, regarding remission and relapse rates. Remission rate following pneumatic dilation was higher than after botulinum toxin injection[38]. A meta-analysis of controlled and uncontrolled studies in the Chinese literature (43 articles with 1791 participants) also showed that myotomy was associated with higher initial and long-term remission rates than pneumatic dilation or botulinum toxin injection. Only 2 studies compared open myotomy with laparoscopic myotomy; there was no difference in remission rate[39]. Since results after any treatment may deteriorate over time, life-long follow-up and objective assessment of the results are recommended.

Medical therapy

Nitrates and calcium channel blockers (e.g. nifedipine) relax the smooth muscles of the LES[40]. These medications are usually taken sublingually 10 to 30 min before meals. Pharmacotherapy for achalasia, however, is often ineffective and frequently associated with side

effects (e.g. headache, hypotension, and tachyphylaxis). Therefore, nitrates and calcium channel blockers are primarily used for patients who are unwilling to undergo or unable to tolerate more effective, invasive forms of therapy[3].

Surgical myotomy

Surgical myotomy was first introduced by Ernst Heller in 1913. Nowadays, a modified technique is commonly used[41]. The standard "open" myotomy can be performed using either an abdominal or, more commonly, a thoracic approach[42,43]. More recently, laparoscopic and thoracoscopic techniques have been used to perform myotomy[39,44-46].

The modified Heller approach results in good to excellent relief of symptoms in 70% to 90% of patients with few serious complications. The mortality rate (approximately 0.3%) is similar to that reported for pneumatic dilation[42]. The major disadvantages of surgery are the high initial cost, long recovery period, and the frequent development of GERD. Reflux esophagitis develops in approximately 10% of patients treated by surgical myotomy[42]; however, the efficacy of proton pump inhibitor treatment minimizes its clinical significance. There is a debate with regard to the need for additional fundoplication in open myotomy. In some studies, gastro-esophageal reflux was relatively frequent even after combining myotomy with anti-reflux procedures[47,48].

There is increasing experience with Heller myotomy performed by minimally invasive techniques (laparoscopy or thoracoscopy), and these techniques have become the procedure of choice by many experienced surgeons for uncomplicated cases in Western countries. These approaches in several trials were more successful in symptom relief than other treatments[37]. They are associated with few major complications[49] and shorten the duration of hospitalization and recovery[44,45]. Some pre- and postoperative findings may be helpful in predicting the outcome. In a study of 407 patients who underwent laparoscopic myotomy, high preoperative LES pressure (> 30 mmHg) was a predictor of a good response, while severe chest pain and the presence of a decompensated sigmoid esophagus (class IV) was associated with poor outcome[50]. In a study of 200 patients who underwent laparoscopic or thoracoscopic myotomy plus a partial fundoplication, low LES pressure, presence of sigmoid esophagus, and longer duration of symptoms were associated with failure of treatment in long-term follow-up[51]. Preoperative LES pressure over 35 mmHg was also a strong predictor of excellent postoperative relief in dysphagia in another study of 200 patients[52].

Objective analyses have shown a high rate of gastro-esophageal reflux in laparoscopic myotomy without an anti-reflux procedure. In a study of 50 patients with achalasia who underwent laparoscopic Heller myotomy without anti-reflux procedures, significant heartburn was reported in 30% of cases. Twenty four-hour pH monitoring revealed abnormal findings in 11 out of 22 patients tested[53]. However, use of a fundoplication procedure with laparoscopic myotomy reduced the rate of gastro-esophageal reflux (8.8% with a fundoplication vs 31.5% without a fundoplication, P = 0.003)[37]. In a study of 20 patients who underwent laparoscopic myotomy and fundoplication, 24-h combined multichannel intra-luminal impedance and pH monitoring did not show any evidence of postoperative pathologic reflux in both upright and recumbent positions[54]. Few randomized trials have investigated the efficacy of different fundoplication techniques used with laparoscopic myotomy in treatment of achalasia. In a randomized, controlled study of 144 patients who underwent laparoscopic myotomy, the outcome when using Dor versus Nissen fundoplication was investigated. Both techniques were successful in long-term control of gastro-esophageal reflux, but the recurrence rate of dysphagia was higher with the Nissen method[55]. Based on the published literature, Dor fundoplication seems to be performed more commonly as the anti-reflux procedure during laparoscopic myotomy than other fundoplication techniques.

Pneumatic dilation

Although some studies reported similar short- and long-term efficacy for myotomy and pneumatic dilation, particularly with graded dilation (1-3 dilations with progressively larger balloons)[56,57], as mentioned earlier, myotomy has been shown to be a more effective treatment for achalasia in several trials[37,38]. However, pneumatic dilation is less expensive than myotomy and still improves symptoms in a substantial number of patients[58-60]. In a meta-analysis of uncontrolled studies, a single pneumatic dilation was found to be effective in 72% of patients during a mean follow-up of 4.9 years[61]. In a study of 150 patients with achalasia, pneumatic dilations were performed until remission was achieved or symptoms recurred, using an "on-demand strategy" based on symptom recurrence, and a long-term remission was achieved in nearly all patients[60]. Pneumatic dilation can also be applied for some patients in whom dysphagia persists after surgery[62,63]. In a study of 27 patients with recurrent dysphagia following surgery, pneumatic dilation improved symptoms in 76% of the patients[63]. If pneumatic dilation fails, laparoscopic myotomy with fundoplication can be performed; the outcome is not affected by previous pneumatic dilation[50,64].

A number of different balloon dilators have been used over the years. A systematic review of the treatment of achalasia compared the results of using different dilators; pneumatic dilation was performed in 2418 patients with "old" dilators, in 234 patients with the "new" Witzel dilator, and in 359 patients with the Rigiflex dilator[65]. Using old dilators and Witzel dilators, two-thirds of patients had good to excellent improvement after one or more dilations during a mean follow-up of 4.6 years and one year, respectively. Using Rigiflex dilators, an equivalent improvement was achieved in up to 90% of patients, depending upon the diameter of the dilator used (74% for 3.0 cm, 86% for 3.5 cm, and 90% for 4.0 cm)[65]. At present, the most popular pneumatic dilator is the Rigiflex balloon, which is passed over a guidewire and positioned

fluoroscopically or endoscopically in the LES. This balloon is available in three different sizes (3.0, 3.5, and 4.0 cm). The thinnest balloon is typically used in the first dilating session. The standard approach to balloon dilation is one dilatation per session; further need for dilation is based on the symptomatic response. Patients are usually referred to a surgeon if three consecutive dilations over a few months do not provide clinical remission. In long-term follow-ups, more than three sessions can be applied if symptoms recur.

In our center we perform all dilations with the Rigiflex balloon dilator. After a clear liquid diet for 24 h and an overnight fast, patients receive intravenous diazepam (5-10 mg) and meperidine (25-50 mg). A guidewire into the stomach is placed under endoscopic visualization. In the first dilating session, a 3.0 cm balloon dilator is passed over the guidewire under endoscopic guidance. The midpoint of the balloon is positioned at the LES. The balloon is gradually inflated to 6 pounds per square inch (psi) over 20 s and then to 8 psi for the next 20 s and finally to 10 psi for 60 s. Then the balloon is deflated and removed along with the guidewire. Patients are discharged after a 6-h observation period. If severe or sustained chest pain occurs, a gastrografin swallow is performed to rule out perforation.

Using the above method we conducted a study of 99 patients to assess therapeutic outcome after pneumatic dilation. Initially, all symptomatic patients underwent pneumatic dilation with a 3.0 cm balloon. If symptoms recurred, dilation was repeated with a 3.5 cm balloon. In the case of further relapse, a third dilation was carried out with a 4 cm balloon. The patients were followed for an average length of 47 (range, 18 to 60) mo. Dilation was repeated in 35 patients; only 6 of them required a third dilation. After the third dilation two patients did not display improvement and underwent myotomy. Over the study period, cumulative remission rate was 65% without re-dilation and 94% with re-dilation. The mean remission period was 44.7 mo[66].

To address the optimal method for performing pneumatic dilation, regarding the amount and rate of inflation pressure and balloon diameter, we conducted a large long-term prospective study and enrolled 262 achalasia patients over 10 years. In the first 62 patients (group A), dilation was done using a 3.5 cm balloon, which was inflated to the pressure of 10 psi over 10 s. In group B (200 patients), we initially used a 3.0 cm balloon with inflation pressure of 10 psi in 30 s. We used a Rigiflex balloon and maintained pressure for 60 s after inflation in both groups. If symptoms recurred, dilation was repeated with incrementally larger balloons (for second dilation, 4.0 cm in group A and 3.5 cm in group B; for third dilation, 4.0 cm in both groups). The cumulative proportional remission rates with single dilation after 6 mo were 83% and 75% in groups A and B, respectively; the corresponding rates decreased to 60% and 57%, respectively, after 30 mo. The difference between the 2 methods was not statistically significant. The remission rate following re-dilation was good; 1 year after the second dilation, it was 88% in group A and 89% in group B,

and 2 years after the second dilation, it was 70% in both groups. All perforations ($n = 3$) occurred in group A at the first dilation (62 dilations) with rapid inflation of balloon (10 psi over 10 s); while there was no perforation in group B (296 dilations), in which gradual increasing of pressure (10 psi over 30 s) and graded dilation method was used[67].

In another Iranian study, 45 patients who underwent pneumatic dilation were compared with 19 patients who underwent open myotomy. Good to excellent relief was achieved in 68% of patients with myotomy and 80% of patients with pneumatic dilation. After over 2 years of follow-up, relapse rates in both groups were not significantly different (39% in surgery group and 25% in pneumatic dilation group). The mean length of hospital stay and days off from work were significantly lower in the pneumatic dilation group; these were discovered to be 9 and 39 d in the myotomy group and 1 and 2 d in the pneumatic dilation group, respectively[57].

Some predictors for the outcome of pneumatic dilation have been suggested, including age of patients[68,69] and a decrease in LES pressure following dilation[69]. In a study of 111 patients, short- and long-term remission rates were good (98% and 75% at months 24 and 60, respectively), but young age ($\leqslant 37.5$ years), high esophageal body pressure, and high LES pressure ($\geqslant 17.5$ mmHg) following first dilation were negative predictive factors. Young patients who required more than 2 dilations seemed not to benefit from this kind of treatment[70]. We did not find any significant association between age, gender, previous treatment, or severity of initial symptoms and the outcome of pneumatic dilation ($P > 0.4$)[66].

Esophageal perforation is the most important complication of pneumatic dilation. It occurs in approximately 3% to 5% of patients in most series, although the range varies from 0% to 21%[3,71]. Patients with esophageal perforation usually present in the first hours after dilation. A high index of suspicion should be maintained in patients complaining of sustained pain or discomfort after the procedure. Some patients respond to conservative treatment with antibiotics and parenteral nutrition but others need a surgical repair. Other complications of pneumatic dilation include development of intramural hematomas, esophageal mucosal tears, and diverticula at the gastric cardia[72]. Severe transient and intermittent post-procedural chest pain has been reported in approximately 15% of patients during the 24-48 h after dilation[73,74]. Although this symptom is disturbing, it is not harmful.

Botulinum toxin injection
Botulinum toxin is a potent inhibitor of acetylcholine release from nerve endings. The toxin theoretically relaxes the LES by decreasing unopposed cholinergic stimulation of the LES[75]. Although the effect of botulinum toxin injection generally is shorter than some other procedures, it can be useful under certain conditions. In patients with multiple medical problems who are poor candidates for more invasive procedures, as well as those unwilling to undergo either surgery or pneumatic dilation, botulinum toxin injection is the preferred approach. Older patients

and those who suffer from vigorous achalasia may benefit more from botulinum toxin[76]. A multicenter randomized study suggested that dose of botulinum toxin may be a predictor of outcome: the higher the dose, the better the response[77].

The use of botulinum toxin in achalasia was first introduced by Pasricha et al[78]. Several studies indicate that 65% to 90% of patients respond to a single injection within 1 mo. The effect of botulinum toxin lasts from 3 mo to more than one year[79-82]. Those who respond to the injection may do equally well after a second or even a third injection. In one series, for example, symptom relief was achieved in 75% of 57 patients who received repeated injections, as needed, during up to 2 years follow-up[31]. However, the effect decreases over time; some studies reported clinical remission rates of 50% and 30% at 6 and 12 mo, respectively, following botulinum injection[83]. Although a few studies reported that botulinum toxin injection could provide an efficacy equal to that of pneumatic dilation over a one year period[84], several randomized clinical trials have shown that while initial symptomatic remission rates by pneumatic dilation and botulinum injection may be similar in some cases, pneumatic dilation is associated with a significantly higher long-term remission rate[85,86]. In some studies, only pneumatic dilation was associated with improvement in objective measures of esophageal function, including esophageal manometry and barium studies[85,87]. In our trial of 40 patients, pneumatic dilation was more efficient than botulinum injection in providing sustained symptomatic relief over a 12 mo period. The remission rates for pneumatic dilation and botulinum injection after 12 mo were 52% and 15%, respectively[88].

Several formulations of botulinum toxin are available. A comparison between 100 U of Botox and 250 U of Dysport showed a similar efficacy for up to 6 mo of follow-up[89]. In the most common method for injection therapy, 1 mL aliquots (20 to 25 units/mL) of the toxin are injected into each of four quadrants, approximately 1 cm above the Z line, using a standard sclerotherapy needle. We use and recommend the following method. After an overnight fast, patients are sedated with intravenous diazepam (5-10 mg) and meperidine (25-50 mg). The LES is identified by visualization of the sphincter rosette at the squamo-columnar junction during upper gastrointestinal endoscopy. Four hundred units of Dysport are diluted in 4 mL normal saline. Two 50-unit aliquots (0.5 mL) of Dysport are injected through a 5 mm sclerotherapy needle into each quadrant of the LES. Patients are discharged when routine post-sedation care is completed and allowed to eat later on the same day. Improvement in symptoms is usually observed after only 24 h; peak effects occur even later in some patients.

Reported complications after botulinum toxin are not major and include post-procedural transient chest pain (25%) and heartburn (5%)[90]. Transient chest pain, the main complication, can be controlled by sedatives. Neutralizing antibodies have been detected in approximately 5% of patients treated chronically with botulinum toxin for skeletal muscle conditions; however,

their significance in relapse of dysphagia in achalasia is uncertain. These antibodies, however, might be a possible cause of the rapid relapse of dysphagia following botulinum toxin injection[91]. Surgical treatment of achalasia in patients who previously received botulinum toxin may encounter some technical problems, but no significant difference in the outcome between patients with and without previous use of the toxin has been reported[92].

Pneumatic dilation after botulinum toxin injection

Only a few studies have investigated the effect of botulinum toxin injection on the outcome of pneumatic dilation. In a retrospective study of the effect of the combined therapy, we studied 12 patients who underwent dilation following botulinum toxin injection and 12 patients with achalasia who underwent only pneumatic dilation (control group). With combined therapy, only one of the patients relapsed 30 mo after dilation, while all the others were in remission for an average of 25.6 mo. In the control group, all the patients relapsed after a mean period of 12.6 mo and needed further dilation. The cumulative remission rate was significantly higher in the combined therapy group than in the control group ($P < 0.01$). One month after dilation, the mean symptom score decreased by 76% in the combined therapy group and by 53% in the control group. Age, sex, duration and severity of symptoms were not correlated with response to treatment[93].

We also conducted a prospective trial. Twenty seven patients were randomly assigned to receive botulinum toxin 1 mo before pneumatic dilation and 27 patients were assigned to undergo pneumatic dilation alone. One-year remission rates of patients in the botulinum toxin-pneumatic dilation group and the pneumatic dilation group were 77% and 62%, respectively ($P = 0.1$). In the pneumatic dilation group, the esophageal barium height significantly decreased at 1 mo ($P < 0.001$), but this reduction did not persist over 1-year follow-up. The botulinum toxin-pneumatic dilation group showed a significant reduction in barium height at both 1 mo and 1 year after treatment ($P < 0.001$). In the botulinum toxin-pneumatic dilation group, 91% of patients older than 40 years were in remission at 1 year, compared with only 55% of this age group in the pneumatic dilation group ($P = 0.07$)[94].

We found an abstract in English that reported a series of 9 patients with achalasia who were treated with application of 250 IU Dysport into the LES and balloon dilation 7 d later. Two patients underwent myotomy because of poor relief of symptoms. Seven other patients, however, were in good symptomatic remission after one year. The remission was even observed for as long as 36 mo, which was the longest follow-up period[95].

Insertion of stents

Only a few studies have investigated the role of expandable stents in treatment of achalasia[96-99]. The results are controversial. Therefore, insertion of stents does not seem to be a currently recommended treatment for achalasia.

CONCLUSION

First stages of achalasia may be misdiagnosed as other diseases, such as GERD. Myotomy, particularly laparoscopic myotomy with fundoplication, is the most effective treatment for achalasia and can be considered as the procedure of choice. Compared to other treatments, however, the initial cost of myotomy is usually higher and the recovery period, particularly following open myotomy, is generally longer. When performing myotomy is not possible for any reason, e.g. medical contraindication, patient's unwillingness, when patients cannot afford surgery, or experienced centers for surgery or post-operative care are not easily accessible (situations that may not be rare particularly in some low- or medium-resource countries), graded pneumatic dilation (using 3.0 cm balloons initially) with slow rate of balloon inflation seems to be an effective and safe initial alternative. The duration of remission can be extended by repeated dilation with larger-sized balloons. Injection of botulinum toxin into the LES before pneumatic dilation seems to increase remission rates. However, this needs to be confirmed in further studies. The timed esophagogram may be used as a non-invasive objective tool for initial and post-operative or post-dilation assessment.

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REVIEW

Treatment modalities for hypersplenism in liver transplant recipients with recurrent hepatitis C

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Abstract

Hepatitis C is the most common indication for orthotopic liver transplantation in the United States. Unfortunately, hepatitis C recurs universally in the transplanted liver and is the major cause of decreased graft and patient survival. The combination therapy of interferon and ribavirin has been shown to be the most effective therapy for recurrent hepatitis C. However, pre- and post-transplant hypersplenism often precludes patients from receiving the antiviral therapy. Splenectomy and partial splenic embolization are the two invasive modalities that can correct the cytopenia associated with hypersplenism. In this report we review the two treatment options, their associated outcomes and complications.

Key words: Hypersplenism; Leukopenia; Recurrent hepatitis C; Thrombocytopenia; Liver transplant

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INTRODUCTION

Liver disease caused by chronic hepatitis C virus (HCV) infection is the most common indication for liver transplantation in the United States. Unfortunately, HCV is universally recurrent in the transplanted liver and is a major cause of graft failure and decreased patient survival[1]. 10%-30% of patients who have recurrent infection develop advanced fibrosis or cirrhosis within the first 5 years post-transplantation[2,3].

The combination therapy of interferon and ribavirin has been shown to be the most effective therapy for HCV recurrence after liver transplantation with sustained virologic response rates between 20%-40%[4]. Further studies are needed to determine whether treatment should be started preemptively, at the time of acute hepatitis, or at the early stages of chronic hepatitis in the graft. The ability to treat patients with adequate doses of interferon and ribavirin or even to initiate treatment is often limited by leucopenia, anemia, and thrombocytopenia. Chang *et al*[5] reported in their study that almost 50% of patients (109 out of 216) had a platelet count below $50\,000/\mu L$ before liver transplantation. At one year of follow-up, 21% of transplanted patients (45 out of 216) continued to have moderate to severe thrombocytopenia. Clinical factors associated with sustained thrombocytopenia were pretransplant severe thrombocytopenia ($< 50\,000/\mu L$) and pretransplant large spleen volume (> 2000 mm^3).

Splenectomy and partial splenic embolization represent two interventional therapies to improve thrombocytopenia which could potentially allow treatment of HCV. In this review we examine the effectiveness and risks of these approaches in liver transplant patients.

SPLENECTOMY

Splenectomy has been performed routinely in the past in liver and kidney transplant patients for immunologic reasons. It allowed patients to tolerate azathioprine therapy for episodes of rejection[6]. This practice continued

until the early 1980s when cyclosporine was introduced. Current accepted indications for post-transplant splenectomy include recurrent ascites, splenic infarction, large aneurysms of the splenic artery, thrombocytopenia secondary to hypersplenism prior to or after liver transplantation, and small-for-size syndrome in recipients of living donors with associated thrombocytopenia and impaired liver function. Splenectomy was also performed at the time of liver transplantation for ABO incompatibility and preemptive HCV treatment with interferon and ribavirin in thrombocytopenic patients[7-9]. Tashiro *et al*[10] advocated performing concurrent splenectomy with liver transplantation in all patients who had a pre-transplant platelet count of less than 60 000 mm^3 so that this group of patients could tolerate preemptive administration of combination therapy in the post-transplant period.

Although successful results have been reported, splenectomy is potentially associated with multiple complications. It is an invasive procedure that can be technically difficult, with a high risk of bleeding in patients with portal hypertension, varices, and enlarged spleen. Portal vein thrombosis and pancreatic leaks requiring surgical reexploration have been described as complications[9,11]. However, in our opinion, the risk of infection post-splenectomy is the most serious and potentially life-threatening complication in the immunosuppressed population. Troisi *et al*[12] reported that 4 out of 10 liver transplant patients who underwent splenectomy developed sepsis, which led to their demise. Samimi *et al*[13] reported 17.5% *vs* 2.7% one-month and 30% *vs* 11.5% one-year sepsis-related mortality in patients who underwent concomitant splenectomy with liver transplantation *vs* those who underwent liver transplantation alone. Neumann *et al*[14] reported an increased risk for opportunistic pneumonia in patients who underwent simultaneous splenectomy and liver transplant. Splenectomy also places patients at risk for overwhelming post-splenectomy sepsis syndrome (OPSS), usually due to encapsulated organisms. It is recommended by the Center for Diseases Control to immunize patients prior to splenectomy to decrease the risk of OPSS from *Streptococcus pneumonia*, *Haemophilus influenzae* type B, and *Neisseria meningitides*[15]. According to guidelines issued by the American Society of Transplantation in 2004, these vaccines are administered to all candidates prior to liver transplantation[16]. Unfortunately, the response rate is only 40%-80%[17,18].

Despite vaccinations, fulminant bacterial sepsis carries a high risk of morbidity and mortality, especially in immunocompromised patients. The risk is greatest in the early months and years after splenectomy, but a period of as long as 45 years after the procedure has been reported in the literature[19].

PARTIAL SPLENIC ARTERY EMBOLIZATION

Partial splenic embolization is a non-surgical, less invasive treatment of hypersplenism. It is usually performed *via* a percutaneous femoral artery approach. The embolization catheter is advanced into the splenic hilum as far as possible in order to avoid injury to the pancreatic circulation. Gelatin sponge slurry suspended in an antibiotic solution, coils, microspheres, and polyvinyl alcohol particles are used for embolization of approximate 60%-70% of spleen parenchyma. Splenic embolization procedures date back to 1973, when the entire spleen parenchyma was ablated. At that time the procedure was associated with high rates of complications, including splenic abscesses, rupture, and pancreatic infarction, resulting in a high mortality rate[20]. Subsequently, this procedure became more successful with selective ablation of the spleen. In 1984, Mozes *et al*[21] showed in a prospective randomized trial that partial splenic embolization was as effective as splenectomy for treatment in renal transplant candidates on hemodialysis with a low platelet count prior to administration of immunosuppression. In this study excessive infarction of the spleen was avoided with a mean of 65% of spleen parenchyma ablated. Partial splenic embolization is an effective method to reduce the platelet pool and improve platelet count and is greatly dependent on the infarcted splenic volume. Hayashi *et al*[22] reported that splenic volume was the best predictive factor for increase in platelet count at one month and one year in patients with liver cirrhosis and hypersplenism.

In several reports in the literature, partial splenic embolization has been described in patients after liver transplantation. It has been successful in patients with thrombocytopenia and recurrent HCV who were able to undergo treatment with interferon and ribavirin as a result of ablation[23-25].

Most patients develop post-embolization syndrome, including symptoms of fever, left upper quadrant pain, pleural effusion, pneumonia, and atelectasis. Splenic abscesses and rupture are infrequent and are more commonly encountered and less tolerated by immunocompromised cirrhotic patients with a greater area of embolization[26]. The risk is greatly reduced with aseptic technique, antibiotic prophylaxis, and careful control of pain. Extent of embolization is important as well, with more complications following greater than 70% area of ablation. In partial splenic embolization, achieving the intended target embolization area remains challenging. Graded partial splenic embolization at several settings has been entertained in order to avoid excessive embolization and severe complications associated with it[27].

DISCUSSION

Hepatitis C is the most common indication for liver transplantation in the United States and Europe, but unfortunately the virus almost always recurs with up to a third of patients developing cirrhosis within the first 5 years. Interferon and ribavirin therapy has been widely accepted as the treatment for recurrent disease. Cytopenia, including thrombocytopenia, which often

afflicts liver transplant patients, leads to failure to initiate this antiviral regimen, dose reduction, or discontinuation of therapy, which ultimately decreases the likelihood of sustained virological response[28].

Although, hemolytic growth factors, such as erythropoietin and growth colony stimulating factors, are used to counter the anemia and neutropenia associated with interferon and ribavirin treatment, there is no approved therapy for low platelet count in HCV infected patients.

A new group of synthetic thrombopoietic agents, including romiplostim and eltrombopag, have been found to be effective in stimulating platelet production[29]. In 2008, romiplostim was approved by the FDA for the treatment of thrombocytopenia in patients with chronic idiopathic thrombocytopenic purpura (ITP). These agents are now being investigated in clinical trials for the treatment of thrombocytopenia in cirrhotic patients with hepatitis C infection[30,31].

CONCLUSION

Currently, splenectomy is the more popular choice of treatment for hypersplenism and thrombocytopenia. The question is whether it is the optimal choice. Partial splenic embolization is an alternative option that is often overlooked. It is less invasive and potentially carries fewer infectious complications since there is a remnant of functional splenic parenchyma remaining after the procedure. Although it diminishes with time, the risk of OPSS in asplenic patients is life-long. It carries a high mortality rate and therefore, we feel, other options should be seriously considered. Thus, further prospective studies are needed to investigate both modalities in this select group of patients.

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REVIEW

Steatosis and insulin resistance in hepatitis C: A way out for the virus?

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Abstract

The hepatitis C virus (HCV) induces lipid accumulation *in vitro* and *in vivo*. The pathogenesis of steatosis is due to both viral and host factors. Viral steatosis is mostly reported in patients with genotype 3a, whereas metabolic steatosis is often associated with genotype 1 and metabolic syndrome. Several molecular mechanisms responsible for steatosis have been associated with the HCV core protein, which is able to induce gene expression and activity of sterol regulatory element binding protein 1 (SREBP1) and peroxisome proliferator-activated receptor γ (PPARγ), increasing the transcription of genes involved in hepatic fatty acid synthesis. Steatosis has been also implicated in viral replication. In infected cells, HCV core protein is targeted to lipid droplets which serve as intracellular storage organelles. These studies have shown that lipid droplets are essential for virus assembly. Thus, HCV promotes steatosis as an efficient mechanism for stable viral replication. Chronic HCV infection can also induce insulin resistance. In patients with HCV, insulin resistance is more strongly associated with viral load than visceral obesity. HCV seems to lead to insulin resistance through interference of intracellular insulin signalling by HCV proteins, mainly, the serine phosphorylation of insulin receptor-1 (IRS-1) and impairment of the downstream Akt signalling pathway. The HCV core protein interferes with *in vitro* insulin signalling by genotype-specific mechanisms, where the role of suppressor of cytokine signal 7 (SOCS-7) in genotype 3a and mammalian target of rapamycin (mTOR) in genotype 1 in IRS-1 downregulation play key roles. Steatosis and insulin resistance have been associated with fibrosis progression and a reduced rate of sustained response to peginterferon plus ribavirin.

Key words: Steatosis; Insulin resistance; Hepatitis C virus

INTRODUCTION

Hepatocyte steatosis, defined as accumulation of fat droplets in hepatocytes, is a histological feature of a group of liver diseases including not only metabolic or alcoholic disorders but also chronic hepatitis C virus (HCV) and drug-induced liver disease[1,2]. Steatosis is a very common lesion in chronic HCV, seen in more than half of patients, with a prevalence of 40% to 86% according to the genotype. The majority of patients show simple steatosis, but features of non-alcoholic steatohepatitis have been found in approximately 10% of patients with chronic HCV[3]. Two main types of steatosis have been defined in HCV: metabolic steatosis found in patients infected by genotype 1 and associated with metabolic syndrome, and viral steatosis reported in patients with genotype 3a, without other known steatogenic cofactors and directly linked to a cytopathic effect of the virus. In the same way, chronic HCV infection can also induce insulin resistance[4,5]. Epidemiological data support an association between HCV infection and a risk for the development of type 2 diabetes mellitus, both in cross-sectional and longitudinal cohorts. The majority of

transgenic mice expressing the HCV core protein developed insulin resistance and type 2 diabetes. Indeed, patients with HCV showed a higher insulin resistance index than healthy controls or patients with liver diseases other than HCV matched by sex, body mass index and age. Insulin resistance could be promoted by viral proteins and the intrinsic mechanism seems to be genotype-specific[5]. In this sense, HCV replicons - genomic or subgenomic constructs expressing the viral replicase complex and capable of autonomous viral replication - constitute a powerful tool to investigate the molecular mechanism leading to steatosis and insulin resistance in host cells.

In this review, we aim to analyse the role of HCV in the pathogenesis of steatosis and insulin resistance as a possible "way out" mechanism of the virus, together with the impact of both metabolic abnormalities on the clinical course of the disease.

PATHOGENESIS OF STEATOSIS IN HEPATITIS C

HCV infection is characterized by a high rate of progression to fibrosis, chronic hepatitis, leading to cirrhosis and ultimately to hepatocellular carcinoma. In addition, various observations suggest that hepatic steatosis is a common histological feature of chronic HCV infection. Furthermore, increasing evidence indicates that hepatic steatosis is a more vulnerable factor that leads to liver inflammation and fibrosis. These suggest that HCV has a direct role in the development of steatosis and/or that the presence of steatosis affects the progression of HCV-related liver disease. The core protein component of HCV is known to contribute to hepatic steatosis[6], hepatic fibrosis, and hepatic carcinogenesis[7]. Some studies suggest that HCV core protein causes hepatic steatosis through inhibition of microsomal triglyceride transfer protein (MTP) activity and very low density lipoprotein (VLDL) secretion, and impairment of the expression and transcriptional activity of peroxisome proliferator-activated receptor (PPAR)α[8]. Sterol regulatory element binding protein 1s (SREBP1s) belong to the basic helix-loop-helix-leucine zipper family of transcription factors. A key role of two SREBP1 isoforms in regulating fatty acid synthesis in liver is suggested by study of transgenic mice overexpressing the constitutively active mature forms of SREBP1 isoforms[9]. The transgenic mice study suggests that SREBP1s increase the transcription of genes involved in hepatic fatty acid synthesis [including fatty acid synthase (FAS); acetyl-CoA carboxylase (ACC); and stearoyl-CoA desaturase (SCD)], inducing massive hepatic steatosis through increased accumulation of triglycerides.

PPARγ is a transcription factor, belonging to the nuclear receptor superfamily, is a master regulator of adipocyte differentiation, and is important in regulation of a number of genes involved in fatty acid and glucose metabolism[10]. There is evidence suggesting that liver PPARγ increases the transcription of genes involved in hepatic fatty acid synthesis (including FAS, ACC and SCD) and fatty acid uptake (including FAT/CD36 and

fatty acid translocase). Thus, liver PPARγ contributes to regulation of lipid synthesis, transport and storage within hepatocytes, causing the development of hepatic steatosis.

Using a cell culture based model, Kim and coworkers[11] have shown that HCV core protein is able to induce the gene expression and transcriptional activity of SREBP1, thereby causing the increase of fatty acid synthesis. They also observed that HCV core protein elevates PPARγ activity, inducing the expression of fatty acid uptake-associated gene. These results suggest that SREBP1 and PPARγ may represent a new potential therapeutic target in the pathogenesis in HCV infection.

Steatosis development is due to both viral and host factors. Viral steatosis is mostly reported in patients with genotype 3a, in whom fat accumulation correlates with HCV replication levels in serum and liver and disappears after successful antiviral therapy, strongly suggesting a direct role of specific viral products in the fat deposition. To address the role of specific HCV genotype on lipid accumulation in cells, Abid and coworkers[12] developed an *in vitro* model to study the effect of the core protein belonging to several viral genotypes (1b, 2a, 3a, 3h, 4h and 5a). They concluded that the pattern observed in Huh7 cells upon expression of the six core proteins largely corroborated the phenotype seen *in vivo*. The genotype 3a-derived core protein was about three-fold more efficient than the corresponding protein from genotype 1b at inducing triglycerides accumulation in transfected cells, and is proposed as the ideal candidate to study the pathogenesis of HCV-induced steatosis. This group also reported the role of PPARγ expression on triglyceride accumulation in Huh7 cells transfected with genotypes 1b and 3a core proteins[13]. They found that the expression of HCV 3a core protein was associated with an increase in triglyceride accumulation and with a significant reduction of PPAR-γ mRNA compared with HCV 1b. Moreover, Fukasawa and coworkers[14] showed that ACC1 and FAS, enzymes responsible for *de novo* lipid biosynthesis, are induced in Huh7 cells transfected with HCV core protein. Using microarray analysis Pazienza *et al*[15] compared the gene expression profile of Huh7 cells transfected with the core protein of HCV genotype 1b and 3a, leading to the conclusion that several genes involving lipid transport and metabolism were up- or down-regulated in a genotype-specific manner. This fact could explain the variable disease expression associated with HCV infection. Taking all these observations together, we can conclude that there is a direct link between virus infection and steatosis development.

It has been shown that genotype 3 infected patients had lower expression of PPARα or MTP mRNA in the liver and fat accumulation was three times higher in comparison with non-genotype 3 patients[16]. Although the mechanism by which genotype 3a induces steatosis more efficiently than others genotypes is not completely understood, some differences in the amino acid sequence in the core protein could explain, at least in part, these differences. Indeed, a specific polymorphism in core protein from genotype 3 has been associated with

lipid accumulation in hepatocytes. Amino acid substitution at positions 182 and 186 caused intracellular lipid accumulation in hepatic cells and contributed to steatosis development[17]. Indeed, single polymorphisms in the core gene promoting an amino acid change from tyrosine to phenylalanine (Y164F) has been associated with greater cumulative lipid droplet area in cultured cells than in cells producing the wild-type core protein[18].

The immune response against HCV releases reactive oxygen species (ROS) from sequestered phagocytes and activated Kupffer cells in the liver. Increased oxidative stress caused by HCV may result in the activation of Kupffer cells[19]. The change in H^+ concentration alters the balance in the Na^+/H^+ exchanger, causing the Kupffer cells to swell and eventually burst. This releases ROS and an arsenal of inflammatory mediators such as TNF-α, TGF-β, IL-6 and IL-8. The rising concentrations of ROS induce lipid peroxidation and damage triglycerides. The process of lipid peroxidation disrupts cellular membranes and can induce mitochondrial dysfunction[20].

Metabolic and viral steatosis share the same pathophysiological pathways, although metabolic abnormalities are more often seen in genotype 3a resulting in greater steatosis. A predominant but not exclusive metabolic or viral mechanism could be associated with each genotype, and steatosis could appear as a consequence of the interplay between both host and viral factors.

Recently, steatosis has been implicated in viral replication. The disruption of the association between core protein and fat droplets impairs viral fitness[21]. In infected cells, core protein is targeted to lipid droplets, which serve as intracellular storage organelles. According to their results, two facts show the relationship between lipids droplets and viral replication: first, the change in the distribution of the core protein from wild sites juxtaposed to lipids droplets at an early stage, which later agreed with a peak production of virus. Secondly, JFH-1DP, a mutant strain obtained from JFH1, which did not give rise to virus progeny, expressed a core protein that was targeted to punctuate sites indistinguishable from those identified for the wild type protein at early times, but JFH1DP core did not proceed to coat lipid droplets. In such cases, an association between core protein and lipid droplets would be essential for virus assembly. Furthermore, alteration of a phenylalanine in domain 2 of the core protein generates an unstable form of protein associated with reduced replication rates. Lastly, Shavinskaya et al[22] identified the lipid droplet binding domain of HCV core as the major determinant for efficient virus production. They show that D2 in HCV core is a critical determinant for efficient virus assembly and that small numbers of variations (mutations) in this highly conserved domain can exert a significant effect on production of infectious HCV. Thus, HCV promotes steatosis as an efficient mechanism for stable viral replication.

From a clinical perspective, the negative impact of severe hepatic steatosis on graft dysfunction during the immediate post-transplant period has long been recognized[23]. Donor livers containing greater than 30%-50% steatosis are at increased risk of developing primary nonfunction and delayed function and are associated with reduced graft and patient survival. Since chronic HCV is the most common indication for liver transplantation (LT), several studies have examined the impact of steatosis within the donor graft on the severity of recurrent HCV and/or survival following LT[24,25]. From those studies, a direct relationship between marginal donors, graft steatosis and more frequent and earlier recurrence for HCV-related cirrhosis has been established[25]. The putative relationship between steatosis and viral replication could explain this fact.

INSULIN RESISTANCE IN HEPATITIS C

Liver fibrosis has been considered for a long time to be responsible for the appearance of insulin resistance and type 2 diabetes in patients with chronic liver diseases. Hyperinsulinemia in liver cirrhosis has been reported to be due to diminished hepatic insulin extraction by liver dysfunction and not to pancreatic hypersecretion. C-peptide (a peptide resulting from the split of proinsulin into insulin and C-peptide) and insulin are secreted in equimolar quantities, and more than 50% of insulin is degraded in the liver at first pass, whereas C-peptide is degraded in the kidneys. Simultaneous measurements of C-peptide and insulin revealed that both insulin resistance and insulin secretion contribute to glucose intolerance in patients with chronic HCV[26]. From a clinical point of view, the insulin resistance index was higher in patients with chronic HCV showing mild or no fibrosis than matched healthy controls. Moreover, insulin resistance was found to be higher in patients with chronic HCV than patients with other causes of chronic hepatitis matched by age, sex, body mass index, family history of type 2 diabetes and fibrosis staging[27]. On the other hand, Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) correlated with HCV RNA level and was found to be higher in patients with HCV than healthy controls in spite of a lower body mass index. Indeed, in patients with HCV, insulin resistance is more strongly associated with viral load than visceral obesity[4]. Insulin resistance is a common metabolic disorder in the pre-diabetic state. Thus, if HCV promotes insulin resistance it should be linked to type 2 diabetes. Indeed, diabetes was more often seen in HCV than other liver diseases (20%-25% in patients with HCV and in 10% of those with hepatitis B)[28]. Moreover, in a recent systematic review an increased risk of type 2 diabetes mellitus in patients with hepatitis C in comparison with non-infected people has been reported[29].

HCV seems to cause metabolic syndrome, but the mechanisms by which HCV promotes insulin resistance have not been completely understood[30]. HCV induces several complex mechanisms that lead to oxidative stress, insulin resistance, steatosis, fibrosis, apoptosis, altered gene expression and hepatocellular carcinoma. HCV seems to lead to insulin resistance through interference of intracellular insulin signalling by HCV proteins, mainly the serine phosphorylation of IRS-1

and impairment of the downstream Akt signalling pathway[31].

Transgenic mice expressing core HCV protein developed insulin resistance, which does not occur in wild-type animals[32]. During HCV replication, core protein has been found to localize to the outer mitochondrial membrane and is also associated with the endoplasmic reticulum (ER)[33]. In the mitochondria, HCV core protein induces mitochondrial permeability transition, calcium accumulation, stimulation of electron transport and ROS production, as well as promoting glutathione depletion and release of cytochrome C[34]. Moreover, HCV proteins are assembled and correctly folded by chaperones in the ER, but in some circumstances the ER fails to export synthesized proteins properly leading to an accumulation of misfolded proteins[35]. The misfolded protein response causes ER dysfunction and promotes inflammation and ER stress[36]. Of the HCV nonstructural proteins, NS3 and NS5A act as key mediators in the induction of oxidative stress and inflammation. The association of NS5A with the ER has been suggested to stimulate mitochondrial ROS production by releasing calcium from the ER[37]. In addition, NS3 has been shown to activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (Nox2) that generates ROS[38]. Nox2 can nitrosylate proteins within cells and can lead to a large number of pathological processes. Consequently, a role for NOX enzymes in hepatic fibrosis, characterized by hepatic stellate cell (HSC) proliferation and accumulation of extracellular matrix proteins, has been suggested[39,40].

HCV core protein inhibits PPAR α and γ expressed in hepatocytes and adipocytes promoting IRS-1 degradation and insulin resistance[13]. HCV core protein induces the over production of TNFα, responsible for phosphorylation of serine residues of IRS-1 and IRS-2 and down-regulation of glucose transporter gene expression. TNF correlates with the hyperinsulinemic state and the blockade of TNF production by anti-TNF drugs like infliximab inhibits the development of insulin resistance. Thus, TNF promotes hyperinsulinemia and hyperglycaemia and has been linked to an increased risk of diabetes development[41]. Moreover, non-structural proteins such as NS3 and NS5 interact with the ER. NS3 enhances Nox2 activity and increases nitrosylated proteins and ROS[42]. NS5A and NS5B proteins activate toll-like receptor-4 and the NFKB pathway enhances TNF and IL-6 production and promotes insulin resistance[43]. IL-6 is a cytokine that is secreted from Kupffer cells, adipocytes, B cells, and hepatocytes. HCV-infected patients are known to have elevated levels of IL-6 due to the virus-induced inflammatory state[44]. Increased IL-6 derived from adipocytes leads to an ongoing acute-phase response that acts on hepatocytes and promotes hepatic insulin resistance. IL-6 is able to inhibit the expression of LPL in mice[45]. Unlike TNF-α, IL-6 circulates at high levels in plasma, perhaps representing a hormonal role of IL-6 that may induce insulin resistance in other tissues besides liver.

The HCV core protein interferes with *in vitro* insulin

Figure 1 Hepatitis C core induces insulin resistance promoting proteasomal degradation of insulin receptor substrate-1 (IRS-1).

signalling by genotype-specific mechanisms (Figure 1). Pazienza *et al*[46] used the previously described model of transient expression of the core protein of different genotypes of HCV to assess the interaction of HCV genotype 3a with the insulin signaling, using as comparison the genotype 1b. They found that Insulin Receptor-1 (IRS-1) protein level was significantly reduced in Huh-7 cells expressing the core protein of both genotypes 3a and 1b. However, while the core protein of genotype 3a promoted IRS-1 degradation through the down-regulation of PPARγ and by up-regulating the suppressor of cytokine signal 7 (SOCS-7), the core protein of genotype 1b activated the mammalian target of rapamycin (mTOR), demonstrating that interaction between viral core protein and IRS-1 degradation is genotype-specific.

Enhanced SOCS production after HCV infection seems to play a crucial role in inducing interferon resistance by inhibiting interferon-α intracellular signalling[47]. Moreover, SOCS inhibit the phosphorylation of Akt and phosphatidyl inositol 3 kinase (PI3K), impairing intracellular insulin signalling, blocking the transactivation of GLUT-4 and avoiding glucose uptake by cells. Over-expression of SOCS-3 has also been linked to interferon and insulin resistance[48]. Indeed, in transgenic mice unable to express SOCS-3 and expressing HCV core protein, insulin resistance did not appear in the absence of SOCS-3[49]. Therefore, as previously reported in steatosis, patients with HCV could suffer from viral or metabolic insulin resistance. HCV itself induces insulin resistance by several factors also implicated in interferon resistance, allowing the virus to resist antiviral treatment and to promote fibrosis progression[50]. However, some controversial results support the presence of additional mechanisms in the development of insulin resistance in patients with chronic HCV and metabolic or viral type insulin resistance. Pazienza and coworkers[46] have shown that SOCS-1 and SOCS-3 mRNA levels did not change following transfection with both core proteins from genotypes 1b and 3a. However, SOCS-7 mRNA levels were found to be significantly higher in cells expressing the core protein 3a (but not in those transfected with core protein 1b). Their results were corroborated at the protein level by immunoblot. The role of SOCS-7 in IRS-1 downregulation in genotype 3a-transfected cells was con-

firmed using siRNA. They found that the mechanism of IRS-1 degradation by genotype 3a seems to be quite different from that possessed by genotype 1b. The latter is apparently mediated neither by PPARγ or SOCS-7 nor by SOCS-1 or SOCS-3, as suggested by other authors.

In chronic HCV infection, steatosis up-regulates hepatocyte CD95/Fas and thus increases apoptosis, which facilitates inflammation and fibrosis[51]. It has been recently shown that adiponectin reduced FFA-induced CD95/Fas expression and apoptosis of HepG2 hepatoma cells, which suggests a protective role for this hormone with promising therapeutic implications[52].

There are still many molecular mechanisms and open questions to uncover on the HCV-host interaction for the development of effective drugs for the treatment of this disease. In this regard, cell culture based replication systems described so far will help in this task. The availability of systems for replication of all known HCV genotypes together with animal models is highly desirable, in order to find out the importance of the virus genome in disease development.

CONCLUSION

Steatosis development is linked to HCV infection. There is evidence for the accumulation of lipids in the infected cell that could play a determinant role for efficient virus assembly. Thus, HCV promotes steatosis as an efficient mechanism for stable viral replication. HCV itself induces insulin resistance by several factors also implicated in interferon resistance, allowing the virus to resist antiviral treatment and to promote fibrosis progression.

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ORIGINAL ARTICLE

Do statins reduce hepatitis C RNA titers during routine clinical use?

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Abstract

AIM: To compare hepatitis C virus (HCV) titers in patients with chronic hepatitis C with and without exposure to 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins).

METHODS: Medical records were reviewed for 6463 patients with documented HCV infection at a single center between March 2004 and September 2006. Patients with confirmed viremia and meeting inclusion criteria were assigned to one of three groups: Group A (n = 50), dyslipidemic patients with statin usage during HCV RNA polymerase chain reaction (PCR) determination; Group B (n = 49), dyslipidemic patients with prior or future statin usage but not at the time of HCV RNA PCR determination; and Group C (n = 102), patients without statin usage during the study period. The primary analysis explored the effect of statin therapy on HCV viremia. Secondary analyses assessed class effect, dose response, and effect of other lipid-lowering therapies on HCV viral titers.

RESULTS: Median HCV RNA titers did not significantly differ among the three groups (Group A: 4550000 IU/mL, Group B: 2850000 IU/mL, Group C: 3055000 IU/mL).

For those subjects with longitudinal assessment of HCV viremia prior to and while on statins, there were no significant differences between pre- and post-HCV viral titers. Additionally, no differences in HCV titers were observed at any dose level of the most prescribed statin, simvastatin. However, hypertriglyceridemia independently correlated with HCV titers, and niacin exposure was associated with significantly lower viral titers ($P < 0.05$).

CONCLUSION: There was no apparent effect of statins on HCV viral replication in this analysis. Further investigation is warranted to explore the possible antiviral properties of triglyceride-lowering agents and their potential role as adjuncts to standard HCV therapy.

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INTRODUCTION

Hepatitis C virus (HCV) infection affects approximately 1.8% of the United States population[1,2]. The burden of disease is markedly increased in the United States Veteran population with 4%-19% of veterans being seropositive for antibodies against the virus[3-5]. Pegylated interferon combined with ribavirin is the current standard therapy for HCV and is curative in approximately 40%-50% of patients. However, the adverse effects and contraindications to therapy limit the applicability and utilization of this regimen in many infected persons[6]. The potential sequelae of chronic HCV infection, the limitations of current therapy, and the large economic burden of this disease provide a critical impetus for the pursuit of novel therapeutic agents.

Recent studies using HCV replicon systems suggest a potential therapeutic role for the lipid-lowering agents 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, commonly referred to as statins, in chronic HCV infection. These studies are predicated on cardinal observations of the HCV life cycle. Firstly, HCV virions circulate in plasma in association with low density lipoprotein (LDL) particles. Secondly, the LDL receptor and the high density lipoprotein (HDL) scavenger receptor B1 putatively facilitate HCV entry into hepatocytes[7]. Thirdly, and perhaps more importantly, HCV replication depends on the formation of a "membranous web" replication complex[8,9]. Within this endoplasmic reticulum-based replication complex, host proteins are found to be closely associated with HCV nonstructural proteins. The process which links these host and HCV proteins, termed prenylation, appears to depend on two distinct host protein pools, farnesyl and geranylgeranyl, both protein products of the cholesterol synthesis pathway. Therefore, statins, agents which block the formation of the lipid precursors for prenylation, could theoretically interfere with viral replication[10].

Indeed, *in vitro* studies using HCV replicon-bearing hepatoma cell lines do suggest that statins inhibit HCV replication by disrupting the formation of viral replication complexes, an effect that can be reversed by the addition of mevalonate or geranylgeraniol, synthetic proteins in the cholesterol pathway[10-12]. Further, the combination of interferon-α and fluvastatin in experimental models exhibited strong synergistic inhibitory effects on HCV RNA replication suggesting that fluvastatin, in particular, but potentially other statins, could be useful as an adjunct to interferon-α based therapy[11]. While the findings from these studies have been invaluable, the applicability to human use remains in question.

The effect of statins on HCV replication in human subjects has been prospectively addressed in two small studies with mixed results[13,14]. O'Leary *et al*[13] found no reduction in HCV RNA titers at week 4 and week 12 relative to baseline levels. In contrast, another study identified a non-sustained, non-dose-related, reduction in HCV RNA titers in 50% of those treated[14]. Neither study, however, explicitly addressed the efficacy of individual statin drugs, viral genotype, or controlled for exposure to non-statin lipid-lowering agents. In order to examine a larger number of exposed subjects, to assess the relative efficacy of individual statin formulations, to control for potential confounders, and to determine whether further prospective trials might be warranted, we performed a cross-sectional and longitudinal analysis of HCV RNA viral loads in chronic HCV patients who received a statin for therapy of dyslipidemia.

MATERIALS AND METHODS

Ethics
This study protocol was reviewed and approved by the Institutional Review Board of the Philadelphia Veterans Affairs Medical Center (VAMC).

Selection of patient groups
We performed a retrospective analysis of chronic HCV-infected patients who were seen at the Philadelphia VAMC from March 14, 2004 to September 14, 2006 and who had at least one quantitative HCV RNA polymerase chain reaction (PCR) test performed using the Taqman® assay (Roche Diagnostics, Indianapolis, IA). Screening for viral hepatitis is part of routine intake for all primary care clinic patients at the Philadelphia VAMC. Patient level data was extracted from the facility's Hepatitis C Registry, an automated system that registers all patients with positive HCV antibody testing from clinical laboratory data and facilitates acquisition of additional clinical information from the Computerized Patient Record System (CPRS) and Veterans Health Information Systems and Technology Architecture databases. Charts of patients identified as HCV antibody-positive were queried as to the presence and type of confirmatory PCR testing. Those without confirmatory PCR testing and those without detectable viremia by quantitative Taqman® HCV PCR assay were excluded. Patients were also excluded if HIV antibody, HIV RNA and/or HBsAg were positive or if antiretroviral drugs were present in the medication profile. Patients with acute HCV, defined as seroconversion within 2 years of exposure, and patients with chronic kidney disease, defined as serum creatinine greater than 2 mg/dL, were also excluded.

Pharmacy records were then examined to identify the start and stop dates of HMG-CoA reductase inhibitors, niacin, clofibrate, ezetimibe, and interferon-α preparations. Interferon-α based therapy was confirmed through review of progress note documentation in the CPRS. HCV RNA titers obtained during interferon-α therapy were annotated. Utilizing refill data and chart review, patients in whom the HCV RNA determination date(s) occurred at least 30 d after initiation of HMG-CoA reductase inhibitor therapy, in whom exposure to the statin drug spanned at least 60 d, and in whom the duration of statin therapy included the date of HCV RNA determination were designated as Group A. Two control groups who also met the inclusion criteria were selected for comparison. Subjects with hypercholesterolemia for whom a statin medication was prescribed but either had HCV RNA titers drawn prior to the statin initiation date or in whom statin therapy was discontinued at least 60 d prior to the HCV RNA determination were selected as dyslipidemic controls Group B. Group C was chosen from a pool of HCV positive subjects without documented statin exposure during the evaluation period. Analysis of this group was performed with and without exclusion of subjects with total cholesterol levels greater than 200 mg/dL. Using the date of closest correlation to the HCV RNA titer obtained, laboratory data, including basic chemistries, liver associated enzymes, coagulation panels, and lipid profiles were extracted for each group. The longitudinal results of HCV RNA titers during ongoing statin therapy were also recorded for those patients in Group A with more than one HCV RNA determination within the study period.

Table 1 Patient characteristics

Variables	Median (range)			P-value		
	Group A (n = 50)	Group B (n = 49)	Group C (n = 102)	All	Group A vs Group B	Group A vs Group C
Gender (male/female)	50/0	49/0	100/2	0.38		
Race (white/black/other/unknown)	15/28/0/7	11/31/0/7	18/64/0/20	0.74		
Ethnicity (hispanic/non-hispanic/unknown)	2/44/4	1/38/3	3/78/21	0.15		
Prior interferon-alpha therapy (%)	14	8	3	0.042	0.32	0.015
HCV genotype (1/2/3/not-typed)	34/4/1/1	32/5/0/0	70/5/0/0	0.26		
Age (yr)	56 (47-87)	55 (42-80)	56 (41-83)	0.60		
Body mass index	29.1 (18.9-47.8)	28.1 (20.8-43.6)	26.8 (17.1-41.8)	0.0021	0.24	0.001
Albumin (g/dL)	4 (3-5)	4 (3-4)	4 (2-5)	0.37		
ALT (U/L)	46 (11-388)	40 (12-207)	53 (15-345)	0.09	0.19	0.035
AST (U/L)	37 (18-199)	36 (16-136)	53 (22-304)	< 0.0001	0.24	0.002
Alkaline phosphatase (U/L)	74 (28-215)	79 (44-162)	77 (40-1106)	0.23		
Total bilirubin (mg/dL)	0 (0-2)	0 (0-1)	0 (0-2)	0.01	0.40	0.060
Creatinine (mg/dL)	1 (0-1)	1 (0-1)	1 (0-2)	0.24		
International normalized ratio	1 (0-2)	0 (0-1)	1 (0-3)	0.48		
Platelets (× 1000/mL)	242 (129-758)	242 (122-450)	208 (55-606)	0.020	0.97	0.024
Total cholesterol (mg/dL)	177 (110-304)	187 (71-277)	161 (75-260)	0.0002	0.21	0.038
HDL cholesterol (mg/dL)	40 (25-70)	42 (26-69)	41 (21-110)	0.16		
LDL cholesterol (mg/dL)	114 (45-222)	122 (64-187)	93 (22-173)	< 0.0001	0.29	0.006
Triglycerides (mg/dL)	120 (35-384)	114 (44-443)	105 (39-467)	0.31		

Group A: Cases (HCV PCR+ with concomitant statin exposure); Group B: Dyslipidemic control (HCV PCR+ with non-coincident statin exposure); Group C: Unexposed control (HCV PCR+, no statin exposure).

Figure 1 Patient assignment algorithm.

Statistical analysis

Comparisons of frequency data were performed with χ^2 or Fisher's exact testing as appropriate. All group-wise comparisons were performed using non-parametric tests including Kruskal-Wallis or Mann-Whitney U tests. Regression analysis was conducted to explore the effect of potential confounders on the primary study endpoint, HCV RNA titers. All analyses were conducted with JMP 7 software (SAS Institute, Cary, NC) and/or STATA 9.2 (College Station, TX). P-values < 0.05 were considered significant.

RESULTS

Patient selection and characteristics

A total of 6463 patients were found to be HCV antibody-positive (+). Fifty HCV-infected patients who met criteria for statin exposure with concomitant HCV RNA determination (Group A), 49 HCV-infected dyslipidemic patients not on a statin at the time of HCV RNA determination (dyslipidemic controls, Group B) and 102 statin-unexposed HCV-infected controls (Group C), were analyzed (Figure 1).

Patients in the three groups were similar in terms of age, gender, race, ethnicity, HCV genotype, serum albumin, alanine aminotransferase (ALT), alkaline phosphatase, total bilirubin, creatinine, international normalized ratio, platelets, HDL cholesterol, triglyceride levels and exposure to non-statin lipid-lowering agents (Table 1). Among Group A, the median duration from first statin exposure to HCV RNA determination was 288 d (range 34-1435, data not shown). Group A, when compared to Group C, had a significantly higher median body mass index (BMI) (29.1 vs 26.8, P < 0.01), platelet count (243 × 1000/mL vs 208 × 1000/mL, P < 0.05), total cholesterol level (177 mg/dL vs 161 mg/dL, P < 0.01), and LDL levels (114.3 mg/dL vs 93.4 mg/dL, P < 0.01). However, there were no significant differences between Group A and Group B. Group C had a significantly higher median aspartate aminotransferase level (54 U/L vs 37 U/L, P < 0.01) and ALT level (53 U/L vs 46 U/L, P < 0.05) when directly compared with Group A.

Figure 2 Hepatitis C RNA titers relative to HMG-coa reductase inhibitor exposure. Log HCV RNA viral load (IU/mL) in Group A, Group B, and Group C subjects. A: Entire cohort; B: Genotype 1 patients; C: Genotype 2 patients; D: Patients in which genotype was not available. Box plots indicate median, 25th and 75th percentiles (box), and 5th and 95th percentiles. *P*-value obtained by Kruskal-Wallis test.

HCV RNA titers and statin usage

Median HCV RNA titer in Group A was 4550000 IU/mL *vs* 2850000 IU/mL in Group B *vs* 3055000 IU/mL in Group C (Figure 2A). The similarity in serum titers suggest that in this cohort there was no evidence that as a class HMG-CoA reductase inhibitors exhibit antiviral properties *in vivo*. To confirm that there was no genotype-specific effect of HMG-CoA reductase inhibitors, we analyzed each known HCV genotype separately for genotype 1, genotype 2 and untyped subjects in all groups. We again found no significant differences between these groups for any genotype (Figure 2B-D).

Statin preparations, HCV RNA titers, and dose response

Among Group A, 42 (84%) received simvastatin, 5 (10%) lovastatin, 2 (4%) pravastatin and 1 (2%) fluvastatin. When comparing HCV RNA titers among patients receiving any of the four statin agents, there were no differences observed (Figure 3A). Furthermore, there was no apparent antiviral effect of simvastatin, the most prescribed statin in this cohort, at any dose level (Figure 3B). However, when comparing the two most commonly used agents in our cohort, there was a trend towards lower HCV RNA titers in subjects who received lovastatin relative to simvastatin. To further investigate whether or not the trend towards lower HCV RNA titers in lovastatin users might be significant, we compared

HCV RNA titers in active lovastatin users in Group A and former or future lovastatin users in Group B. However, no significant trend towards a lower HCV viral load could be identified (Figure 3C). Thus, there was no class effect of HMG-CoA reductase inhibitors and further conclusions regarding specific statin formulations cannot be made.

Serum total cholesterol and HCV RNA titers

The lack of apparent anti-viral effect of statins theoretically could have resulted from neutralization from a pro-viral effect of hypercholesterolemia rather than a lack of effect of the statin in individual patients. To control for this potential confounder, we correlated viral titers and total cholesterol for Group A (Figure 4A), Group B (Figure 4B), and Group C subjects (Figure 4C) and found no evidence of a relationship between total serum cholesterol and HCV RNA titers in any group. Further, there was no correlation of HCV RNA titers and HDL, LDL, or triglyceride levels (data not shown). Thus, hypercholesterolemia itself did not appear to mediate a pro-viral effect or to be related to cholesterol metabolism in a fashion that may negate any anti-viral effect of statin preparations.

BMI and HCV RNA titers

As noted above, Group A subjects had a significantly

Figure 3 Antiviral effects of individual HMG-CoA reductase inhibitors. A: Log HCV RNA viral load (IU/mL) for Group A subjects who received simvastatin ($n = 42$), lovastatin ($n = 5$), fluvastatin ($n = 2$) and pravastatin ($n = 1$); B: Log HCV RNA viral load (IU/mL) for simvastatin-exposed subjects who were receiving 10 mg ($n = 4$), 20 mg ($n = 20$), 40 mg ($n = 20$), 80 mg ($n = 10$). *P*-value obtained by Kruskal-Wallis Test; C: Pairwise comparison of median HCV RNA titers in lovastatin-exposed Group A subjects and Group B subjects who received lovastatin but were not being treated at the time of the RNA determination. *P*-value from Wilcoxon sign rank test.

higher median BMI (29.1 *vs* 26.8, $P < 0.01$) than Group C, but no significant difference in BMI was found between Group A and B. To assess any potential interaction of BMI on HCV RNA titers, we performed regression analysis that demonstrated no association between BMI and HCV RNA titer in either Group A or Group C.

Hypertriglyceridemia, other lipid lowering agents and HCV RNA titers

In order to assess serum triglyceride levels as a confo-

unding variable, we analyzed the association between serum triglycerides as well as triglyceride-directed therapy on HCV RNA titers. As shown in Table 1, 6/50 in Group A, 2/49 in Group B and 5/102 in Group C received non-statin lipid lowering therapy at the time of HCV RNA determination. Of these, a total of 10 exposures were to niacin (5/6 in Group A, 2/2 in Group B, and 3/5 in Group C). After excluding patients on niacin and gemfibrozil, the presence of triglyceride levels greater than 250 mg/dL was associated with a higher median HCV RNA titer (> 250 mg/dL: 6760000 IU/mL *vs* < 250 mg/dL: 3130000 IU/mL, $P < 0.05$, Figure 4D) and triglycerides were weakly but significantly correlated with HCV viral titers ($R^2 = 0.023$, $P < 0.05$, data not shown). After excluding two patients taking gemfibrozil, niacin-exposure, irrespective of statin exposure, was associated with lower median HCV RNA titers (exposed: median 835000 IU/mL *vs* unexposed: 3350000 IU/mL, $P < 0.05$, Figure 4E). Compared to 13 patients with untreated hypertriglyceridemia (defined as triglycerides > 250 mg/dL), niacin-exposure was associated with a 1.16-log reduction in median HCV RNA titer ($P < 0.05$). Similar analyses for fibric acid derivatives or ezetimibe were not possible due to the limited number of exposed patients.

Longitudinal effect of statin therapy

Twenty-eight Group A subjects (23 simvastatin, one fluvastatin and four lovastatin) had more than one determination of HCV RNA titer, allowing for longitudinal assessment of changes in HCV viremia during the duration of HMG-CoA reductase exposure. In the vast majority of non-interferon-treated subjects, there was either a modest increase or no change in HCV RNA titers during such therapy (Figure 5). In 11 of these 28 subjects, an HCV RNA determination was made prior to and after the institution of statin therapy. Within this subgroup, there was no significant difference between pre- and post-statin levels of HCV viremia. In two of three lovastatin-exposed subjects with initial HCV RNA testing within 1 mo (range: -15 to +17 d) of initiation of interferon, HCV RNA titers declined by 0.7-1.1 log between 9-16 mo. Only two subjects with repeated assessments were exposed to interferon during the observation period. These data imply that statin exposure in this cohort was not associated with significant inhibition of viral replication for individuals with longitudinal follow-up.

DISCUSSION

The sustained virologic response rate of chronic hepatitis C in registration trials with currently approved interferon-α based antiviral therapy ranges from 44%-54%[15,16], but varies widely by genotype, ethnicity and underlying histology. In practice, at centers similar to the study site, HCV treatment response rates range from 20% for genotype 1 to 43%-52% for genotypes 2-3[17]. Suboptimal response and significant toxicity continue to spur the development of novel anti-HCV therapies.

The capacity of HMG-CoA reductase inhibitors

Figure 4 Lack of correlation between serum cholesterol and HCV RNA viral load, but apparent association with hypertriglyceridemia. Linear correlation of log HCV RNA titers (IU/mL) and serum total cholesterol (mg/dL) in Group A (A), Group B (B), and Group C (C). *P*-values obtained by Spearman correlation; D: Log HCV RNA titers for patients with serum triglycerides greater than or below 250 mg/dL excluding patients on gemfibrozil or niacin (> 250 mg/dL: median 6 760 000 IU/mL *vs* < 250 mg/dL: median 3 130 000 IU/mL, *P* = 0.042); E: Log HCV RNA titers for patients with or without exposure to niacin, excluding patients on gemfibrozil (exposed: median 835 000 IU/mL *vs* unexposed: median 3 350 000 IU/mL, *P* = 0.041); F: Log HCV RNA titer among patients with hypertriglyceridemia, excluding gemfibrozil therapy, with and without exposure to niacin shows lower HCV RNA viral load in niacin-treated patients (niacin-exposed: median 835 000 IU/mL *vs* unexposed: median 6 760 000 IU/mL, *P* = 0.013).

Figure 5 Longitudinal effect of statin therapy. Log HCV RNA titer plotted against days relative to the statin initiation date for simvastatin (black lines, *n* = 21), simvastatin plus interferon (*n* = 2), lovastatin (grey lines, *n* = 4), and fluvastatin (big dotted line, *n* = 1) for patients with more than one determination of HCV RNA titer. Eleven subjects had HCV RNA titers measured before and after the initiation of statin therapy.

to impede HCV replicon replication *in vitro* in a dose-dependent fashion raised hope that this commonly prescribed and acceptably safe class of medications could serve as an adjunct to standard interferon-based therapy. Data from such *in vitro* studies further demonstrated a hierarchy of statin-induced viral inhibition, with the greatest effect demonstrated with fluvastatin followed by atorvastatin, simvastatin, and lovastatin, respectively. These *in vitro* findings prompted a pilot study in which

10 subjects with chronic hepatitis C and laboratory evidence of dyslipidemia were treated with atorvastatin 20 mg daily. However, no significant changes in HCV RNA titers were demonstrated at the atorvastatin dose administered though such subjects did have a significant lowering of LDL and total cholesterol[13]. This first study did not examine the effect of fluvastatin, the most potent statin identified in *in vitro* experiments. A more recent small, uncontrolled study of 22 patients given fluvastatin at doses ranging from 20-80 mg/d found transient, 0.5-log reductions in HCV RNA titers in 50% of treated subjects[14], but did not correlate these responses with the lipid lowering therapeutic effect of statins or specifically explore viral genotypes and other medications used.

In order to validate the need for further prospective study of the effect of statins on HCV viral replication in clinical practice, we performed a cross-sectional study of a larger number of subjects and controlled for potential confounders such as viral genotype, cholesterol levels, triglyceride levels and exposure to other lipid lowering medications. We found exposure to different statin preparations, primarily simvastatin, during routine clinical use, was not associated with a change in HCV viral titers. In a limited number of subjects with longitudinal measures of HCV viral load pre- and post-initiation of statin therapy, our data suggested that there was no evidence of clinically significant change in HCV

RNA titers. Specifically for simvastatin, the statin for which we had the most data, we were unable to show a dose-dependent association with reduced HCV titers, contrasting with *in vitro* studies[11]. Given the small number of fluvastatin-exposed subjects at our institution ($n = 2$), we were unfortunately not able to analyze the effect of exposure to the most potent *in vitro* inhibitor.

A plausible explanation for the discrepancy between our *in vivo* and others' *in vitro* results may rest with the pharmacokinetic properties of statins. There is a significant first pass effect for all statins with the exception of pravastatin[18]. Intrahepatic concentrations of statins with routine use, however, have not been to our knowledge well documented in the literature. Serum levels of such agents after prolonged therapy are significantly lower than the statin concentrations that were used in replicon systems. For example, the maximal serum concentration of fluvastatin dosed at 40 mg daily is approximately 0.589 μmol/L[19], approximately 10-fold lower than effective statin concentrations for inducing viral inhibition in the replicon systems[10-12].

Additionally, replicon-bearing cell lines are highly adapted and behave quite differently from *in vivo* hepatocytes. For instance, Huh7 cells exposed to interferons are exquisitely responsive to the agent regardless of viral genotype[20]. This is in direct contrast to the disparate rates of sustained virologic response observed clinically with interferon therapy. It is possible that the adaptations that confer interferon sensitivity also confer statin sensitivity, explaining the *in vitro* results. Alternatively, the specific dependence on prenylation of nonstructural proteins for establishment of the viral replication complex might be a feature of cell-culture models for which alternative pathways may be present *in vivo*. The HCV might also develop resistance mutations under selection pressure induced by statin therapy, an effect that could be demonstrated *via* sampling at regular intervals early after initial statin exposure for early viral load changes and sequence evolution. Lastly, pro-viral effects of statins might occur *via* induction of LDL-receptor expression which may paradoxically facilitate viral uptake into uninfected hepatocytes[21].

Another possible explanation for the lack of difference between our study groups could be the presence of a significant confounding variable. For example, obesity, which is associated with non-response to interferon-based antiviral therapy[22], and hypercholesterolemia, were significantly more prevalent in Groups A and B than C. We, however, did not find within any of the groups an association of HCV RNA titers with height, weight or body-mass index (data not shown) nor with total cholesterol; therefore we do not believe that differences in these variables could explain our negative findings. Since hypertriglyceridemia was directly associated with viral titer, the excess of hypertriglyceridemia cases in Group A could contribute to a type II error. However, when we controlled for triglycerides in the regression analysis, statin exposure remained insignificantly associated with HCV RNA titer.

While not powered for an analysis of non-statin lipid lowering medications and their effect on HCV

viral load, our analysis unexpectedly identified a possible direct association of triglycerides and viremia and a suggestion that niacin may have antiviral properties *in vivo*. Recent work with human serum[23] and with primary hepatocytes[24] suggests that HCV is co-secreted with VLDL, implicating triglyceride metabolism as an additional critical step in the viral lifecycle. Given these preliminary findings as well as a biologically plausible mechanism for the action of triglyceride lowering medications on HCV replication, these findings merit further investigation in a larger dataset and, if confirmed, in a prospective clinical trial.

There are several limitations of this analysis that we would like to acknowledge. First, the cross-sectional design inherently precludes determining causality and is inherently weaker than prospective evaluation. Further, given the limited number of subjects with measurements prior to and after the initiation of statin therapy, we cannot definitively rule out an association between statins and reduction in HCV viral replication. Secondly, the sample size remained relatively small after applying our exclusion criteria, raising the possibility of a type II error and specifically that of finding no association when one does indeed exist. Thirdly, there were no direct measurements of patient adherence with prescribed statin therapy, an important factor since poor adherence may make the medication in question appear to be less efficacious. Fourthly, the preferred statin agent on the formulary during the observation period was not the most highly active agent *in vitro*. Lastly, there is a small possibility that patients in Group B could have received a statin agent prescribed by a non-VA physician, which was not recorded in the VA electronic medical record. Since there is significant financial incentive for most veterans to obtain medications through the VA, we believe the impact of this factor is quite small.

In summary, in this single center, retrospective analysis, there was no evidence of an apparent effect of statins on HCV viral replication. Unexpectedly, we found that triglyceride lowering agents such as niacin may have HCV antiviral properties *in vivo*. Therefore, we suggest exploration of this result. Additionally, the potential antiviral efficacy of drugs such niacin in chronic hepatitis C as adjuncts to interferon-based therapies merit further investigation.

ACKNOWLEDGMENTS

COMMENTS

Background
Interferon and ribavirin are the mainstay of treatment for chronic hepatitis C

infection. Unfortunately, this combination is only effective in approximately 50% of all-comers. Therefore, novel therapies must be explored. Cardinal observations of the hepatitis C virus (HCV) life cycle have demonstrated that the lipid metabolism pathway is implicated in viral entry and replication within the hepatocyte. 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, referred to commonly as statins, have been shown to not only disrupt lipid metabolism within the hepatocyte but also halt HCV viral replication in *in vitro* models of chronic HCV infection. Relatively few studies have explored the effect of statins on HCV viral replication *in vivo*.

Research frontiers

Though *in vitro* studies using HCV replicon-bearing hepatoma cell lines do suggest that statins inhibit HCV replication, the applicability of use in humans with chronic hepatitis C infection remains in question. In this study, the authors attempt to, but are unsuccessful in, demonstrating a significant reduction in HCV viral titers with routine use of statins in a cohort of patients infected with hepatitis C.

Innovations and breakthroughs

The results did not suggest an effect of statins on HCV viral replication. However, the investigators did find preliminarily that triglyceride lowering agents may in fact lower levels of HCV viremia. Though significant, this finding was noted in a small number of patients and given that the study was not powered for this question, this should be viewed as hypothesis generating.

Applications

By continuing to explore potential adjunct therapies to current HCV anti-viral therapies, it is likely that some highly effective adjunct(s) will be discovered. This study, while not demonstrating a significant effect of statins in this cohort, does provide some preliminary data to support further investigation of lipid altering medications and their potential effect on HCV.

Terminology

The process which links host and HCV proteins during intrahepatic viral replication is termed prenylation. Prenylation is dependent on two distinct host protein pools, farnesyl and geranylgeranyl, both protein products of the cholesterol synthesis pathway.

Peer review

This is a well written manuscript. The study suffers from the limitations of a retrospective cohort study and may have not detected a difference in HCV viral titers because longitudinal assessment was not made for all subjects prior to and after initiation of statin therapy. The authors need to comment on these limitations in the discussion.

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ORIGINAL ARTICLE

Interventional treatment for symptomatic acute-subacute portal and superior mesenteric vein thrombosis

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Abstract

AIM: To summarize our methods and experience with interventional treatment for symptomatic acute-subacute portal vein and superior mesenteric vein thrombosis (PV-SMV) thrombosis.

METHODS: Forty-six patients (30 males, 16 females, aged 17-68 years) with symptomatic acute-subacute portal and superior mesenteric vein thrombosis were accurately diagnosed with Doppler ultrasound scans, computed tomography and magnetic resonance imaging. They were treated with interventional therapy, including direct thrombolysis (26 cases through a transjugular intrahepatic portosystemic shunt; 6 through percutaneous transhepatic portal vein cannulation) and indirect thrombolysis (10 through the femoral artery to superior mesenteric artery catheterization; 4 through the radial artery to superior mesenteric artery catheterization).

RESULTS: The blood reperfusion of PV-SMV was achieved completely or partially in 34 patients 3-13 d after thrombolysis. In 11 patients there was no PV-SMV blood reperfusion but the number of collateral vessels increased significantly. Symptoms in these 45 patients were improved dramatically without severe operational complications. In 1 patient, the thrombi did not respond to the interventional treatment and resulted in intestinal necrosis, which required surgical treatment. In 3 patients with interventional treatment, thrombi re-formed 1, 3 and 4 mo after treatment. In these 3 patients, indirect PV-SMV thrombolysis was performed again and was successful.

CONCLUSION: Interventional treatment, including direct or indirect PV-SMV thrombolysis, is a safe and effective method for patients with symptomatic acute-subacute PV-SMV thrombosis.

INTRODUCTION

With the development of imaging technology, the rate of diagnosis of portal and superior mesenteric vein thrombosis has gradually increased[1]. When thrombosis occurs only in the portal vein, clinical symptoms such as abdominal pain, nausea, anorexia, and weight loss are usually not serious. When thrombosis involves the superior mesenteric vein, patients may have cramps in the upper middle or lower middle abdomen, nausea, anorexia, vomiting, diarrhea, and/or bloody stool[2-4]. Conservative medical treatment is often unsatisfactory. Surgical treatment is accompanied by more tissue damage, more complications, and a high recurrence rate. Therefore, it is seldom used as a standard treatment unless intestinal necrosis occurs. Recently, interventional and minimally invasive techniques were introduced to treat portal vein and superior mesenteric vein thrombosis (PV-SMV) thrombosis. The results have been

excellent[5,6]. Herein we summarize the interventional treatment of 46 patients with symptomatic acute-subacute PV-SMV thrombosis. Our goal was to describe how to choose a specific interventional treatment, and how to enhance the effect of the treatment and reduce complications.

MATERIALS AND METHODS

General information

We reviewed the case histories of 46 patients including 30 males and 16 females, with an average age of 48 years (range: 17-68 years). All patients had different degrees of abdominal pain, fullness, and anorexia. Other symptoms and signs included diarrhea (25 cases), vomiting (11 cases), and ascites (8 cases). There was no obvious abdominal rigidity and rebound tenderness. Causes of the disease were clearly defined in 42 patients, including 18 who had splenectomies within one year, 8 who had a recent history of pancreatitis, 14 who had liver cirrhosis with portal hypertension (6 of them accompanied by esophageal and gastric varices), 1 who had the condition secondary to duodenal surgical repair for traumatic injury within the last 24 h, and 1 who had appendicitis within the last month.

The period of the disease included 28 acute cases (thrombosis occurred within 1 wk of onset) and 18 subacute cases (1-3 wk from onset). The number of platelets was higher (500-980×10^9/L) than standard scores in 28 patients. The number of white cells was higher (12-20×10^9/L) than normal standards in 17 patients. Red cell number and hemoglobin levels were in the normal range. Liver function tests showed 42 with Child A, and 4 with Child B. Renal function and plasma electrolytes were in the normal range. All patients were clearly diagnosed as having PV-SMV thrombosis by Doppler ultrasound scan, computed tomography (CT) and magnetic resonance imaging (MRI). They were fast and gastrointestinal decompressed.

Interventional treatment

Direct PV-SMV thrombolysis: Twenty four patients were seen within 1 wk of the onset of disease and diagnosed as having acute PV-SMV thrombosis without obvious lateral branch angiogenesis by imaging. Eight patients were seen between 1 and 2 wk from the onset of disease and diagnosed as subacute PV-SMV thrombosis without obvious lateral branch angiogenesis by imaging.

Thrombolysis through transjugular intrahepatic portosystemic shunts (TIPS): In 26 patients, a 10F long sheath (RUPUS 100 system, COOK Corp, USA) was used to puncture the right internal jugular vein according to Seldinger's method and then advanced into the hepatic vein *via* the superior vena cava, right atrium, and inferior vena cava. A shell-type needle was introduced into the hepatic vein through the sheath and it was advanced into the right branch of the PV under X-ray guidance. When PV branches were filled with thrombi and blood flow was interrupted, it was not possible to determine whether PV was punctured or not using the aspirate puncture-cannula. Under such conditions, the contrast should be injected slowly *via*

the puncture-cannula while the puncture-cannula is withdrawn gradually. When the puncture was successful, the contrast remained in the PV branches and the shape of vessels could be seen clearly. An ultra-smooth guide wire was easily introduced into the PV-SMV system. Once it was in the right branch of the PV, a 4F Cobra Catheter (Cordis Corp. USA) or an inferior vena cava angiographic catheter (COOK Corp, USA) was advanced to the distal SMV for direct PV-SMV angiography.

Before thrombolysis, 30-50 mg sodium heparin was injected intravenously into patients without any contraindications. An 8F large thin-walled introducer catheter (90 cm Cordis Corp., USA, or a 100 cm Boston Scientific Corp. USA) was used to aspirate the thrombi and a 4F pigtail catheter (Cordis Corp. USA) was used for mashing the thrombi. A 4F inferior vena cava catheter with multiple lateral holes (COOK Corp. USA) was inserted into the PV-SMV and used to deliver sodium heparin and urokinase (average doses of 0.8 MIU, 0.5-1.5 MIU) intermittently for local thrombolysis. After most of the thrombi in the PV-SMV had been removed, the catheter was maintained for continuous thrombolysis with urokinase (0.5-1.5 MIU/d) and sodium heparin (30-200 mg/d) for 3-13 d. Clopidogrel (75-150 mg/d) or enteric-coated aspirin (100-150 mg/d) were administered to patients with platelet counts greater than 300×10^9/L. During treatment, thrombin time (TT) and activated partial thromboplastin time (APTT) were monitored to maintain TT at 1.5-2.5 times the normal range, and APTT at 2-2.5 times the normal range. In the meantime, levels of D-dimer were monitored[7]. The duration of indwelling catheters, which depended on the improvement of symptoms and thrombolysis, usually lasted less than 2 wk. After the catheter was withdrawn, intravenous sodium heparin was continued for 2 additional weeks, and then changed to oral warfarin sodium (for not less than 1 year). Treatment with warfarin and heparin should overlap for 3 d. After being discharged from the hospital, patients were monitored with standard blood tests and we maintained the international normalized ratio (INR) at 2.0-3.0. Abdominal ultrasound scans were repeated every 1-3 mo and CT or MRI scans as necessary.

Thrombolysis through percutaneous transhepatic portal vein cannulation: In 6 patients, a percutaneous transhepatic puncture was performed with a 22-gauge Chiba needle (COOK Corp, USA) into the right branch of the PV, at the midline through the right armpit. Then an 8F artery sheath (Cordis Corp. USA) was introduced into the PV for thrombolysis. The following thrombolysis procedure was the same as the TIPS method. After treatment, the punctured tract was broken by a gelatin sponge and a steel ring (COOK Corp, USA) was used to prevent secondary liver bleeding.

Indirect PV-SMV thrombolysis

Patients who received indirect PV-SMV thrombolysis included: (a) 4 with acute PV-SMV thrombosis diagnosed by imaging tests within one week of the onset of the disease and who refused direct PV-SMV thrombolysis, (b) 4 with subacute PV-SMV thrombosis diagnosed within 1-2 wk,

Figure 1 The figure shows a male, 43-year-old patient, 2 mo after splenectomy, with abdominal fullness for 6 d. He had thrombolysis through a transjugular intrahepatic portosystemic shunt (TIPS). A: Direct PV-SMV angiography showed extensive thrombosis in the PV and SMV. Contrast agent remained. No lateral branch angiogenesis (arrow); B: Mash and suck thrombi with intermittent injection of urokinase and heparin sodium. Repeated angiography 30 min after thrombolysis showed blood reperfusion in the PV-SMV and normal PV-SMV branches (arrow). Symptoms disappeared.

including 3 who refused and 1 who failed direct PV-SMV thrombolysis, (c) 6 with subacute PV-SMV thrombosis diagnosed within 2-3 wk, including 3 showing lateral branch angiogenesis around the PV-SMV system in the CT scan, suggesting a long duration of the disease.

Thrombolysis through the femoral artery to the superior mesenteric artery cannulation

In 10 patients, a 4F artery sheath (Terumo Corp. Japan) was used to puncture the right femoral artery according to a modification of Seldinger's technique, and then a 4F Cobra Catheter was inserted to perform celiac artery, superior mesenteric artery and indirect PV-SMV angiography. When angiography was completed, 3-6 holes were drilled at the front of the Cobra Catheter with a microdriller. An intensive dose of urokinase (0.2 MIU) was administered into the superior mesenteric artery through the catheter. Afterward, the catheter remained in the superior mesenteric artery in order to continue the thrombolysis with urokinase (0.75-1.5 MIU/d) and sodium heparin (30-200 mg/d) for 3-11 d. All treatments during and after indwelling catheters and review were the same as those performed in the TIPS method.

Thrombolysis through the radial artery to superior mesenteric artery catheterization

The left radial artery in 4 patients was punctured with a radial puncture system (COOK Corp, USA) using a modified Seldinger's method. A 5F artery sheath (COOK Corp, USA) was inserted and then an extended (120 cm) 5F Cobra Catheter (Terumo Corp. Japan) was introduced for celiac artery, superior mesenteric artery and indirect PV-SMV angiography. All other procedures were the same as those through the femoral artery to the superior mesenteric artery cannulation thrombolysis methods.

RESULTS

Direct PV-SMV thrombolysis was performed in 32 pa-

tients, taking 80-180 min each. In 22 of the 32 patients, direct PV-SMV angiography showed extensive thrombosis in the PV and SMV with contrast agent remaining in the vessels and without lateral branch angiogenesis (Figures 1A, 2B, 3C). Ten patients had thrombosis mainly in the PV, SMV and splenic vein. After thrombolysis, repeated angiography showed normal PV-SMV systems in 26 patients (Figure 1B) while thrombosis partially remained in the PV-SMV in 6 patients. Symptoms such as abdominal pain, fullness, and diarrhea disappeared or were significantly relieved. Hospital stays lasted from 2 wk to 2 mo. One patient whose thrombosis was secondary to duodenal surgical repair had PV-SMV reperfusion and symptomatic improvement but died because of an abdominal abscess and multiple organ failure. In 3 patients with interventional treatment, thrombosis recurred (at 1, 3 and 4 mo, respectively) after the treatment. For these 3 patients, indirect PV-SMV thrombolysis was performed again and repeated angiography showed increased lateral branch angiogenesis in the PV-SMV branches. Their symptoms were improved. All other patients had good PV-SMV circulation without abnormal symptoms. Ultrasound and CT scans did not show signs of recurrence.

In 14 patients treated with indirect PV-SMV thrombolysis, one patient with acute thrombosis and another one with subacute thrombosis had partial recovery of PV-SMV flow, partial thrombolysis, and complete symptomatic relief (Figure 2C). Eleven patients had intact thrombosis in the PV-SMV and obvious lateral branch angiogenesis. Their symptoms were dramatically improved with only slight abdominal pain and fullness, and less diarrhea remaining (Figure 3D). One patient with acute thrombosis had no improvement in symptoms 3 d after continuous thrombolysis and developed exudation around the remaining sheath. Ileum segmental necrosis was found on laparotomy 2 d after the catheter was withdrawn. Bowel resection was performed and treatment was continued with an anticoagulant treatment. Six patients with esophageal-gastric varices had obvious improvement confirmed by gastroscope examination after treatment. During follow-up, one patient had upper gastrointestinal bleeding 22 mo after the interventional treatment and was treated with endoscopic sclerotherapy (EIS). Other patients did not develop symptoms of PV-SMV thrombosis again.

DISCUSSION

PV-SMV thrombosis has a concealed onset without any specific symptoms and signs. The diagnosis is therefore, easily delayed. If the following conditions occur, the possibility of PV-SMV thrombosis should be considered in order to achieve early diagnosis and treatment. (1) unexplained abdominal pain, abdominal distension, especially with nausea, vomiting, and bloody stool; (2) intractable ascites; (3) unexplained bloody ascites; (4) unexplained portal hypertension; (5) unexplained upper gastrointestinal bleeding or progressive spleen en-

Figure 2 The figure shows a female, 22 year old patient who had abdominal pain for 17 d. A: A CT scan showed a high density of superior mesenteric vein thrombosis (characteristic of subacute thrombosis) (arrow); B: A Cobra catheter was inserted *via* the femoral artery into the superior mesenteric artery. Indirect angiography showed extensive thrombosis in the PV-SMV. Angiographic contrast agent remained (arrow); C: Indwelling catheters for 8 d. Angiography showed partial blood reperfusion in the PV-SMV. PV branches in the liver were intact (arrow). Symptoms were relieved.

Figure 3 A 24 year old male with abdominal pain for 8 d. A: The CT scan showed a low density SMV thrombosis (arrow); B: An enhanced CT scan still showed a low density of thrombosis (arrow). This fulfilled the symptoms and signs of acute thrombosis; C: An extended Cobra catheter was introduced into the superior mesenteric artery via the radial artery. The indirect angiography shows extensive thrombosis in the PV-SMV without lateral branch angiogenesis (arrow); D: Indwelling catheters for 10 d. The indirect angiography showed that the lateral vessels of the PV-SMV had significantly increased (arrow). Symptoms were relieved.

largement without apparent splenic hyperfunction; (6) unexplained paralytic intestinal obstruction, necrosis or peritonitis, *etc*[8,9]. The diagnosis of PV-SMV thrombosis relies on imaging. Color Doppler ultrasound is very simple, noninvasive, and has a high negative predictive value. It should be chosen first. If a positive result is found, CT or MRI scans should be considered for further investigation. Accurate judgment of the PV-SMV thrombosis duration - acute, subacute, or chronic - is extremely important for disease management[10]. According to our experience, thrombosis shown in a CT scan has a low density during the acute period (within 1 wk of the onset of the disease) (Figure 3A). It has a high density during the subacute period (1-3 wk after disease onset) with a CT value of 5-15 HU which is higher than values for the abdominal aorta and inferior vena cava (the so-called CT scan mesenteric vein angiographic phenomenon, an important piece of diagnostic evidence). It has a low density during the chronic period (> 3 wk) and is accompanied by lateral branch angiogenesis. The density of the thrombosis is not increased with contrast (Figure 3B)[11]. In the MRI scan, PV-SMV thrombosis is shown as a T1WI low signal and a T2WI high signal during the acute period. During the sub-acute period, both T1WI

and T2WI signals are high. During the chronic period, T1WI gives mixed signals and T2WI gives low signals. After Gd-DTPA injection, the signal for thrombosis is not increased[12].

Traditional PV-SMV thrombolysis treatment includes conservative internal treatment and surgical treatment. Medical treatments include thrombolysis and anticoagulation, and others, which can improve symptoms in some patients. But conservative treatment cannot directly remove the obstruction due to the thrombosis. Therefore, its efficiency is very limited and the mortality rate due to gastric bleeding is high[13]. The application of surgical treatment is limited by tissue damage and additional complications[14-17]. With the development of interventional radiology, minimally invasive technology has become one of the predominant means of treating acute-subacute PV-SMV thrombosis without obvious intestinal necrosis, perforation, and peritonitis[18-26]. The method includes direct and indirect PV-SMV thrombolysis[27]. In our study, 46 patients with acute-subacute PV-SMV thrombosis were treated interventionally. The thrombolysis was effective without severe complication.

Our study showed that the effect of direct thrombolysis is better than indirect treatment. Injecting throm-

bolytic agents directly into a PV-SMV thrombus can dramatically increase the effect of thrombolysis, reduce the dose of thrombolytic agent, and reduce the complication of bleeding. Using a mechanical method such as aspirating and mashing to eliminate the thrombi, balloon expanding, stent implantation, etc. can result in reperfusion within a short time and recover blood circulation[28]. However, when the disease duration is too long (> 2 wk) or angiography shows some lateral branch angiogenesis around the main vessels, there is no indication for TIPS and surgical treatment; or, if the treatment via TIPS fails, indirect PV-SMV thrombolysis is still an option[24]. Evaluation of the efficacy of indirect PV-SMV thrombolysis should rely not only on reperfusion of the main vessels. The improvement in clinical symptoms and lateral branch angiogenesis with treatment are also important indications of efficacy. We found that indirect PV-SMV thrombolysis is simple and easy. When direct thrombolysis is difficult to perform, indirect thrombolysis can dissolve some thrombi, promote lateral branch angiogenesis and relieve symptoms for acute-subacute patients. With thrombolysis through the radial artery, one can achieve superior mesenteric artery indwelling catheters. Patients with such indwelling catheters do not need to rest in bed. Complications such as bleeding at the puncture point and infection are significantly reduced. Therefore, the procedure does not introduce any inconveniences into the patient's daily life and is easily accepted by patients.

Regarding the choice of pathways for direct PV-SMV thrombolysis, the way through TIPS does not pass through the intraperitoneal cavity. Hence, it is suitable for patients with existing ascites, coagulative dysfunction and catheters that have been indwelling for a long time[22]. It has a specific advantage in prevention of bleeding, not only avoiding hepatic surface injury, but also reducing bleeding due to thrombosis being aspirated through the big vessel sheath (> 7F) because the cannula is inside the liver. Moreover, cannulation through TIPS can also divert portal blood flow and effectively relieve portal hypertension[29,30]. Therefore, it is very suitable for patients requiring a portosystemic shunt for portal hypertension and embolism for esophageal-gastric varices. The disadvantages of TIPS are its complexity and difficulties in performing it[31]. The procedure for percutaneous transhepatic portal vein cannulation is simpler, easier and cheaper than TIPS[25,32]. It is suitable for patients without ascites and coagulative dysfunction[33]. Recently, this procedure has been improved in several ways. Ultrasound was used to guide a fine needle to the puncture. A steel coil and a gelatin sponge were used to fill the puncture channel to reduce intraperitoneal haemorrhage[25,34]. This method can be an alternative for cases of unsuccessful TIPS or cases that are unsuitable for TIPS but that require direct PV-SMV thrombolysis.

During the time that the catheter is indwelling, determining safe and effective doses of urokinase and heparin is fundamental for the success of the treatment. Our study suggests doses for urokinase (0.5-1.5 MIU/d) and heparin sodium (30-200 mg/d). Urokinase should be rapidly injected through the catheter within half an hour, twice a day. Heparin sodium can be admitted via peripheral veins when urokinase treatment is performed via the catheter. It must be administered via the catheter to the thrombosis in the time gap between urokinase treatments. Patients with PV-SMV thrombosis usually have complications of chronic hepatic disease, clotting factor insufficiency, and generally low coagulation conditions but high coagulation conditions in the portal vein. The direct injection of urokinase and heparin into the PV is more effective. Clopidogrel or enterically-coated aspirin are administered for patients with platelet counts more than 300×10^9/L. In our study, one patient had a platelet count of $600\text{-}970 \times 10^9$/L while they had indwelling catheters after splenectomy and was treated with hydroxycarbamide to inhibit platelet formation.

Urokinase is a plasminogen activator. Its half-life is short (15-20 min). Quickly administering urokinase can instantly lead to its penetration into the thrombosis and cause thrombolysis. In our study, urokinase was injected within 30 min. If the injection is too slow, the effect of thrombolysis becomes weak. Urokinase can also cause degeneration of some clotting factors such as fibrinogen (FIB). FIB is an important clotting factor and a reflective index for activation of the fibrinolysis system. It is also a fundamental factor for plasma viscosity and platelet aggregation. The elevation of plasma FIB can promote blood coagulation and form clots. Because the half-life of urokinase is very short, FIB will increase after 24 h of urokinase administration and return to previous levels after 48 h. If it is not combined with other anticoagulation treatments, it will increase the incidence of vascular thrombosis obstruction after thrombolysis. Therefore, anticoagulation treatment during thrombolysis is necessary.

Heparin sodium affects blood clotting by inhibiting synthesis of fibrous protein factors and the extension of existing clots within the blood. In our study, all patients had anticoagulation treatment with a combination of urokinase and heparin sodium. The prevalence of hemorrhage is high (23%-28%)[21,35-38]. Therefore, an emergency treatment including ECG monitoring and blood pressure control for bleeding must be prepared. All indexes for coagulation and anticoagulation should be monitored. Presently, most researchers suggest that maintaining TT at 1.5-2.5 times the normal range and APTT at 2-2.5 times the normal range can not only achieve the best treatment efficacy, but also avoid severe bleeding. In our study, only three patients had exudation around the catheter sheath. Their symptoms were relieved with a compression bandage without any severe organic bleeding. This is related to understanding the time window of urokinase and heparin, and monitoring the dose for both medications according to all factors involved in coagulation and anticoagulation.

COMMENTS

Background

With the development of imaging technology, the rate of diagnosis of portal and superior mesenteric vein thrombosis has gradually increased. Thrombosis of

portal vein and superior mesenteric vein thrombosis (PV-SMV) is a severe disease. The consequences of these thromboses can be severe, including mesenteric ischemia and variceal bleeding, with high mortality rate. There are no uniform protocols for the effective treatment of PV-SMV thrombosis. Conservative medical treatment is often unsatisfactory. Surgical treatment is accompanied by more tissue damage, more complications, and a high recurrence rate.

Research frontiers
The treatment of symptomatic acute thrombosis of the PV and SMV is controversial due to unsatisfactory results obtained in some cases with medical treatment, as well as the difficulty in performing surgical procedures in some cases. Recently, interventional and minimally invasive techniques were introduced to treat PV-SMV thrombosis.

Innovations and breakthroughs
The authors summarize the interventional treatment of 46 patients with symptomatic acute-subacute PV-SMV thrombosis, which demonstrated the feasibility of this method in the management of this challenging illness. Compared to conservative medical and surgical treatment, interventional treatment has the least tissue damage, complications, invasive and a high success rate.

Applications
Interventional endovascular thrombectomy with direct or indirect thrombolysis can offer a non-surgical alternative for the treatment of symptomatic acute-subacute PV-SMV thrombosis. This technique can be performed in patients who do not present with bowel ischemia and infarction, or who are not at risk for bleeding, and have persistent symptoms or worsening of symptoms despite anticoagulation.

Terminology
PV-SMV: Portal vein and superior mesenteric vein thrombosis. TIPS: Transjugular intrahepatic portosystemic shunts. TT: Thrombin time. APTT: Activated partial thromboplastin time. INR: International normalized ratio.

Peer review
This study summarizes the interventional treatment of 46 patients with symptomatic acute-subacute PV-SMV thrombosis.

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ORIGINAL ARTICLE

Expression of γ-synuclein in colorectal cancer tissues and its role on colorectal cancer cell line HCT116

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Abstract

AIM: To investigate the expression pattern of γ-synuclein in colorectal cancer (CRC) tissues, and to study the effects of γ-synuclein on CRC cell line HCT116 biological features *in vitro*.

METHODS: The expression pattern of γ-synuclein was determined in 54 CRC tissues and 30 tumor-matched nonneoplastic adjacent tissues (NNAT) 5 cm away from the tumor *via* real-time quantitative reverse transcription PCR (RT-PCR) and immunohistochemistry. The relationship between γ-synuclein protein expression and clinicopathological factors of CRC tissues was analyzed. Three small interfering RNA (siRNA) targeting γ-synuclein mRNA plasmids were constructed and transfected into the CRC cell line HCT116. The stable cell lines were selected with G-418 for 28 d, and the biological features of these cells were examined by cell growth curve, soft agar assay, and cell migration and invasion assays *in vitro*.

RESULTS: The expression of γ-synuclein mRNA and protein was much higher in CRC tissue samples than in NNAT samples ($P = 0.02$, $P = 0.036$). There was a significant correlation between the γ-synuclein protein expression and clinical stage and lymph node involvement of CRC ($P = 0.02$, $P = 0.033$). In functional analysis we found that down-regulation of γ-synuclein expression in HCT116 cells could inhibit the growth, colony formation rate, and migration and invasion ability of HCT116 cells.

CONCLUSION: Increased expression of γ-synuclein in CRC tissues and the biological effects of reduced γ-synuclein expression on HCT116 cells suggest that γ-synuclein may play a positive role in the progression of CRC.

Ye Q, Feng B, Peng YF, Chen XH, Cai Q, Yu BQ, Li LH, Qiu MY, Liu BY, Zheng MH. Expression of γ-synuclein in colorectal cancer tissues and its role on colorectal cancer cell line HCT116. *World J Gastroenterol* 2009; 15(40): 5035-5043 Available from: URL: http://www.wjgnet.com/1007-9327/15/5035.asp DOI: http://dx.doi.org/10.3748/wjg.15.5035

INTRODUCTION

The synucleins are a family of small, soluble, highly conserved neuronal proteins that consist of α-, β-, and γ-synuclein. They are a natively unfolded group of proteins that are characterized by 5-6 repeats of the amino acid motif (KTKEGV), constituting most of the N-terminal half of the proteins[1-3]. The synucleins

have attracted considerable attention due to their involvement in neurodegenerative diseases. α-synuclein is the major component of Lewy bodies in Parkinson's disease and has also been identified as the non-amyloid component of amyloid deposition, the hallmark of Alzheimer's disease[4,5]. β- and γ-synuclein are assumed to have a neuroprotective role by inhibiting α-synuclein aggregation and toxicity[6,7].

γ-synuclein gene [also referred to as breast carcinoma specific gene 1 (BCSG1)] initially was cloned from infiltrating breast carcinoma cells by using the expressed sequence tag-based differential cDNA sequencing approach[8]. γ-synuclein maps to chromosome region 10q23, and is composed of five exons and transcribed into an mRNA of about 1 kb, coding 127 amino acids[9]. γ-synuclein expression is usually highly tissue-specific and restricted to brain tissue and presynaptic terminals[2]. However, the tissue-specificity appears to be lost, and γ-synuclein is abnormally expressed in a high percentage of advanced breast and ovarian cancers, but not in normal or benign tissues[10]. Furthermore, overexpression of γ-synuclein can stimulate proliferation, and induce invasion and metastasis of breast cancer cells[11]. γ-synuclein has also been shown to compromise normal mitotic checkpoint controls, resulting in multinucleation as well as faster breast cancer cell growth[12,13]. Liu et al[14] found that γ-synuclein protein was also abnormally expressed in a high percentage of tumor tissues of other cancer types, including liver, gastric, lung, prostate, cervical etc., but rarely expressed in tumor-matched nonneoplastic adjacent tissues (NNAT). However, Zhou et al[15] had an opposite conclusion in esophagus cancer, in which low expression levels of γ-synuclein in human esophageal squamous cell carcinoma (ESCC) and biological effects of γ-synuclein overexpression on ESCC 9706 cells suggested that γ-synuclein might play a role as a negative regulator in the development of human ESCC. Therefore, further study in cancer tissues and cell line culture is needed to understand the roles of γ-synuclein in the development of other human neoplastic diseases.

Recent reports demonstrate that colorectal cancer (CRC) has been the third most common malignancy and the third leading cause of cancer-related deaths worldwide[16]. The conventional therapies involving surgery and adjuvant therapy seem to give rise to improvements in progression-free and overall survival; nevertheless about 50% of patients die within 5 years owing to metastasis or recurrent disease. Patients with early stage CRC have an estimated 5 year survival rate of 91%, compared to only 6% for those with later stage disease. Early detection remains the most important factor in improving long-term survival. Furthermore, tumor invasion and regional lymph node metastasis are important factors for determining CRC prognosis[17-19].

To further determine whether aberrant expression of γ-synuclein is involved in the development of CRC, and identify a new biomarker or a potential target for diagnosis and treatment, we examined expression patterns of γ-synuclein in CRC tissues, analyzed the relationship between γ-synuclein protein expression and clinicopathological factors of CRC, and then studied the effects of γ-synuclein down-regulation on colorectal cancer cell line HCT116 biological features in vitro.

MATERIALS AND METHODS

Tissue samples and cell lines

Fifty-four CRC samples and 30 NNAT samples 5 cm away from the tumor were obtained from patients undergoing CRC surgery between January 2005 and October 2008 at the Department of General Surgery, Ruijin Hospital, Shanghai, China. After washing with RNase-free 9 mL/L NaCl to remove blood after surgery, one half of each sample was snap-frozen in liquid nitrogen immediately and stored at -80°C for RNA extraction, and the other half was fixed in 40 g/L formalin for histological assessment. For tumor samples, non-tumor portions were trimmed off from the frozen tumor blocks and the selected areas had more than 80% tumor cells as shown by histological assessment. Tumors were staged using the TNM and World Health Organization classification systems. The ethics committee at Ruijin Hospital approved the use of these tissues for research purposes. The colorectal cancer cell line HCT116 (ATCC No. CCL-247) was grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Life Technologies Inc, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO, USA). The HCT116 cells were maintained in a humidified incubator at 37°C with 50 mL/L CO_2, fed every 3 d with complete medium, and subcultured when confluence was reached.

Total RNA extraction and real-time quantitative reverse transcription PCR (RT-PCR) of γ-synuclein mRNA

Cultured cells were washed twice with phosphate-buffered saline (PBS) and harvested, and tissues were ground into fine powder in liquid nitrogen before extraction of RNA. Total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis from 1 μg of RNA was performed with a reverse transcription system kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Real-time quantitative PCR was carried out in 96-well polypropylene microplates on an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) using SYBR Green Realtime PCR Master Mix (TOYOBO, Tokyo, Japan) according to the manufacturer's instructions. Amplification was carried out with the following profile: 1 cycle at 95°C for 1 min, and 40 cycles each at 95°C for 15 s, 59°C for 15 s, 72°C for 45 s. All PCR reactions were performed in triplicate wells. Specificity of the resulting PCR products was confirmed by melting curves. H_2O was used as a negative control. Data were analyzed by using the comparative Ct (ΔCt) method and the amount of γ-synuclein relative to GAPDH was expressed as $10\,000 \times 2^{-(\Delta Ct)}$. Table 1 provides the sequences of the primers used in these studies.

Table 1 Sequences of γ-synuclein gene-specific primers

Primer	Sequence (5'-3')	Product size (bp)
γ-synuclein-5'	GGAGGACTTGAGGCCATCTG	73 (339 to 411)
γ-synuclein -3'	CTCCTCTGCCACTTCCTCTTTC	
GAPDH-5'	GGACCTGACCTGCCGTCTAG	100 (831 to 930)
GAPDH-3'	GTAGCCCAGGATGCCCTTGA	

Immunohistochemistry (IHC) analysis

Unstained 4 mm sections were cut from the tissue paraffin-embedded block and deparaffinized in xylene, and the slides were bathed in 0.01 mol/L sodium citrate and heated in a microwave oven for 12 min. The sections were incubated with mouse anti-γ-synuclein monoclonal antibody (SantaCruz, CA, USA) at a dilution of 1:100 and kept at 4℃ overnight. Negative control slides were treated with non-immunized mouse immunoglobulin fraction under equivalent conditions. For the secondary developing reagents, a labeled streptavidin-biotin kit (DAKO, CA, USA) was used. Slides were developed with diaminobenzaminidine and counterstained with hematoxylin. Positive cases were defined by the presence of intracellular staining with red/brown color in epithelial cells. Negative cases were defined by the absence of specific intracellular staining as seen in negative controls. Samples were evaluated under light microscopy independently by two pathologists without prior knowledge of the patients' clinical data.

Plasmid construction

The pGCsi-U6/neo/GFP plasmid (Shanghai GeneChem., Ltd, Shanghai, China), a siRNA expression vector containing a green fluorescent protein gene (GFP) under a separate promoter for tracking the transfection efficiency, was used for the cloning of small synthetic oligonucleotides that encode two complementary sequences of 19 nucleotides separated by a short spacer region of 9 nucleotides. Three sequences (as shown in Table 2) unique to the coding region of γ-synuclein were designed and inserted between the *Bam*H I and *Hin*dⅢ sites of the pGCsi-U6/neo/GFP plasmid. The positive clones were confirmed by sequencing.

siRNA transfection and selection of HCT116 stable transfectants

One day before transfection, HCT116 cells were plated in a six-well plate with 1×10^5 cells per well using culture medium without antibiotics. The cells were transfected with 3.0 μg/well of pGCsi-U6/neo/GFP-vector and pGCsi-siRNA plasmids, respectively, using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Cells transfected with medium but lacking DNA served as controls. Fresh growth medium was replaced after 4 h of transfection. Cells were passaged at a 1:10 dilution at 24 h after transfection and cultured in medium supplemented with G418 (Promega, Madison, WI) at 1000 μg/mL for 4 wk. Stably transfected clones were picked and main-tained in medium containing 400 μg/mL G418 for further study.

Western blotting analysis

Cells were harvested and lysed with mammalian protein extraction reagent (Pierce Rockford, IL, USA). Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Pierce Rockford, IL, USA). Samples containing 50 μg of protein were mixed with 2 × sodium dodecyl sulfate (SDS) gel-loading buffer (100 mmol/L Tris-CL, 200 mmol/L dithiothreitol, 4% SDS, 0.2% bromophenol blue, and 20% glycerol), boiled for 5 min, loaded onto each lane of 15% acrylamide gel in a minigel apparatus (Bio-Rad, Richmond, CA, USA), and separated by SDS-PAGE. The separated proteins were electrophoretically transferred to a Sequi-blot PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). After being incubated with mouse anti-γ-synuclein monoclonal antibody (SantaCruz, CA, USA) (1:500), and goat anti-mouse IgG-AP antibody (SantaCruz, CA, USA) (1:5000) respectively, immune complexes were detected using BCIP/NBT Alkaline Phosphatase Color Development Kit (Sigma, St. Louis, MO). GAPDH served as a loading control.

Cell proliferation analysis

Cells were seeded onto 96-well plates at a density of 2×10^3 cells per well in 100 μL medium containing 10% FBS. The number of viable cells was determined daily with WST-8 cytotoxicity assay using the Cell Counting Kit-8 (Dojindo, Japan). Briefly, 10 μL of the CCK-8 solution was added to each well of the microplate, and the absorbance at 490 nm was measured by a microplate reader (μQuant, Bio-Tek, USA) after 4 h incubation.

Soft agar colony formation assay

Cells (1×10^3) were trypsinized to a single-cell suspension and then plated in triplicate onto six-well plates in complete culture medium containing 0.3% agar on top of 0.6% agar in the same medium. Cultures were maintained at 37℃ in the 50 mL/L CO₂ incubator for 15 d. The colonies were fixed with 70% ethanol, and stained with 0.2% crystal violet. The colonies containing at least 50 cells were counted. Colony formation rates were calculated as the number of colonies relative to that of cells initially plated in a well (1×10^3), and expressed as mean ± SD.

Cell migration and invasion assay

Boyden chambers with 8 μm polycarbonate membranes in 24-well dishes (Nucleopore, Pleasanton, CA) were used for migration assay, and chambers coated with 4 mg/mL growth factor reduced Matrigel (50 μg; Collaborative Biomedical, Becton Dickinson Labware) were used for the invasion assay. Cells (1×10^5) were resuspended in serum-free DMEM and added to the upper chamber in triplicate. Consecutively, DMEM with 10% FBS was added to the lower chamber. Chambers were incubated at 37℃ in the 50 mL/L CO₂ incubator for 24 h. After incubation, the chambers were fixed with 70% ethanol, and stained with 0.2% crystal violet. Cells on the surface of the upper chamber were removed by

Table 2 Sequences of small synthetic oligonucleotides unique to the coding region of γ-synuclein

Oligonucleotides	Sequence (5'-3')
siRNA1: Target sequence	AAGACCAAGGAGAATGTTGTA
Sense strand	5'-GATCCGACCAAGGAGAATGTTGTATTCAAGAGATACAACATTCTCCTTGGTCTTTTTGGAAA-3'
Antisense strand	5'-AGCTTTTCCAAAAAAGACCAAGGAGAATGTTGTATCTCTTGAATACAACATTCTCCTTGGTCG-3'
siRNA2: Target sequence	AAGGAGAATGTTGTACAGAGC
Sense strand	5'-GATCCGGAGAATGTTGTACAGAGCTTCAAGAGAGCTCTGTACAACATTCTCCTTTTTTGGAAA-3'
Antisense strand	5'-AGCTTTTCCAAAAAAGGAGAATGTTGTACAGAGCTCTCTTGAAGCTCTGTACAACATTCTCCG-3'
siRNA3: Target sequence	AATGTTGTACAGAGCGTGACC
Sense strand	5'-GATCCGTGTTGTACAGAGCGTGACCTTCAAGAGAGGTCACGCTCTGTACAACATTTTTTGGAAA-3'
Antisense strand	5'-AGCTTTTCCAAAAAATGTTGTACAGAGCGTGACCTCTCTTGAAGGTCACGCTCTGTACAACACG-3'

Table 3 The expression of γ-synuclein in CRC and NNAT

Group	n	γ-synuclein mRNA expression		P value	γ-synuclein protein expression		P value
		Median (Range)	mean ± SD		Positive	Negative	
CRC	54	11.06 (38.24)	14.15 ± 10.14	0.02[1]	26	28	0.036[2]
NNAT	30	6.11 (29.4)	9.46 ± 8.47		7	23	

[1]Calculated by the Mann-Whitney U-test; [2]Calculated by the Fisher's exact test. CRC: Colorectal cancer; NNAT: Nonneoplastic adjacent tissues.

Table 4 Correlation between γ-synuclein protein expression and clinicopathological factors of colorectal cancer patients

Variable	n	γ-synuclein protein expression		P value[1]
		Positive	Negative	
Age				
≥ 60	28	14	14	
< 60	26	12	14	0.793
Sex				
Male	32	14	18	
Female	22	12	10	0.580
Histological types				
Differentiated	47	21	26	
Undifferentiated	7	5	2	0.243
Stage				
I	13	2	11	
II	11	5	6	
III	24	14	10	
IV	6	5	1	0.020
Lymph node invision				
Positive	29	18	11	
Negative	25	8	17	0.033
Distant metastasis				
Positive	6	5	1	
Negative	48	21	27	0.095

[1]Statistical significance was determined with Fisher's exact test.

swiping with cotton swabs. The amount of migration and invasion cells in the lower chamber was determined under light microscopy. The data are means ± SD of counting ten random fields of vision.

Statistical analysis

Statistical analyses were performed using SPSS11.0 software (Shanghai jiaotong University School of Medicine, Shanghai, China). Mann-Whitney U-test was used to analyze γ-synuclein mRNA expression in paired CRC and NNAT samples. The Fisher's exact test was used to test the significance of the difference in frequency of γ-synuclein protein expression between CRC and NNAT samples, and to assess the relationship between the protein expression and clinicopathological characteristics of CRC. Two-way analysis of variance (ANOVA) was performed to detect the effects of γ-synuclein knockdown on cell proliferation, soft agar colony formation, cell migration and invasion, and Student-Newman-Keuls test was used to detect the difference between any two groups. $P < 0.05$ was selected as the statistically significant value.

RESULTS

Examination of γ-synuclein mRNA and protein expression in CRC and NNAT samples

γ-synuclein mRNA expression in 54 CRC and 30 NNAT samples was examined using Q-RT-PCR. Table 3 shows the results of Q-RT-PCR. γ-synuclein mRNA expression in CRC samples ranged from 1.12 to 39.36 with a median value of 11.06, while in matched NNAT samples it ranged from 0.81 to 30.21 with a median value of 6.11. The γ-synuclein mRNA expression levels in CRC samples were significantly higher than those in NNAT samples ($P = 0.02$).

The expression and subcellular localization of γ-synuclein protein were evaluated *via* IHC in 54 CRC and 30 NNAT samples. NNAT sections showed either no protein expression ($n = 23$) or relatively weak protein expression ($n = 7$) in the cytoplasm of epithelium cells (Figure 1A). Conversely, the immunoreactive patterns of γ-synuclein were predominantly positively identified in the cytoplasm, sometimes in the nucleus of cancer cells (Figure 1B) with a relatively high frequency of 48.1% (Table 3, $P = 0.036$).

In the analysis of γ-synuclein protein expression in CRC tissues and various CRC patients' clinicopathologic variables, the results clearly showed a close association of γ-synuclein staining with clinical stage and lymph

Figure 1 γ-synuclein protein expression detected by IHC. A: Negative γ-synuclein immunostaining in NNAT; B: Positive γ-synuclein immunostaining in the cytoplasm of CRC cells (original magnification × 200). IHC: Immunohistochemistry; NNAT: Nonneoplastic adjacent tissues; CRC: Colorectal cancer.

Figure 2 The siRNA plasmid can specifically knock down γ-synuclein expression in the HCT116 cells. A: γ-synuclein mRNA expression detected by Q-RT-PCR after transient transfection. Values were γ-synuclein/GAPDH expression of one group cells relative to that of parental HCT116 cells; B: The vision of HCT116 stable transfectants in fluorescence microscopy and light microscopy; C: γ-synuclein protein expression detected by western blotting after selection of stable transfectants. Control: Parental HCT116 cells; pGCsi-vector: HCT116/vector cells; siRNA1 (2,3): HCT116/siRNA1 (2,3) cells.

node involvement (Table 4). The frequency of positive γ-synuclein staining was much higher in later stage tumors than in earlier stage tumors ($P = 0.02$), and was much higher in lymph node-positive tumors than in lymph node-negative ones ($P = 0.033$). However, there was no significant correlation between the γ-synuclein protein expression and other clinicopathologic characteristics.

Identification of the effective siRNA target sequence

We first investigated three recombinant γ-synuclein-specific siRNA plasmids, pGCsi-siRNA1, pGCsi-siRNA2, and pGCsi-siRNA3. These γ-synuclein-specific siRNA plasmids and pGCsi-U6/neo/GFP-vectors were transfected into HCT116 cells. After 24 h, these cells were examined for γ-synuclein expression by Q-RT-PCR. As shown in Figure 2A, γ-synuclein levels were different in the transfected HCT116 cells containing siRNA1, siRNA2, siRNA3 and the vector. There were no significant changes of γ-synuclein mRNA expression in pooled HCT116/vector, HCT116/siRNA1, and HCT116/RNA3 cells. However, in HCT116/siRNA2 cells, γ-synuclein mRNA levels were significantly low, compared with parental HCT116 cells and HCT116/vector cells. Then stable transfected clones of HCT116/siRNA2 cells were selected with G418. After 4 wk of the selection, stable transfected clones were established (Figure 2B). These clones were examined for γ-synuclein expression by western blotting, the result of which suggested that pGCsi-siRNA2 plasmid could specifically

knock down γ-synuclein protein expression in the stable transfected HCT116 cells (Figure 2C).

Inhibition of cell proliferation and colony formation by γ-synuclein knockdown

In vitro cell proliferation tests, two clones derived from stable transfectants with control vector, siRNA2 plasmids, and parental HCT116 cells were chosen for further study. As shown in Figure 3A, γ-synuclein knockdown suppressed cancer cell growth significantly in regular medium. The number of pooled HCT116/siRNA2 cells was significantly reduced by 48, 72, 96, and 120 h after plating, respectively, compared with the HCT116/vector and parental HCT116 cells ($P < 0.05$).

Subsequent soft agar colony formation assay was

Figure 3 Inhibition of cell proliferation and colony formation by γ-synuclein knockdown. A: The role of γ-synuclein in regulating HCT116 cell proliferation was determined by CCK8 assay. Values were the mean ± SD of absorbance at 490 nm for five independent experiments; [a]P < 0.05; B: The colony formation rates were analyzed by soft agar assay. Values were the mean ± SD for three independent experiments; [a]P < 0.05; C: The colonies were stained with 0.2% crystal violet and photographed. Control: Parental HCT116 cells; pGCsi-vector: HCT116/vector cells; siRNA2: HCT116/siRNA2 cells.

done to evaluate the tumorigenicity of γ-synuclein down-regulated cells *in vitro*. Colony formation rates were 29.4% ± 4.5%, 32.1% ± 5.8% and 9.6% ± 2.9% in parental HCT116 cells, HCT116/vector and HCT116/siRNA2 cells (Figure 3B, P < 0.05). The size of colonies formed by HCT116/siRNA2 cells was much smaller than that of two control cells, and there were no significant differences between parental HCT116 cells and HCT116/vector cells colonies both in number and size (Figure 3C).

Inhibition of cell migration and invasion by γ-synuclein knockdown

The close correlation of γ-synuclein protein expression and CRC staging suggests that γ-synuclein might be involved in advanced stage tumor progression and metastasis. We used an *in vitro* reconstituted basement membrane (Matrigel) invasion assay to determine the effect of γ-synuclein on cell migration and invasion

(Figure 4A). The results showed that the amount of migration HCT116/siRNA2 cells in the lower chamber was much less than that of parental HCT116 cells, and HCT116/vector cells (Figure 4B, P < 0.05). It was demonstrated that γ-synuclein down-expression led to suppression of cell motility in HCT116 cells. Similarly, we observed that γ-synuclein down-expression led to decreased cell invasion in HCT116 cells. HCT116/siRNA2 cells showed a significant decrease in the number of invasive cells compared to that of two control cells (Figure 4C, P < 0.05).

DISCUSSION

γ-synuclein belongs to the synuclein protein family, consisting of α-, β-, and γ-synuclein, which are abundantly expressed in nervous tissues[9]. Although there has been a report that showed down-regulation of γ-synuclein in human ESCC, more studies support the statement of over-expression of γ-synuclein in more types of cancer[14,15,20-22]. The loss of tissue-specificity raises questions about the involvement of γ-synuclein in the process of tumorigenesis and metastasis, and presents the possibility to use γ-synuclein as a potential target for early diagnosis and treatment. However, little is known about the expression and biological effects of γ-synuclein in CRC.

In the current study, we showed that γ-synuclein expression levels were higher in CRC tissues than those in matched NNAT. IHC analysis also confirmed that CRC tissues exhibited abundant γ-synuclein expression in the cytoplasm of cancer cells, in contrast to NNAT, which did not appear to exhibit γ-synuclein expression or exhibit faint expression.

To further investigate how γ-synuclein contributes to the biological behavior changes in CRC, we constructed specific γ-synuclein siRNA plasmids and established permanent transfected HCT116 cells to investigate the potential role of γ-synuclein in the progression of CRC. Consistent with our observation that the γ-synuclein expression levels were lower in NNAT than in CRC tissues, the cell growth and colony formation rate decreased in HCT116/siRNA2 cells with reduced expression of γ-synuclein, compared with parental HCT116 cells, and HCT116/vector cells, which gave evidence that γ-synuclein indeed had the ability to promote cell growth. Previous studies have demonstrated that ectopic expression of γ-synuclein increased breast cancer cell growth in anchorage dependent and independent conditions through interaction with BubR1, a mitotic checkpoint kinase, which led to inhibition of the mitotic checkpoint control[12,13,23]. It has also been shown that γ-synuclein could constitutively activate ERK1/2, and increase ER-α transcriptional activity through an HSP-based multiprotein chaperone complex, which led to an increase in breast cancer or ovarian cell survival and proliferation[24-28].

In addition to the effects on cell growth, γ-synuclein is associated with cell invasion and metastasis. In previous *in vitro* studies, retinoblastoma cell lines

A

B

C

Figure 4 Inhibition of cell migration and invasion by γ-synuclein knockdown. A: Boyden chambers with 8 μm polycarbonate membranes coated with or without 4 mg/mL growth factor-reduced Matrigel were used for migration or invasion assay. The chambers were stained with 0.2% crystal violet and analysed by photography, and the stained cells were migration or invasion cells in the lower chamber; B, C: The migration or invasion cells were counted in ten random fields of vision. Values were the number of cells relative to that of parental HCT116 cells, and expressed as mean ± SD for three independent experiments; [a]$P < 0.05$. Control: Parental HCT116 cells; pGCsi-vector: HCT116/vector cells; siRNA2: HCT116/siRNA2 cells.

overexpressing γ-synuclein were shown to have higher MMP9 protein levels and activity, which were enhanced in cell motility and invasion[29]. In *in vivo* studies, γ-synuclein was also shown to cause metastasis in nude mice on implanting γ-synuclein expressing MDA-MB 435 breast cancer cells in fat pads of these mice. IHC results showed mice given implants of γ-synuclein positive cells displayed an increase in tumor growth, and metastasis into axillary lymph nodes and lungs, compared with mice given control implants[11]. In the current study, we presented the clinical evidence and experimental data to indicate that γ-synuclein played a key role in CRC invasion and metastasis. We analyzed the relationship between γ-synuclein protein expression in 54 CRC tissues and the clinicopathologic characteristics of patients with CRC, and found that the frequency of positive γ-synuclein staining was much higher in tumors with lymph node-positive or later stage than in lymph node-negative or earlier stage tumors ($P < 0.05$).

Our results also showed that there was a tendency for high γ-synuclein expression with metastasis. However, this difference is not significant ($P = 0.095$), possibly because of our relatively small sample size. Consistent with the clinical evidence, we also observed that reduced γ-synuclein expression led to decreased cell motility and invasion in HCT116 cells. All these results gave evidence that γ-synuclein may indeed function as key mediators of cancer cell growth and metastasis and will be a promising target for CRC treatment. Biological treatment targeting γ-synuclein has been studied in breast cancer, and Singh *et al*[30] have designed and characterized a γ-synuclein targeting peptide inhibitor, which associates with γ-synuclein and enhances sensitivity of breast cancer cells to antimicrotubule drugs.

In summary, we have shown that strong expression of γ-synuclein occurred in CRC tissues and correlated with the advancement of tumor stage and lymph node involvement. With a vector-based siRNA method, we

showed that stable down-regulation of γ-synuclein expression inhibited CRC cell growth, colony formation, motility and invasion. Therefore, γ-synuclein is likely to play an important role in the progression of CRC. Further study is needed to prove the value of γ-synuclein as a biomarker or molecular target for CRC diagnosis, prognosis evaluation and therapy. The following research may encompass: 1. Examination of γ-synuclein expression in serum or stool samples from patients with CRC; 2. Relationship between γ-synuclein expression and 5-year survival rate of CRC patient; 3. Delineation of the interaction between γ-synuclein and other proteins, and γ-synuclein targeting biotherapy in cell culture and animal model.

ACKNOWLEDGMENTS

COMMENTS

Background

Colorectal cancer (CRC) is the third most common malignancy and the second leading cause of cancer-related deaths worldwide. The conventional therapies involving surgery and adjuvant therapy do not significantly prolong the survival period. It is necessary to identify a reliable biomarker or a potential target for diagnosis and treatment.

Research frontiers

γ-synuclein, a member of synuclein protein family, is abundantly expressed in brain tissue and presynaptic terminals. However, the tissue specificity of γ-synuclein expression appears to be lost in some types of cancer. Particularly in breast cancers, γ-synuclein promotes malignancy of breast cancer cell lines in *in vitro* studies and animal models. However, little is known about γ-synuclein in colorectal cancer.

Innovations and breakthroughs

The results of this study provide strong evidence suggesting that γ-synuclein expression is up-regulated in CRC tissues, and is significantly correlated with clinical stage and lymph node involvement of CRC. The authors also constructed specific γ-synuclein siRNA plasmids and established a permanent transfected colorectal cancer cell line HCT116, and found that down-regulation of expression of γ-synuclein in HCT116 cells could inhibit the growth, colony formation rate, and migration and invasion ability of HCT116 cells.

Applications

These results demonstrate that γ-synuclein indeed may function as a key mediator of cancer cell growth and metastasis and will be a promising target for CRC diagnosis, prognosis evaluation and biotherapy.

Terminology

Mitotic checkpoint is a cellular inherent mechanism, which strictly controls the cell division cycle and makes sure there is faithful cell replication. G418, a kind of aminoglycoside antibiotic, is a most common resistance selection reagent, used for stable transfectants in molecular biology tests.

Peer review

The paper investigated expression pattern of γ-synuclein in CRC tissues, and the effects of γ-synuclein on CRC cell line HCT116 biological features also were studied *in vitro*. The study is well conducted and the results is clear.

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ORIGINAL ARTICLE

Effects of LY294002 on the invasiveness of human gastric cancer *in vivo* in nude mice

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Abstract

AIM: To investigate the effects of class I phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 on the invasiveness and related mechanisms of implanted tumors of SGC7901 human gastric carcinoma cells in nude mice.

METHODS: Nude mice were randomly divided into model control groups and LY294002 treatment groups. On days 5, 10 and 15 after treatment, the inhibitory rate of tumor growth, pathological changes in tumor specimens, expression levels of matrix metalloproteinase (MMP)-2, MMP-9, CD34 [representing microvessel density (MVD)] and vascular endothelial growth factor (VEGF), as well as apoptosis indexes in tumor samples were observed.

RESULTS: In this study, we showed that treating the tumors with LY294002 could significantly inhibit carcinoma growth by 11.3%, 29.4% and 36.7%, after 5, 10 and 15 d, respectively, compared to the control group. Hematoxylin & eosin staining indicated that the rate of inhibition increased progressively (23.51% ± 3.11%, 43.20% ± 3.27% and 63.28% ± 2.10% at 5, 10 and 15 d, respectively) along with apoptosis. The expression of MMP-2 was also downregulated (from 71.4% ± 1.6% to 47.9% ± 0.7%, 31.9% ± 0.9% and 7.9% ± 0.7%). The same effects were observed in MMP-9 protein expression (from 49.4% ± 1.5% to 36.9% ± 0.4%, 23.5% ± 0.9% and 7.7% ± 0.6%), the mean MVD (from 51.2% ± 3.1% to 41.9% ± 1.5%, 30.9% ± 1.7% and 14.9% ± 0.8%), and the expression of VEGF (from 47.2% ± 3.1% to 25.9% ± 0.5%, 18.6% ± 1.2% and 5.1% ± 0.9%) by immunohistochemical staining.

CONCLUSION: The class I PI3K inhibitor LY294002 could inhibit the invasiveness of gastric cancer cells by downregulating the expression of MMP-2, MMP-9, and VEGF, and reducing MVD.

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INTRODUCTION

Gastric cancer is the fourth most frequently diagnosed malignancy worldwide, accounting for 12% of all cancer-related deaths. In Asia and parts of South America in particular, gastric cancer is the most common epithelial malignancy and a leading cause of cancer-related death[1,2]. The major cause of death from gastric cancer

is metastasis that is usually resistant to conventional treatment.

Invasiveness and metastasis are the leading biological characteristics of a malignant tumor, and have a close relation with factors such as movement of tumor cells, apoptosis and metastasis-associated genes. Matrix metalloproteinase (MMP)-2, MMP-9, intratumoral microvessel density (MVD) and vascular endothelial growth factor (VEGF) are important angiogenic factors, which have a higher expression in tumor tissues, may induce angiogenesis in the tumor and play an important role in metastases, invasion and prognosis of gastric cancer[3-7]. However, the underlying mechanism remains uncertain.

The phosphatidylinositol 3-kinase (PI3K) pathway plays a central role in the regulation of cell proliferation, growth, differentiation and survival[8,9]. Dysregulation of this pathway is frequently observed in a variety of tumors, including brain tumors and breast, ovarian and other carcinomas[10-12]. Therefore, inhibition of PI3K signaling is under investigation as a potentially useful approach for cancer treatment. However, the detailed mechanisms are poorly understood.

LY294002 is a specific inhibitor of class I PI3K. The antitumor activity of LY294002 may be related to the induction of apoptosis of tumor cells, but the precise mechanism of its antitumor activity is not well understood.

In the present study, we investigated whether the inhibition of class I PI3K by LY294002 restricted the growth and invasiveness of implanted tumors of SG7901 cells in nude mice. Through this research, we would suggest that the class I PI3K inhibitor LY294002 could play an important role in the inhibition of progression of gastric carcinoma, and may be of value in effective clinical antitumor therapy.

MATERIALS AND METHODS

Materials

SG7901 cells and female Balb/c nude mice (4 wk old, 16-18 g) were purchased from the Chinese Academy of Sciences (Shanghai, China). RPMI-1640 medium was purchased from Gibco (Rockville, MD, USA). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Material Co. (Hangzhou, China), anti-MMP-2 (sc-13595), anti-MMP-9 (sc-21733), anti-CD34 (sc-19621) and anti-VEGF (sc-57496) monoclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (USA). SP kit was purchased from Maixin Biotechnology (Fuzhou, China).

Drug preparation

LY2940029 was purchased from Cell Signaling Technology (Beverly, MA, USA) and diluted in phosphate buffered saline (PBS) to create a stock solution that was stored according to the manufacturer's instructions. The final concentration of the LY294002 solution used was 500 μmol/L. This concentration of LY294002 was selected on the basis of our experiments on implanted tumors of human SGC7901 cells in nude mice.

Cell culture

SGC7901 cells were maintained in RPMI-1640 medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Hangzhou Sijiqing Biological Engineering Material Co.), 0.03% L-glutamine (Sigma) and incubated in a 5% CO_2 atmosphere at 37℃. Cells in the mid-log phase were used in the experiments.

Inhibitory rate of tumor growth

A transplanted tumor model was established by injecting 1×10^9 cells/mL human SGC7901 into the subcutaneous tissue in the armpit of nude mice. After 10 d, 25 nude mice were randomly divided into four groups, and 0.2 mL normal saline solution, LY294002 (500 μmol/L) was directly injected adjacent to the tumor, twice at a 2 d interval (5 d group), three times at 2 d intervals (10 and 15 d group). There were six nude mice given saline, 19 nude mice given LY294002 (six in the 5 d group, six in the 10 d group, seven in the 15 d group). Changes in tumor volume = $(\pi/6) \times$ abc (a: the length of the tumor, b: the width of the tumor and c: the depth of the tumor) were measured at 5, 10, 15 d after LY249002 treatment and the tumor inhibition rate of each group was calculated.

Inhibitory rate of tumor growth = $[C(V_1 - V_0) - T(V_1 - V_0)]/C(V_1 - V_0)$

Where C is the control group, T is the treated group, V_1 is the volume before treatment (mm^3), and V_0 is the volume after treatment (mm^3).

Hematoxylin and eosin (HE) and immunohistochemical staining

Tumor specimens were taken from areas next to the margin of the tumors as well as from more central areas. All the specimens were formalin-fixed, paraffin-embedded and pathologically diagnosed to be gastric carcinoma and evaluated using HE for conventional histological assessment. Histological characteristics were reviewed by two pathologists.

The tumor samples were cut into 4 μm thick slices and fixed in acetone. After washing in PBS, slices were incubated in 0.3% H_2O_2 solution at room temperature for 5 min. Slices were then incubated with anti-MMP-2 or anti-MMP-9 or anti-MVD or anti-VEGF monoclonal antibody at a 1:300 dilution at 4℃ overnight. After washing in PBS, the second antibody, biotinylated anti-rat IgG, was added and the cells were incubated at room temperature for 1 h. After washing in PBS, avidin-biotin complex was added and then incubated at room temperature for 10 min. Diaminobenzidine was used as the chromogen. After 10 min, the brown color signifying the presence of antigen bound to antibodies was detected by light microscopy. Controls were prepared in the same manner as the experimental group, except for incubation with the primary antibody. The positive rate (PR) was calculated as follows: PR = (number of positive cells/total number of cells) × 100%.

Immunohistochemical assessment

The cytoplasm with MMP-2, MMP-9 and VEGF

appeared as brown in color. Immunohistochemical staining was independently evaluated by two pathologists, who were blinded to the experimental data. Two hundred cells were chosen under the microscope to evaluate the stained cell number against the total cell number in the field. Based on the positive cell number, the criteria were set as follows: negative (-) having a positive cell number < 10%; (+) having 11%-50% positive cells; (++) having 51%-75% positive cells; and (+++) having > 75% positive cells. The results of staining of MMP-2, MMP-9 and VEGF were classified into negative (staining of $\leqslant$ 10% of cells) or positive (staining of > 10% of cells).

Evaluation of MVD

MVD-CD34 of tumor tissues were evaluated at low power field ($\times$ 40). The tissue sections were screened and five areas with the most intense neovascularization (hot spots) were selected. Microvessel counts of these areas were performed at high power field ($\times$ 200). Cases with discrepancies in scores between the two investigators were evaluated jointly to obtain a common consensus score. Any positively stained endothelial cell or endothelial cell cluster that was clearly separated from the adjacent microvessels, tumor cells and connective elements was counted as one microvessel, irrespective of the presence of a vessel lumen. An automated microvessel count/field was computed in each hot spot and the mean microvessel count of the 5 most vascularized areas was taken as the MVD-CD34, which was expressed as the absolute number of microvessels per 0.74 mm^2 ($\times$ 200 field). The microvessel count that was higher than the median of microvessel count was taken as high MVD-CD34 and the microvessel count that was lower than the median of microvessel count was taken as low MVD-CD34.

Statistical analysis

All data are presented as mean% ± SD. Statistical analysis was carried out using ANOVA followed by Dunnett's t-test, with $P < 0.05$ taken to indicate significance.

RESULTS

Effect of the LY294002 on tumor growth

SGC7901 cells (1×10^9) were injected subcutaneously into the armpit of nude mice. Within 1 wk, visible tumors had developed at the injection sites. To determine the therapeutic effectiveness of LY294002, intratumoral injection of LY294002 was started after the volume of the implanted tumor reached 20 mm^3, and was repeated every 2 d for a total of three times. As shown in Table 1, LY294002 markedly suppressed tumor growth compared with PBS alone ($P < 0.01$). No gross adverse effects, i.e. the loss of body weight, were observed during the experimental periods. Moreover, LY294002 inhibited the proliferation of the implanted tumor of human SGC7901 cells in nude mice in a time-dependent fashion. The inhibition rate of the tumors

Table 1 Comparison of different pathological indexes for volume of tumors (mean ± SD)

Groups	Number of animals		Volume of tumors (mm³)		Inhibition rate (%)
	Beginning	End	Beginning	End	
Control	5	5	20.6 ± 1.1	536.3 ± 12.4	
PBS	5	5	20.5 ± 1.2	525.7 ± 6.9	
LY294002	15	15			
5 d	5	5	20.9 ± 1.4	476.2 ± 6.7	11.3 ± 1.13[a]
10 d	5	5	20.8 ± 1.6	435.9 ± 7.4	29.4 ± 1.47[a]
15 d	5	5	20.4 ± 1.7	379.2 ± 9.3	36.7 ± 2.12[a]

[a]$P < 0.05$ vs control.

was 11.31% ± 13% on day 5, 29.4% ± 1.47% on day 10 and reached 36.7% ± 2.12% on day 15.

LY294002 reduced cell viability and induced apoptosis of transplanted SGC7901 tumor cells

Treatment with LY294002 for 5, 10 and 15 d in SGC7901 cells produced intensive HE staining indicating apoptosis. A significant increase in apoptosis was observed along with the time that tumors were treated (Figure 1). After 5 d, the rate of inhibition reached 23.51% ± 3.11%. The rate of inhibition rose when the experimental time increased, reaching 43.20% ± 3.27% on day 10 and 63.28% ± 2.10% on day 15 after LY294002 treatment. All the results indicated that LY294002 induced apoptosis (Figure 1E-H).

Similarly, in the lymphatic vessels, the effect of the drug increased with time. The invasion rate of the tumor cells in the lymphatic vessels decreased from 75.69% ± 3.5% to 63.78% ± 4.3%, 45.62% ± 3.1% and 28.17% ± 2.7% at 5, 10 and 15 d, respectively, after treatment with LY294002 (Figure 1A-D).

LY294002 inhibited the expression of MMP-2 and MMP-9 proteins

Positive staining was distributed in the cell membrane and cytoplasm (Figures 2 and 3). The PR of MMP-2 protein downregulated was from 71.4% ± 1.6% in the control group to 47.9% ± 0.7%, 31.9% ± 0.9% and 7.9% ± 0.7% at 5, 10 and 15 d, respectively, after treatment with LY294002 for (Figure 2E-H). Significant differences in the expression were observed between the 500 μmol/L LY249002 group and the control group at every time point ($P < 0.05$). Similarly, the expression of MMP-9 decreased from 49.4% ± 1.5% to 36.9% ± 0.4%, 23.5% ± 0.9%, 7.7% ± 0.6%, respectively (Figure 3E-H).

In the lymphatic vessels, we found that the expression of MMP-2 in the tumor cells also decreased from 59.23% ± 6.2% to 45.46% ± 7.3%, 32.14% ± 1.9%, 14.01% ± 3.9% at the various time points (Figure 2A-D). Interestingly, MMP-9 shared the same characteristics in expression as that of MMP-2, which fell from 39.95% ± 5.7% to 25.32% ± 6.5%, 12.84% ± 2.8%, 4.01% ± 5.9% (Figure 3A-D).

LY294002 decreased the expression of MVD and VEGF

Positively stained particles of MVD-CD34 were mainly distributed in the plasmalemma of cells, though some

Figure 1 Pathological changes in lymphatic vessels and SGC7901 tumor cells in the model control groups (A, E) and treatment groups (B-D, F-H). Lymphatic vessels (A-D) (HE, × 200) and tumor cells (E-H) (HE, × 100); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

were expressed in the cytoplasm (Figure 4). The mean MVD was much lower in the tumors treated with LY294002 than in the control group.

According to the PR of MVD protein of the control group and the experimental group after treatment with LY294002 5, 10, 15 d, the expression of MVD was downregulated from 51.2% ± 3.1% to 41.9% ± 1.5%, 30.9% ± 1.7% and 14.9% ± 0.8% after 5, 10 and 15 d, respectively (Figure 4E-H). A significant difference in positive expression was observed between the 500 μmol/L

LY294002 group and the control group at every time point ($P < 0.05$). Similarly, the expression of VEGF decreased from 47.2% ± 3.1% to 25.9% ± 0.5%, 18.6% ± 1.2% and 5.1% ± 0.9%, respectively (Figure 5E-H).

In the vascular area, we found that the effect of the agent increased with time, and the MVD of the tumor cells decreased from 37.65% ± 2.7% to 27.24% ± 5.3%, 10.64% ± 3.5%, 5.26% ± 1.3% at 5, 10 and 15 d, respectively (Figure 4A-D). The VEGF expression was also downregulated from 34.25% ± 1.7% to 25.21% ±

Figure 2 Pathological changes of the expression of MMP-2 in lymphatic vessels and SGC7901 tumor cells in the model control groups (A, E) and treatment groups (B-D, F-H). Lymphatic vessels (A-D) (HE, × 200) and tumor cells (E-H) (HE, × 100); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

2.3%, 11.34% ± 5.1% and 3.26% ± 0.3%, respectively (Figure 5A-D).

DISCUSSION

Currently only a few chemotherapeutic drugs are effective for the treatment of human gastric carcinoma[13] and there is an increasing interest in the use of drugs to prevent its occurrence or invasiveness. The lipid kinase PI3K is a proto-oncogene that generates 3'-phosphoinositides at the cell membrane. The best-characterized inhibitors of PI3K are LY294002 and wortmannin. LY294002 effectively inhibits the growth of many types of tumor cells *in vitro* and *in vivo*, *via* the inhibition of PI3K and downstream components of the pathway[14-17]. It is possible that LY294002 inhibits proliferation and induces apoptosis in SGC7901 cells through inhibiting class I PI3K. However, the downstream molecules involved in the apoptotic death of tumor cells following inhibition of PI3K/Akt by LY294002 remain to be identified[1,18].

Figure 3 Pathological changes of the expression of MMP-9 in lymphatic vessels and SGC7901 tumor cells in the model control groups (A, E) and treatment groups (B-D, F-H). Lymphatic vessels (A-D) (HE, × 200) and tumor cells (E-H) (HE, × 100); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

Tumor metastasis involves a series of complex processes in which many gene products take part in regulating MMPs, which play an important role in breaking the extracellular matrix, are overexpressed in malignant tumors and are believed to contribute to tumor proliferation, invasion, and metastasis[19]. Among the MMPs, MMP-2 and MMP-9 are closely related to the metastasis of tumors, and have been specifically considered to be important factors in facilitating lymphatic invasion and metastases in gastric carcinoma[20-22].

MVD is a quantitative index for carcinoma angio-genesis, which indicates the regulation of the progress and metastasis of primary gastric carcinoma, and can therefore serve as a marker for carcinoma prognosis[23-25]. VEGF is one of the agents accelerating the formation of blood vessels, and plays a vital part in tumor-associated microvascular invasion[26-28]. It has been found that tumor metastasis is speeded up by VEGF, which is highly expressed in gastric carcinoma, thus may be used as an index for poor prognosis of gastric carcinoma[29-31].

Figure 4 Pathological changes of the expression of MVD in lymphatic vessels and SGC7901 tumor cells in the model control groups (A, E) and treatment groups (B-D, F-H). Lymphatic vessels (A-D) (HE, × 200) and tumor cells (E-H) (HE, × 100); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

In the present study, there was a significant difference in the expression of MMP-2, MMP-9 and VEGF, and in MVD between the transplanted gastric tumor tissues treated by the class I PI3K inhibitor LY249002 and that of the control group ($P < 0.05$), indicating that LY249002 may inhibit the gene expression of MMP-2, MMP-9, MVD, and VEGF. We also showed that LY294002 reduced the viability and induced apoptosis in the implanted tumor of human SGC7901 cells, thus demonstrating the cytotoxic effects of LY294002. Both the *in vitro* invasion assay and the *in vivo* nude mice assay suggested that LY249002 had the potential to inhibit the invasion and metastasis of gastric cancer. This may be the result of the decrease of the expression of MMP-2, MMP-9, MVD and VEGF together with the cytotoxicity towards the tumor cells, both induced by LY294002. Moreover, no gross adverse effects, i.e. loss of body weight, were observed during our experimental periods. These findings suggest that inhibition of the class I PI3K signaling pathway is a potent and safe strategy for

Figure 5 Pathological changes of the expression of VEGF in lymphatic vessels and SGC7901 tumor cells in the model control group (A, E) and treatment group (B-D, F-H). Lymphatic vessels (A-D) (HE, × 200) and tumor cells (E-H) (HE, × 100); control groups (A, E); LY294002 groups on days 5 (B, F), day 10 (C, G), day 15 (D, H).

managing gastric cancers, and further indicated that development of LY249002-based therapies may be an emerging approach for the next step in gastric cancer management.

COMMENTS

Background

The phosphatidylinositol 3-kinase (PI3K) pathway plays a central role in the regulation of cell proliferation, growth, differentiation, and survival. Dysregulation of this pathway is frequently observed in a variety of tumors, including brain tumors and breast, ovarian and other carcinomas. Currently only a few chemotherapeutic drugs are effective for the treatment of human gastric carcinoma and there is an increasing interest in the use of drugs to prevent its occurrence or invasiveness.

Research frontiers

LY294002 is a specific inhibitor of class I PI3K. The antitumor activity of LY294002 may be related to the induction of apoptosis of tumor cells, but the precise mechanism of its antitumor activity is not well understood.

Applications

The research indicated that development of LY249002-based therapies may be an emerging approach for the next step in gastric cancer management.

Terminology

Matrix metalloproteinases (MMPs) are a family of structurally related zinc-endopeptidases. MMP-2 contributes to cell proliferation, migration, and matrix invasion in a number of cell types such as tumor cells and fibroblasts MMP-9 is directly involved in tumor metastasis, and more recently MMP-9 activity has been linked with the process of tumor cell invasiveness. High MVD is an indirect measure of tumor aggressiveness. Vascular endothelial growth factor is a potent regulator of placental vascular function. Endothelial dysfunction is a key factor associated with preeclampsia.

Peer review

Overall, the experiment was well performed and the authors have presented interesting data to provide a mechanistic insight into the antitumor effect of LY294002.

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BRIEF ARTICLE

Liver tumor infiltrating lymphocytes: Comparison of hepatocellular and cholangiolar carcinoma

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Abstract

AIM: To investigate the role of tumor infiltrating lymphocytes (TIL) in primary hepatocellular and cholangiolar carcinomas of the liver.

METHODS: Immunohistochemical analysis was performed including antibodies to CD3, CD4, CD8, CD20, CD56 and TIA-1 in formalin-fixed and paraffin-embedded tissue of 35 liver resection specimens of hepatocellular or cholangiocellular carcinomas. Semiquantitative evaluation was performed with emphasis on the area of the tumor itself and of the tumor/liver interface.

RESULTS: All hepatocellular carcinomas showed infiltration of lymphocytes predominantly around the tumor in the tumor/liver interface consisting mainly of CD3+ CD4+ T lymphocytes [164.3/10 high power fields (HPF)] and in the tumor itself of CD8+ cells (54.9/10 HPF). Cholangiocarcinomas contained a heterogeneous amount of TIL, composed mainly of CD3+ T cells with a predominance of CD8+ cells in the tumor tissue (52.6/10 HPF) and of CD4+ cells in the interface region (223.1/10 HPF). CD56+ cells of the innate immune system were scarce. There was no significant difference between hepatocellular or cholangiolar carcinoma. No correlation with the clinicopathological data was seen.

CONCLUSION: Liver TIL consists of intratumoral CD8+ T cells and peritumoral CD4+ T cells independent of histogenetic origin. Different functions of lymphocytes in these regions seem possible.

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INTRODUCTION

Tumor infiltrating lymphocytes (TIL) are part of the tumor surveillance system[1]. This immune response is thought to be a result of changes of surface components of tumor cells. The innate as well as the adaptive immune system is involved in tumor destruction with cell-mediated mechanisms playing the main role. They are frequently present in human solid tumors. Some CD8+ T cells are seen as final effector cells with the ability to induce apoptosis of cancer cells. Subpopulations of CD4+ T cells have a helper function, activating other immune cells *via* cytokine secretion or antigen processing. TIL are a target for immunotherapeutic strategies[2].

The liver can be regarded as an immunological organ, specially equipped with liver-associated lymphocytes, mainly T lymphocytes and natural killer cells[3]. They play an important role in the barrier function of the liver between the gastrointestinal tract and an organism. They

do not only function as part of the defense system but also as a regulator of immune tolerance.

Hepatocellular carcinoma is the leading cause of malignant cancer deaths worldwide and the morbidity is increasing year on year. It accounts for approximately 6% of all human cancers and up to 1 million deaths per year. The second most common primary malignancy of the liver, cholangiolar carcinoma also has a bad prognosis. Its resectability rate is very low, but surgical resection is the only treatment which can change outcome significantly[4,5].

We studied the frequency and composition of TIL in primary liver cancers with special attention to the morphological distribution. The subtyping was performed to clarify their putative role in host response, immunotolerance and as a therapeutic target.

MATERIALS AND METHODS

Patients
Formalin-fixed and paraffin-embedded tissue of 35 liver resection specimens were investigated. The specimens were obtained from 8 women and 27 men with a median age of 60.5 years (38-82 years). Twenty seven of the cases were diagnosed as hepatocellular carcinoma (8 × T1, 3 × T2, 12 × T3 4 × T4; 6 × G1, 14 × G2, 7 × G3) and 8 as cholangiolar carcinoma (2 × T1, 1 × T2, 2 × T3, no T-stage available in 3; 6 × G2, 2 × G3) with a mean diameter of 7.9 cm and 8.3 cm, respectively.

Immunohistochemistry
A panel of immunohistochemical stains was performed including antibodies to CD3, CD4, CD8, CD20, CD56 and TIA-1. The specifications and titers are given in Table 1.

Routinely processed formalin-fixed and paraffin-embedded tissue sections of the tumor and the tumor/liver interface with a thickness of 4 μm were used. Sections were mounted onto capillary gap slides (DAKO, Glostrup, Denmark), dried overnight at 30℃, deparaffinized with xylene and rehydrated with ethanol in a graded series to distilled water. Staining was performed using an automated immunostainer (Techmate, DAKO, Glostrup, Denmark) with AEC (3-amino-9-ethylcarbazole) for visualization. The slides were counterstained with hemalaun and a coverslip placed on top. Appropriate positive and negative tissue control samples were used throughout. Tonsils served as positive controls.

Statistical analysis
Ten high power fields (HPF) of the tumor and the tumor-liver interface were randomly selected and the frequency of TIL was counted using an ocular grid. Results are reported as mean value and standard deviation per 10 HPF. Comparison of the groups was performed using the two-tailed Students t-test (SPSS for windows). The significance level was set as $P < 0.05$.

RESULTS

TIL in hepatocellular carcinoma
All hepatocellular carcinomas showed an infiltration of

Table 1 List of antibodies

Name	Clone	Pre-treatment	Titer	Manufacturer
CD3	PS1	10 min autoclave 120℃, citrate buffer pH 6	1:50	DAKO, Glostrup, Denmark
CD4	1F6	10 min autoclave 120℃, citrate buffer pH 9	1:10	Novocastra, Newcastle, UK
CD8	C8/144B	10 min autoclave 120℃, citrate buffer pH 6	1:100	DAKO, Glostrup, Denmark
CD20	L26	none	1:500	DAKO, Glostrup, Denmark
CD56	123C3 SCLC	2 × 7 min microwave citrate buffer pH 6	1:500	Zymed, San Francisco, CA, USA
TIA-1	TIA-1	2 × 7 min microwave citrate buffer pH 6	1:500	Coulter Immunol., Hinleah, FL, USA

lymphocytes which was mainly localized around the tumor in the tumor/liver interface, with less among the tumor cells (Table 2, Figure 1). The TIL consisted mainly of CD3+ T lymphocytes. CD20+ cells and CD56+ cells were rarely found. In the tumor itself, the infiltration was dominated by CD8+ cells. In contrast, in the peritumoral area the amount of CD4+ cells was higher than the amount of CD8+ cells. TIA-1 containing cells were more frequent in the peritumoral region.

TIL in cholangiolar carcinoma
Cholangiocarcinomas contained a heterogeneous amount of TIL, composed mainly of CD3+ T cells. The relationship of the subpopulations was comparable to that of hepatocellular carcinoma, with a predominance of CD8+ cells in the tumor tissue and of CD4+ cells in the interface region. CD56+ and CD20+ cells were found only in a minor proportion. Cells containing the cytotoxic granula TIA-1 occurred often in the interface region. The details are summarized in Table 2 and Figure 2.

No statistical differences were found in the frequency and distribution according to age, sex, size or grade of the tumor.

DISCUSSION
Liver carcinoma evolves over a long period of time from precursor lesions to invasive cancer and metastases. The exact mechanisms of this pathway are not fully understood[6]. It is known, however, that TIL have the potential to modulate this process. The extent of this modulation and the real effects of tumor growth and dissemination remain unclear.

Several methodical approaches have been used to measure TIL. Most often flow cytometry and immunohistology is used. Flow cytometry is a rapid method, in which a higher amount of cells and a panel of markers can be investigated. Fresh tissue, however, is necessary. Therefore retrospective studies, especially with human tissue from pathological files, are not possible. An exact localization of the TIL cannot be determined. Immunohistochemistry can identify different cell populations and has the advantage of direct localization of the lymphocyte subsets. Manual counting and automated counting

Table 2 Frequency of TIL in hepatocellular and cholangiolar carcinoma

TIL	Hepatocellular carcinoma			Cholangiolar carcinoma		
	Intratumoral (/10HPF)	Peritumoral (/10HPF)	Level of significance	Intratumoral (/10HPF)	Peritumoral (/10HPF)	Level of significance
CD20	3.2 (± 9.7)	26.4 (± 49.5)	$P < 0.001$	0.1 (± 0.3)	11.1 (± 11.8)	$P = 0.035$
CD3	85.1 (± 78.2)	256.5 (± 90.5)	$P < 0.001$	52.6 (± 28.5)	310.4 (± 202)	$P = 0.008$
CD4	37.9 (± 42.5)	164.3 (± 26.4)	$P < 0.001$	18 (± 22.3)	223.1 (± 43.2)	$P = 0.043$
CD8	54.9 (± 57.9)	131.5 (± 86.8)	$P \leqslant 0.001$	40.7 (± 30.5)	118.7 (± 35.5)	$P \leqslant 0.001$
CD56	0.2 (± 0.6)	0.5 (± 1.1)	$P = 0.058$	0.4 (± 1.0)	1.9 (± 2.7)	$P = 0.088$
TIA-1	50.2 (± 40.5)	80 (± 64.5)	$P = 0.036$	41.1 (± 41.8)	72.5 (± 37.4)	$P = 0.071$

HPF: High power fields; TIL: Tumor infiltrating lymphocytes.

Figure 1 Lymphocytic infiltration in the tumor tissue of hepatocellular carcinoma. A-D: Intratumoral region; E-H: Tumor/liver interface (peritumoral); A and E: CD3+ T cells are the main infiltrate with a higher amount in the interface region; B and F: CD4+ cells were mainly located in the peritumoral area; C and G: In the tumor tissue, CD8+ cells were more often seen; D and H: CD20+ cells were scarce.

Figure 2 The distribution of tumor infiltrating lymphocytes in cholangiocellular carcinoma. A-D: Intratumoral region; E-H: Tumor/liver interface (peritumoral); A and E: CD3+ T cells were the dominant infiltrate; B and F: The quantity of CD4+ and CD8+ cells were opposite with a higher level of CD4 cells in the peritumoral region; C and G: CD8+ cells were more often found in the tumor tissue; D and H: CD20+ cells were scarce.

of digital images are possible[7,8].

Using a histological approach we were able to localize TIL and could differentiate between lymphocytes in the tumor itself and in the environs of the tumor. In the tumor, CD8+ cells were more frequent, showing a closer contact with the tumor cells. As these cells can have a cytotoxic function, a direct apoptotic effect *via* different pathways such as the FAS/FAS-ligand pathway seems

possible. Large numbers of CD8+ TILs are associated with a favorable prognosis in several solid carcinomas such as colorectal, ovarian, pancreatic and esophageal carcinoma[9-14]. Other entities such as nasopharyngeal cancer, renal cell carcinoma and non-small cell lung cancer show opposite behavior[15-17]. For hepatocellular carcinoma, further investigation is necessary.

In contrast, TIL in the interface are mostly of the CD4+ type. As CD4+ cells include helper T cells, their infiltration could activate cytotoxic killer cells by cytokine secretion or antigen presentation. Other authors, however, found an involvement of these cells, also known as regulatory cells, in immune tolerance[18]. Tumor cells adapting to these mechanisms of the CD4+ lymphocytes could use these cells to induce a tumor-friendly environment. The infiltration of CD4+ T-cells could be a sign of tumor adaptation known as enhancement[19].

Unitt et al[20,21] indicated that lymphocytic infiltration of the tumor and a high CD4+/CD8+ T cell ratio were associated with a reduced risk of tumor recurrence after liver transplantation for hepatocellular carcinoma. This ratio was beneficial in hepatocellular carcinoma. A decreased ratio was shown to imply poor tumor response[22]. CD4+ T lymphocytes can activate monocytes, macrophages and natural killer cells and can help CD8+ T lymphocytes to kill tumor cells. Thus, a positive effect of those infiltrations seems to exist. On the other hand, however, CD4+ T cells were also involved in tolerance mechanisms. As the liver is particularly involved in tolerance induction to food antigens, a supportive role of these infiltrates cannot be ruled out.

A lower quantity of CD8+ cells in the tumor and in the tumor periphery could signal a low level of T cell activation by tumor specific antigens in the liver. Primary reasons for this can be in the immune system itself such as development of self tolerance in the thymus or in the periphery[23]. The tumor can also develop various evasion strategies like converting T helper cells or lowering the expression of tumor specific antigens to below the required threshold[24-26]. These mechanisms would lead to a low quantity of TIL. Janicki et al[27], however, could show that TIL can lose their effector function even after infiltrating the tumor. One reason could be a loss of adhesion capability[28]. These results have critical implications for vaccination studies.

The liver normally contains a higher quantity of cells of the innate immune system such as natural killer cells. The lack of CD56+ cells in the tumor and in the interface to the liver is surprising. This cell type is thought to be involved in tumor defense. This is in contrast to a recent study investigating human glioblastomas[29]. Half of all TIL in these tumor types were CD56+ cells, in particularly CD4+CD56+ T cells. Whether this cell type is unique for brain tumors has to be clarified by further studies.

In the liver, a primary defect or lack of cells of the innate immune systems could be regarded as an aid to tumor development. On the other hand, there is the possibility that a manifest tumor can suppress the innate immune system.

For cholangiolar carcinoma, only scarce data are available regarding TIL. Takagi and coworkers found that patients with high numbers of TIL (CD8+ or CD4+) had significantly better prognosis[30]. In our study, the amount of TILs was heterogeneous. In the tumor tissue itself, the CD8+ cells can act as cytotoxic cells. In this entity, regulatory cells are also found in the tumor environs. As in hepatocellular carcinoma, there is a lack of CD56+ cells of the innate immune system.

Earlier we investigated the lymphocytic reaction of the liver with liver metastases from different primary neoplasms[31]. In this investigation, the results were similar to those demonstrated here. Also, in liver metastases, the frequency of TIL in the interface between the liver and tumor was higher than in the tumor itself. The infiltrate in the interface was also composed mainly of CD4+ T cells. Thus, a general rule can be seen in the reaction of the liver. A manifest tumor is accompanied by a CD4+ reaction in the liver. Whether this is a defense mechanism or more like an induced protection by the tumor cells needs further clarification.

In summary, we could demonstrate for hepatocellular carcinoma and cholangiolar carcinoma that TIL consists, in the tumor itself, of CD8+ T cells and, in the peritumoral region, of CD4+ T cells such as helper cells or regulatory cells. CD20+ cells and TIA-1+ cells were scarce. There was a lack of CD56+ cells of the innate immune system. The functional interaction of TIL in liver carcinomas needs further investigation especially if considered as a target for immunotherapeutic strategies.

COMMENTS

Background
Tumor development is based on an interaction between the tumor cells and the immune system of the body. Tumor infiltrating lymphocytes (TIL) are part of the tumor surveillance system. This immune response is thought to be a result of changes of surface components of tumor cells. TIL are a target for immunotherapeutic strategies in cancer treatment.

Research frontiers
Immune therapy is one of the newer strategies in cancer therapy. The main aim is vaccination against carcinomas. A prerequisite for the development of these vaccines is an understanding of the immunological tumor reaction.

Innovations and breakthroughs
Recent reports have shown different functions of lymphocyte subsets in tumor surveillance. These cells not only have antitumor potential but growth promotion by certain lymphocytes is also documented.

Applications
This study suggests that the immune reaction to liver cancer consists of different lymphocyte subtypes. Some can enhance tumor growth, others can destroy tumor cells. A complex strategy would be necessary for successful immune therapy of liver cancer.

Peer review
The authors investigated the role of TIL in primary hepatocellular and cholangiolar carcinomas of the liver. They found that liver TIL consist of intratumoral CD8+ T cells and peritumoral CD4+ T cells independent of histogenetic origin. This article is well written and deserves publication.

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BRIEF ARTICLE

UGT1A1 gene polymorphism: Impact on toxicity and efficacy of irinotecan-based regimens in metastatic colorectal cancer

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Abstract

AIM: To investigate the correlation between *uridine diphosphate glucuronosyl transferase 1A1* (*UGT1A1*) gene polymorphisms and irinotecan-associated side effects and parameters of drug efficacy in patients with metastatic colorectal cancer (mCRC) receiving a low-dose weekly irinotecan chemotherapeutic regimen.

METHODS: Genotypes were retrospectively evaluated by gene scan analysis on the ABI 310 sequencer of the TATAA box in the promoter region of the *UGT1A1* gene in blood samples from 105 patients who had received 1st line irinotecan-based chemotherapy for mCRC.

RESULTS: The distribution of the genotypes was as follows: wild type genotype (WT) (*6/6*) 39.0%, heterozygous genotype (*6/7*) 49.5%, and homozygous genotype (*7/7*) 9.5%. The overall response rate (OR) was similar between patients carrying the (*6/7*, *7/7*) or the WT genotype (*6/6*) (44.3% *vs* 43.2%, *P* = 0.75). Neither time to progression [(TTP) 8.1 *vs* 8.2 mo, *P* = 0.97] nor overall survival [(OS) 21.2 *vs* 18.9 mo, *P* = 0.73] differed significantly in patients who carried the

(*6/6*) when compared to the (*6/7*, *7/7*) genotype. No significant differences in toxicity were observed: Grade 3 and 4 delayed diarrhoea [(*6/7*, *7/7*) *vs* (*6/6*); 13.0% *vs* 6.2%, *P* =0.08], treatment delays [(*6/7*, *7/7*) *vs* (*6/6*); 25.1% *vs* 19.3%, *P* = 0.24] or dose reductions [(*6/7*, *7/7*) *vs* (*6/6*); 21.5% *vs* 27.2%, *P* = 0.07].

CONCLUSION: This analysis demonstrates the non-significant influence of the *UGT1A1* gene polymorphism on efficacy and rate of irinotecan-associated toxicity in mCRC patients receiving low-dose irinotecan based chemotherapy.

Schulz C, Heinemann V, Schalhorn A, Moosmann N, Zwingers T, Boeck S, Giessen C, Stemmler HJ. *UGT1A1* gene polymorphism: Impact on toxicity and efficacy of irinotecan-based regimens in metastatic colorectal cancer. *World J Gastroenterol* 2009; 15(40): 5058-5066 Available from: URL: http://www.wjgnet.com/1007-9327/15/5058.asp DOI: http://dx.doi.org/10.3748/wjg.15.5058

INTRODUCTION

Irinotecan (Camptosar®, CPT-11, Pfizer Oncology, New York, NY, US) is one of the most effective chemotherapeutic agents in the treatment of metastatic colorectal cancer (mCRC)[1,2]. Data from phase III studies indicated an improved clinical outcome of patients who had received an irinotecan-based regimen when compared to those who had received 5-FU/LV alone. As reported by Saltz *et al*[3], the irinotecan-based combination therapy not only improved the response rate (39% *vs* 21%), but also the progression free (PFS) (7.0 *vs* 4.3 mo; *P* = 0.004) and overall survival (OS) (14.8 *vs* 12.6 mo; *P* = 0.04).

Irinotecan is a semisynthetic derivative of camptothecin and acts as an inhibitor of intracellular topoisomerase- I [4]. *In vivo*, the pro-drug is metabolized by carboxylesterase into its active metabolite SN-38. SN-38 is inactivated by uridine disphosphate glucuronosyl transferase 1 (UGT1A1) into SN-38G which is excreted with bile[5].

The most common side effects of irinotecan include neutropenia, febrile neutropenia, nausea, alopecia and delayed diarrhoea which particularly often represents the main and dose-limiting toxicity. This major side effect is because betaglucuronidase in the bowel re-activates SN-38G into the active metabolite SN-38[6-8].

UGT1A1, an essential enzyme for the inactivation of SN-38, is also involved in the metabolism of bilirubin. Changes in the metabolism of bilirubin result in several clinical disorders. They range from the clinically harmless condition of mild jaundice to the deadly disease Crigler-Najjar that may have a lethal outcome in adolescence[9]. Patients with Gilbert's syndrome present with a mild unconjugated hyperbilirubinaemia without any structural liver disease or haemolysis. The activity of UGT1A1 is reduced in these individuals compared to those without a comparable defect[10,11].

Molecular analyses have revealed that in the Caucasian population Gilbert's syndrome is most commonly caused by a polymorphism in the *UGT1A1* gene[12]. It consists of a TA insertion in the TATAA element of the 5'-promotor region. Genotypes are defined *(6/6)*, *(6/7)* and *(7/7)* according to the number of TA repeats. Therefore patients with the wild type (WT) genotype *(6/6)* (33% in the Caucasian population) are homozygous with 6 repeats of the TA insertion. Patients with the *(7/7)* genotype are homozygous with 7 TA repeats while the heterozygous genotype *(6/7)* consists of 1 allele with 6 TA repeats and of 1 with 7 TA repeats. Patients being heterozygous or homozygous for this variant allele (also named UGT1A1*28) show a reduced expression of the UGT1A1 enzyme resulting in lower rates of bilirubin and SN-38 glucuronidation[13]. Compared to the wild type genotype *(6/6)* patients carrying the *(7/7)* genotype displayed a 70% reduction in transcriptional activity and are, by the attenuated expression of UGT1A1, theoretically predisposed to SN-38 associated side effects[14]. Apart from that, there are rare genotypes with less than five or more than seven TA repeats leading to variable enzyme levels.

A number of trials have provided evidence of an association between UGT1A1 gene polymorphism and increased toxicity in patients who received irinotecan[15-18]. Comparability and appliance of the results of these trials is difficult because of the various regimens and irinotecan dosages used. Due to the widespread use of irinotecan and the associated risk of severe side effects the question of defining subgroups of patients susceptible to irinotecan-related toxicities is of eminent clinical importance.

This retrospective analysis included a subgroup of 105 patients with mCRC who were treated with an irinotecan-based chemotherapy within a large prospective randomized multicenter phase III study which investigated the role of a low-dose irinotecan-based chemotherapy in patients with metastatic or advanced colorectal cancer (FIRE-trial)[19]. Patients underwent UGT1A1 genotyping in order to evaluate UGT1A1 as a predictor for drug efficacy and/or toxicity for patients with low-dose irinotecan-based chemotherapy and to provide a future tool for a patient tailored chemotherapy.

MATERIALS AND METHODS

Patient selection

One hundred and five Caucasian patients with mCRC were included in this analysis. The study population represents a subgroup of patients treated within the FIRE-trial. In the FIRE-trial a total of 492 patients from 56 centres were included, of which 478 patients could be evaluated for efficacy and toxicity. After an amendment UGT1A1 genotyping was offered and 105 patients were included in this subgroup analysis within the ongoing main study. The local ethics committees of the participating centres approved both the study protocol of the FIRE-trial and the amendment for UGT1A1 genotyping. Since the trial was a multicentre trial, monitoring for consistency was done by an external monitoring expert (ClinAssess GmbH, Leverkusen, Germany). Patients gave written informed consent prior to any study-specific procedures.

Patients with known Gilbert's syndrome or known DPD-deficiency were not allowed to enter the trial.

Treatment regimen

Patients within the FIRE-trial were randomly assigned to a modified FOLFIRI (mFOLFIRI) or modified IROX (mIROX) protocol. Randomisation was done by the external monitoring expert after an eligibility check. Inclusion criteria included serum bilirubin $\leq$ 1.25 or $\leq$ 1.5 of the upper institutional limits without or with hepatic metastasis. The modified FOLFIRI consisted of irinotecan 80 mg/m^2 i.v. over 30 min, folinic acid 500 mg/m^2 i.v. over 120 min, followed by 5-FU 2000 mg/m^2 i.v. over 24 h weekly for 6 wk. The modified IROX consisted of oxaliplatin 85 mg/m^2 i.v. over 120 min every 2 wk and irinotecan 80 mg/m^2 i.v. over 30 min weekly. For both arms, treatment cycles were repeated on day 50. Pre-medication with atropine (s.c.) was given routinely to prevent acute anti-cholinergic syndrome. Antiemetics were given according to good clinical practice and the local standards of the participating centres (generally 5-HT3 antagonists). Treatment continuation was intended until progression, until inacceptable toxicity or confirmed complete response (CR).

Therapy was postponed for at least one week until bone marrow recovery or resolution of side effects (diarrhoea $\geq$ grade 1, mucositis $\geq$ grade 1, leucocytopenia $\geq$ grade 2, thrombocytopenia $\geq$ grade 1 or any other toxicity $\geq$ grade 2). In case of toxicity, defined as diarrhoea $\geq$ grade 3, mucositis $\geq$ grade 3, leucocytopenia grade 4, thrombocytopenia $\geq$ grade 3, severe obstipation $\geq$ 96 h or hand-foot-syndrome $\geq$ grade 3, a dose reduction of irinotecan and 5-FU to 80%

Figure 1 Electropherograms/gene scanning: Example results of TA promoter region of the *UGT1A1* gene. A: Control heterozygous genotype (*6/7*); B: Patient homozygous genotype (*6/6*); C: Patient homozygous genotype (*7/7*); D: Negative control.

was mandatory. Doses of irinotecan and oxaliplatin were reduced to 80% for obstipation $\geq$ grade 3. Moreover, a dose reduction of oxaliplatin was required in cases of persistent paraesthesia (dose reduction 25%), painful paraesthesia with duration of > 7 d (dose reduction 25%) or paraesthesia with functional impairment with duration of > 7 d (dose reduction 50%).

In case of any toxicity as described above, following cycles were begun at a reduced dose level as defined by the scheme above.

UGT1A1 genotyping

Genotyping studies were performed by an independent laboratory (OncoScreen GmbH, Jena, Germany). Prior to any chemotherapy blood samples were collected for DNA isolation and determination of genotypes. DNA extraction, preparation and genotyping was performed using the methods as previously described[12,14,16]. DNA was extracted from peripheral blood leucocytes using standard methods. A two-extra-nucleotide insertion (TA) within the TATA box resulting in the sequence (TA)7TAA (-39 to 53) was researched. Genotypes were identified by gene scan analysis on the ABI 310 sequencer of the TATAA box in the promoter region of the *UGT1A1* gene. Figure 1 displays an electropherogram with typical overlapping peaks to distinguish the WT genotype from the heterozygous and homozygous genotype (Figure 1). The design of the primers to specifically amplify the TA expansion was done with support of GenBank AF297093.

Data collection

Standard evaluation by history, physical examination and routine laboratory tests (including complete blood count, chemical profile and electrolyte determination) were performed prior to the beginning of each cycle.

Drug administration, performance status and toxicity or adverse events were recorded after each cycle of chemotherapy. Toxicity was graded according to the NCI-CTC Classification (version 3.0). Imaging studies using computed tomography (CT) or magnetic resonance imaging (MRI) were performed prior to the beginning of each following cycle as well as for confirmation 4 wk after the end of chemotherapy. Data collection was monitored by an independent monitoring expert (ClinAssess GmbH, Leverkusen, Germany) and was done by an external data manager (Estimate GmbH, Augsburg, Germany).

Response evaluation

Patients' response was assessed by standard WHO criteria, as follows: complete response (CR) was defined as the disappearance of all known disease, documented by at least two observations not less than 4 wk apart, while partial response (PR) was defined as a decrease by at least 50% of the sum of the products of the largest perpendicular diameters of all measurable lesions, as determined by two observations not less than 4 wk apart. Stable disease (SD), lasting for at least 6 wk from the start of the study (i.e. first drug administration), was defined as a < 50% decrease and < 25% increase in the sum of the products of the largest perpendicular diameters of all measurable lesions. Progressive disease (PD) was defined a > 25% increase in the size of at least one bidimensional or unidimensional measurable lesion, or the appearance of a new lesion.

Endpoints and statistical methods

The primary endpoint of the FIRE-trial was time to progression (TTP). Secondary endpoints included response rate (RR), overall survival (OS), resectability rate, toxicity and quality of life. TTP was defined as the interval

Table 1 Patient characteristics and UGT1A1 status (%)

	Total (*n* = 103)	WT (*6/6*) (*n* = 41)	(*6/7*) (*n* = 52)	(*7/7*) (*n* = 10)
Age (yr) median (range)	64 (41-79)	63 (41-76)	65 (42-79)	65 (52-74)
Gender (M/F)	70.5/29.5	70.7/29.3	67.3/32.7	80.0-20.0
Colon cancer	59.0	51.2	63.5	80.0
Rectal cancer	41.0	48.8	36.5	20.0
Adjuvant pre-treatment	35.2	43.9	30.8	20.0
KPS 70%-90%	43.8	29.3	51.9	70.0
KPS 100%	56.2	70.7	48.1	30.0
LDH ⩽ 240 U/L	55.2	61.0	48.1	70.0
LDH > 240 U/L	44.8	39.0	51.9	30.0

KPS: Karnofsky perfomance status; LDH: Lactate dehydrogenase.

between the start of therapy and first documentation of disease progression. OS was measured from the date of starting treatment to the date of death from any cause (intent-to-treat). Probability of survival and TTP were estimated using the Kaplan-Meier method[20]. Statistical comparisons between different genotypes were determined by using the χ^2-test. Differences in OS and TTP were analyzed using the log-rank test. A difference of *P* < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics
The median age was 64 years with a gender distribution of 70.5% male and 29.5% female patients. 59% of the patients suffered from metastatic colon and 41% from metastatic rectal cancer. Performance status and adjuvant pre-treatment was similar between WT and heterozygous genotype patients. Detailed patient characteristics are provided in Table 1. Patients in the main trial were stratified according to performance status, lactate dehydrogenase (LDH) and adjuvant pre-treatment. These patients were well balanced between the two treatment arms.

Distribution of the UGT1A1 status
The distribution of the *UGT1A1* genotypes was analysed in 105 of 478 patients evaluated within the FIRE-trial. The majority of the patients (49.5%) had the heterozygous genotype (*6/7*), 39.0% showed the WT genotype (*6/6*) and the homozygous genotype (*7/7*) was found in 9.5% of the analyzed patients. There were also two single cases of the rare genotype (*5/7*) which were not included for further evaluation due to the low frequency. Results of the distribution of the genotypes are given in Table 2.

Response to chemotherapy with regard to UGT1A1 genotype
The overall response rate (ORR = CR + PR) was similar between the WT genotype (*6/6*) and the (*6/7*, *7/7*) genotypes (43.2% *vs* 44.3% *P* = 0.75). However, the disease control rate (DCR = CR + PR + SD) appeared to be lower within patients carrying the WT genotype (*6/6*) as compared to those patients with the (*6/7*,

Table 2 Genotype distribution with regard to treatment *n* (%)

UGT1A1 status	FOLFIRI	IROX	All
WT (*6/6*)	19 (34.5)	22 (44.0)	41 (39.0)
(*6/7*)	28 (50.9)	24 (48.0)	52 (49.5)
(*7/7*)	7 (12.7)	3 (6.0)	10 (9.5)
(*5/7*)	1 (1.8)	1 (2.0)	2 (1.9)

FOLFIRI: 5-fluorouracil, leucovorin, irinotecan; IROX: Irinotecan, oxaliplatin.

Table 3 Response to treatment with regard to UGT1A1 status *n* (%)

	WT (*6/6*)	(*6/7, 7/7*)	All	χ^2 test [WT *vs* (*6/7, 7/7*)]
CR	3 (8.1)	7 (11.5)	10 (10.2)	
PR	13 (35.1)	20 (32.8)	33 (33.7)	
SD	11 (29.7)	29 (47.5)	40 (40.8)	
PD	7 (18.9)	5 (8.2)	12 (12.2)	
NA	3 (8.1)	-	3 (3.1)	
OR	16 (43.2)	27 (44.3)	43 (43.9)	*P* > 0.05
DCR	27 (73.0)	56 (91.8)	83 (84.7)	*P* < 0.05

CR: Complete remission; PR: Partial remission; SD: Stable disease; PD: Progressive disease; OR: Overall response rate (CR + PR); DCR: Disease control rate (CR + PR + SD); NA: Not available.

7/7) genotypes (73.0% *vs* 91.8%, *P* = 0.008). Detailed response data are presented in Table 3.

There are no efficacy data for the 2 patients carrying the rare genotype (*5/7*). One patient quit the trial while the other died early during therapy.

Toxicity with regard to UGT1A1 genotype
Overall toxicity according to genotype and treatment arm is given in Table 4. The incidence of grade 3-4 toxicity did not differ significantly between the treatment arms mFOLFIRI and mIROX both for patients with the WT (*6/6*) and those carrying the (*6/7*), (*7/7*) and (*5/7*) genotypes.

Haematological toxicity was generally mild (grade 3 and 4 < 5%). Comparing the incidence of grade 1-2 and grade 3-4 haematotoxicity in patients with the WT (*6/6*) genotype to those with the (*6/7, 7/7*) genotypes, there were no significant differences regarding leucocytopenia, anaemia or thrombocytopenia (*P* > 0.05) (Table 5).

Regarding non-haematological toxicity, the incidence of grade 3-4 delayed diarrhoea appeared higher in patients with the (*6/7, 7/7*) genotypes compared to the WT (*6/6*) genotype even though this difference did not reach the statistical level of significance (13.0% *vs* 6.2%, *P* = 0.08) (Table 6).

Both cases with the rare genotype (*5/7*) were excluded from the statistical evaluation. However, they experienced grade 3-4 toxicity in the course of the treatment (delayed diarrhoea and leucocytopenia, per cycle analysis).

There were no significant differences regarding toxicity-related treatment discontinuations or dose adjustments. Dose delays in patients with the WT (*6/6*) were observed in 27.2%, compared to 21.5% in patients with the (*6/7, 7/7*) genotypes (*P* = 0.071). Dose reductions

Table 4 Toxicity and UGT1A1 status according to treatment arm n (%)

Toxicity WHO	mFOLFIRI				mIROX				χ^2 test
	Grade 1	Grade 2	Grade 3	Grade 4	Grade 1	Grade 2	Grade 3	Grade 4	
WT (6/6)	2 (33.3)	10 (33.3)	6 (37.5)	1 (33.3)	-	17 (53.1)	3 (23.1)	2 (50.0)	P > 0.05
(6/7)	3 (50.0)	16 (53.3)	7 (43.8)	2 (66.7)	1 (100)	14 (43.8)	7 (53.8)	2 (50)	P > 0.05
(7/7)	1 (16.7)	4 (13.3)	2 (12.5)	-	-	1 (3.1)	2 (15.4)	-	P > 0.05
(5/7)	-	-	1 (6.3)	-	-	-	1 (7.7)	-	P > 0.05

Table 5 Haematological toxicity and UGT1A1 status (per cycle analysis) cycles (%)

	WT (6/6)			(6/7, 7/7)			χ^2 test
	Grade 0	Grade 1-2	Grade 3-4	Grade 0	Grade 1-2	Grade 3-4	
Leucocytes	69 (61.1)	43 (38.1)	1 (0.9)	102 (52.8)	85 (44.0)	6 (3.1)	P > 0.05
Neutropenic fever	113 (100.0)	-	-	191 (98.9)	1 (0.5)	1 (0.5)	P > 0.05
Anaemia	30 (26.5)	80 (70.8)	3 (2.7)	47 (24.3)	142 (73.6)	4 (2.1)	P > 0.05
Thrombocytes	89 (78.8)	23 (20.4)	1 (0.9)	157 (81.3)	36 (18.7)	-	P > 0.05

Table 6 Non-haematological toxicity and UGT1A1 status (per cycle analysis) cycles (%)

	WT (6/6)			(6/7, 7/7)			χ^2 test
	Grade 0	Grade 1-2	Grade 3-4	Grade 0	Grade 1-2	Grade 3-4	
Nausea	35 (30.9)	77 (68.1)	1 (0.9)	69 (35.8)	122 (63.2)	2 (1.0)	P > 0.05
Vomiting	77 (68.1)	35 (31.0)	1 (0.9)	123 (63.7)	69 (35.8)	1 (0.5)	P > 0.05
Diarrhoea early	81 (71.7)	30 (26.5)	2 (1.8)	149 (77.2)	42 (21.8)	2 (1.0)	P > 0.05
Diarrhoea delayed	51 (45.1)	55 (48.7)	7 (6.2)	78 (40.4)	90 (46.6)	25 (13.0)	P > 0.05
Mucositis	97 (85.8)	16 (14.2)	-	160 (82.9)	31 (16.1)	2 (1.0)	P > 0.05

Table 7 Dose reductions, treatment delays and UGT1A1 status (per cycle analysis) n (%)

	WT (6/6)	(6/7, 7/7)	χ^2 test
Dose reduction	22 (19.3)	48 (25.1)	P > 0.05
Dose delayed	31 (27.2)	41 (21.5)	P > 0.05

were more frequently observed in patients with the (6/7, 7/7) genotypes compared to patients who carried the WT (6/6) genotype (25.1% vs 19.3%, P = 0.24) (Table 7).

TTP and survival with regard to UGT1A1 genotype

TTP was similar between the WT (6/6) and the (6/7, 7/7) genotypes with 8.1 mo and 8.2 mo respectively (P = 0.971) (Figure 2). Moreover, there was no difference in survival between the WT compared to the genotypes [WT (6/6) vs (6/7, 7/7); 21.2 mo vs 18.9 mo, P = 0.725] (Figure 3).

DISCUSSION

More than 50 genetic alterations of the UGT1A1 gene locus have previously been described[21]. Among the Caucasian population the UGT1A1*28 genotype (7/7) plays the most important role for the development of Gilbert's syndrome and an increased toxicity related to irinotecan-based chemotherapy.

The distribution of the UGT1A1 genotype in this retrospective analysis was comparable to that described by Iyer and co-workers[15]. They reported a frequency of the WT (6/6) genotype in 45% of the patients (39% in the

present analysis), the (6/7) genotype in 35% (49.5% in the present analysis), and the (7/7) genotype in 20% (9.5% in the present analysis) respectively[15]. Moreover, another analysis of 51 patients suffering from non-small cell lung cancer (NSCLC) showed a comparable distribution of the genotypes WT (6/6) 49%, (6/7) 36% and (7/7) 15%[22].

Prior to discussion of an association of toxicity and UGT1A1 genotype the following preliminary remarks are inevitable: patients randomized within the FIRE-trial received different irinotecan-based regimes. The incidence of delayed diarrhoea, neurotoxicity, leucocytopenia and thrombocytopenia was more frequent in the IROX arm compared to the FOLFIRI arm[19]. Differences in the frequency of adverse events might therefore partly influence the present analysis. Moreover, 7.3% of the patients (mainly with rectal cancer) had previously received locoregional radiotherapy prior to study entry. Local irradiation may also play a role in the development of severe diarrhoea or may at least worsen it, independent of the UGT1A1 genotype[23]. Finally, in analogy to the study of Ando et al[14], there is a certain bias by excluding patients with elevated pre-treatment bilirubin levels.

Among non-haematological toxicities, the incidence of grade 3 and 4 delayed diarrhoea was twice as high in our study population with a homozygous (7/7) or heterozygous (6/7) genotype compared to those carrying the wild type genotype WT (6/6), even though the difference did not reach the level of significance (13.0% vs 6.2%; P = 0.08). Nevertheless, this trend is supported by the data provided by Marcuello et al[23] who found a significant correlation of the UGT1A1 genotype and

Figure 2 Time to progression and UGT1A1 status. WT (*6/6*) 8.1 mo (range = 5.7-10.5) *vs* (*6/7, 7/7*) 8.2 mo (range = 6.3-9.4), *P* > 0.05; WT: Solid line, (*6/7, 7/7*): Dashed line.

Figure 3 Overall survival and UGT1A1 status. WT (*6/6*) 21.2 mo (range = 12.3-41.0) *vs* (*6/7, 7/7*) 18.9 mo (range = 16.4-34.1), *P* > 0.05; WT: Solid line, (*6/7, 7/7*): Dashed line.

the frequency of severe delayed diarrhoea [WT (*6/6*) 17%, (*6/7*) 33%, (*7/7*) 70%; *P* = 0.005]. Moreover, Ando *et al*[14] reported on a 3.5-fold higher frequency of the UGT1A1*28 genotype in patients who suffered from severe diarrhoea and leucocytopenia during irinotecan-based chemotherapy. Taking the UGT1A1*28 genotype as a significant risk factor for the development of severe toxicity the authors suggest that UGT1A1 genotyping serves as a useful tool in predicting toxicity in patients receiving irinotecan. It is critical to state that the majority of these analyses were done retrospectively. In contrast, an exploratory analysis presented by Seymour *et al*[24] could not confirm such an association of UGT1A1 to irinotecan-related toxicity. Moreover comparability is hampered by the different doses of irinotecan applied within these trials. The interpretation of our data is limited by a slight imbalance with more patients with the (*6/7*) and (*7/7*) genotype being treated with the modified FOLFIRI protocol. Therefore, a tendency towards an increased rate of diarrhoea which might be associated with exposure to 5-FU can not be ruled out.

Comparing the incidence of severe haematotoxicity in patients with the WT (*6/6*) genotype to those with the (*6/7, 7/7*) genotype in our analysis, there were no significant differences regarding leucocytopenia, anaemia or thrombocytopenia (*P* > 0.05). The incidence of hae-matological toxicity was generally low, as expected from other trials with comparable doses of weekly scheduled irinotecan[25,26]. Nevertheless, these findings are in contrast to previously published results of patients with mCRC treated with IRIFUFOL or FOLFIRI[18]. In the study of Rouits *et al*[18] grade 3-4 neutropenia was significantly associated with the genotypes (*6/7, 7/7*). The results of a retrospective analysis of 128 Chinese patients with mCRC who received biweekly irinotecan indicate that the heterogenous and homogenous genotype UGT1A1 (*6/7*) and (*7/7*) predicts severe neutropenia and diarrhoea but not treatment efficacy[27]. Necessity for dose reduction was significantly associated with the (*6/7*) or (*7/7*) genotype (42.3% *vs* 12.7%; *P* < 0.01).

Moreover, Innocenti *et al*[16] also observed no case of grade 4 neutropenia among patients with the wild type genotype WT (*6/6*), whereas 50% among patients with the homozygous genotype (*7/7*), and 12.5% of those with the heterozygous genotype (*6/7*) experienced grade 4 neutro-

penia. In their study irinotecan was administered at a dose of 350 mg/m^2 every 3 wk. Accordingly, data from Roth *et al*[28] indicated that the risk of severe neutropenia was higher among patients carrying the homogenous genotype (*7/7*). Interestingly, female sex was superior to UGT1A1 in predicting grade 4 neutropenia. Contrary, Marcuello *et al*[23] found an increased, but not significantly increased, haematological toxicity among patients with the genotypes (*6/7, 7/7*) during single-agent or combination chemo-therapy with irinotecan given at a dosage of 350 mg/m^2 every 3 wk or 180 mg/m^2 every 2 wk.

The literature regarding UGT1A1 genotyping and irinotecan-related toxicity is heterogeneous and to some extent conflicting which may be partly explained by the retrospective character of most studies[14,16,18,23,24]. Another aspect contributing to the heterogeneity of the results is due to the variety of irinotecan dosages and schedules which also applies for the present study. Patients within our trial received dose reduced and modified FOLFIRI and IROX. Patients in both treatment arms received weekly scheduled irinotecan (80 mg/m^2) in combination with 5-FU 2000 mg/m^2 weekly or oxaliplatin 85 mg/m^2 biweekly. We assume that the mild toxicity and the absent impact of UGT1A1 genotype on toxicity in our study are a result of the low irinotecan dose. Stewart *et al*[29] con-cluded from their study of low-dose protracted irinotecan in pediatric patients that UGT1A1 genotyping is not a useful prognostic factor in predicting toxicity. Hoskins *et al*[30] advised a genotype-tailored therapy only for those patients who receive irinotecan at higher doses. Patients carrying the genotype UGT1A1*28 who receive irinotecan up to a dose of 150 mg/m^2 are at the same risk of expe-riencing severe neutropenia as any patients. Consequently, UGT1A1 genotyping is not generally recommended.

The response rate in the present analysis was similar between the WT (*6/6*) genotype and the (*6/7, 7/7*) geno-types (43.2% *vs* 44.3%, *P* > 0.05). Interestingly, the disease control rate appeared to be higher in patients with the (*6/7, 7/7*) genotypes compared to those with the WT (*6/6*) genotype (91.8% *vs* 73.0%, *P* = 0.008). However, this find-ing did not result in any advantage regarding TTP or OS.

Douillard *et al*[31] reported a TTP of 6.7 and an OS of 17.4 mo in previously untreated patients with mCRC who had received irinotecan and FU/FA. A large Italian study reported a comparable TTP of 7 mo and an OS of 14 mo

for the FOLFIRI regimen given as 1st line chemotherapy for mCRC[32]. Ashley et al[33] have found a TTP of 6.7 mo and an overall survival of 17.3 mo in previously untreated patients with mCRC who had received an IROX regimen consisted of oxaliplatin 85 mg/m^2 and irinotecan 200 mg/m^2 every 3 wk. These data are supported by the findings of Goldberg et al[34] who found a similar TTP of 6.5 mo and an overall survival of 17.4 mo for patients who were treated with IROX. Comparing these data to those of our study a loss of activity due to the dose modifications with low-dose irinotecan dosage can be ruled out.

In a large prospective study conducted by Toffoli et al[35], the response rate was higher in mCRC patients with the homozygous genotype (7/7) compared to the wild type genotype WT (6/6). These patients experienced a slightly improved survival of approximately 2 mo ($P >$ 0.05). One may argue that in patients with the (6/7) or (7/7) genotype there is less detoxification of SN-38 resulting in higher blood levels of the active compound, more antitumor effect and therefore a better response rate. However, Marcuello et al[23] observed no statistically significant impact of UGT1A1 gene polymorphism on response rate but a trend towards an improved OS in patients with the WT (6/6) genotype compared to patients with the (6/7) or (7/7) genotype (33 vs 21 mo; $P = 0.09$). This is partly explained by dose reductions in patients with homozygous or heterozygous genotypes (6/7, 7/7) because of severe diarrhoea. Another recent study reported on prospectively genotyped patients suffering from mCRC who had received either 1st line irinotecan/capecitabine or 2nd line single-agent irinotecan. Response rates, numbers of dose reductions and applied chemotherapy cycles were similar within the different genotypes[36].

An important factor contributing to irinotecan metabolism is the existence of several UGT1A1 isoforms and their distribution among different patient populations[37]. There exist an increasing number of reports on genetic variants of UGT1A1 as well as SNPs in the coding region of the gene locus potentially influencing drug metabolism. Moreover, other enzymes of the UGT1 family like UGT1A7 and UGT1A9 are also involved in the glucuronidation of SN-38[38-40]. Patients of African, Caucasian and Asian descent show a different gene frequency of the UGT1A1*28 gene variant[12]. When analysing UGT1A1 genotypes and irinotecan-related adverse events the different variants of the UGT1A1 gene locus among an ethnic population must be taken into consideration[41]. Moreover, there is strong evidence that several other individual factors apart from ethnic affiliation may influence the irinotecan metabolism[42].

In conclusion, UGT1A1 genotyping alone does not allow characterizing subgroups of patients who are at an increased risk of life threatening toxicity during low-dose irinotecan-based chemotherapy. Due to the incoherent findings of several small trials, a larger prospective phase Ⅲ trial is warranted. The metabolism of irinotecan is highly complex with different UGT enzymes and drug transporters involved. Distinction between high-dose and low-dose irinotecan is of eminent importance when considering the use of UGT1A1 genotyping. There is a need for a diagnostic panel including the testing of multiple gene polymorphisms that more reliably predicts toxicity. On the other hand, beside a comprehensive patient education, and the escalation of supportive therapy, dose modifications and alternative treatment schedules may help to provide patients who are at high risk with a safe irinotecan-based chemotherapy.

COMMENTS

Background
Irinotecan is one of the most effective chemotherapeutic agents in the treatment of colorectal cancer. Among other side effects irinotecan can lead to neutropenia and delayed diarrhoea. The active metabolite of irinotecan, SN-38, is inactivated by uridine disphosphate glucoronosyl transferase 1 (UGT1A1), the same enzyme by which bilirubin is metabolised. Genetic polymorphisms of the UGT1A1 gene result in variable levels of the enzyme. According to the number of TA repeats in the enhancer region of the gene a wild type genotype (6/6) can be differentiated from the heterozygous genotype (6/7) and the homozygous genotype (7/7).

Research frontiers
Preclinical and clinical studies have revealed a close dependency of the activity of UGT1A1 enzyme and the occurrence of irinotecan-associated side effects. Existing data are contradictory to some extent or are derived from heterogenous or small study populations. Therefore and against the background of other metabolic ways of toxification and detoxification the research hotspot is how to apply genetic testing of UGT1A1 gene polymorphisms to predict toxicity and efficacy of an irinotecan-based chemotherapy.

Innovations and breakthroughs
Recent studies have emphasized the impact of UGT1A1 genotyping to predict toxicity and outcome in patients undergoing an irinotecan-based chemotherapy. Distribution of the genotype in this study was well in line with data in the literature. No significant differences could be noticed between the homozygous and the heterozygous genotype compared to the wild type genotype in terms of efficacy, toxicity of higher grades, treatment delay or dose reduction. This is a retrospective study to report that UGT1A1 genotyping appeared not to be useful for predicting treatment efficacy and irinotecan-associated side effects in patients receiving low-dose irinotecan for mCRC. Furthermore, our analysis supports the idea that UGT1A1 genotyping should be considered when using irinotecan at a higher dosage.

Applications
By understanding the limitations of UGT1A1 genotyping and by understanding the complexity of irinotecan metabolism, this study may just add another piece of the puzzle for the future development of a patient-tailored chemotherapy with genotyping as one tool among others.

Terminology
The chemotherapeutic agent irinotecan is metabolised by the enzyme UGT1A1, the same enzyme by which bilirubin is metabolised. Genetic polymorphisms resulting in a decreased amount of the enzyme can lead to enhanced irinotecan-associated toxicity. Pre-existing data have partly shown genetic subgroups of patients with different toxicity and treatment efficacy under chemotherapy with irinotecan.

Peer review
The manuscript by Schulz et al describes the impact of UGT1A1 gene polymorphism on toxicity and efficacy of irinotecan-based regimens in metastatic colorectal cancer. The authors demonstrate that the genetic polymorphism of the UGT1A1 gene does not influence treatment efficacy. The manuscript is well written, the methods are adequately chosen.

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BRIEF ARTICLE

Patient-reported outcomes in subjects with neuroendocrine tumors of the pancreas

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Abstract

AIM: To assess the patient-reported outcomes (PROs) of pancreatic neuroendocrine tumor (PNET) patients.

METHODS: Fifty-one consecutive patients (21 male, 30 female, 61.0 ± 10.3 years) with proven PNETs were studied. An SF-12 questionnaire capable of exploring the physical (PCS) and mental (MCS) aspects of daily life was used. Four questionnaires were also used [12 items General Health Questionnaire (GHQ-12) for non-psychotic psychiatric disorders, State Trait Anxiety Inventory (STAI) Y-1 and Y-2 for anxiety and BDI-II for depressive symptoms] to explore the psychological aspects of the disease. Forty-four sex- and age-matched Italian normative subjects were included and evaluated using the SF-12, STAI Y-1 and Y-2 questionnaires.

RESULTS: Seven patients refused to participate to the study; they were clinically similar to the 44 participants who agreed to complete the questionnaires. PNET patients had a PCS score (44.7 ± 11.0) were not significantly different from the norms (46.1 ± 9.9, P = 0.610), whereas the MCS score was significantly lower in patients (42.4 ± 13.0) as compared to the norms (48.2 ± 9.8, P = 0.036). GHQ-12 identified 11 patients (25.0%) as having non-psychotic psychiatric disorders.

The STAI scores were similar in the patients and in the normative population. Finally, BDI-II identified eight patients (18.2%) with moderate depression and 9 (20.5%) with mild depression whereas 27 patients (61.4%) had no depression.

CONCLUSION: The PNET patients had a good physical but an impaired mental component of their quality of life; in addition, mild or moderate depressive symptoms are present in about 40% of PNET patients.

INTRODUCTION

Pancreatic neuroendocrine tumors (PNETs) are a heterogenous group of rare neoplasms, occurring in fewer than one in 100 000 people per year[1]. These tumors have attracted considerable attention in recent years, both because they are relatively easy to palliate and because they demonstrate the chronic effects of the particular hormone whose level is elevated. In about 10%-50% of cases, the tumors are not associated with obvious signs or symptoms of hormone hypersecretion and are called non-functioning tumors[2]. We have recently demonstrated that radical surgery continues to have a central role in the therapeutic approach to PNETs[2], and

that medical treatment has a precise role in functioning neuroendocrine tumors. Even if the survival rate is good, especially in those patients who were resected, there is no extensive data available on the quality of life (QoL) in these patients compared to the general population. In addition, the majority of studies published on this topic until now examined a Scandinavian population[3-10], and it is well known that some differences in perceiving well-being exist among different cultures[11]. Finally, there are no studies specifically focusing on the localization site of neuroendocrine tumors such as PNETs. Therefore, we carried out this prospective study on a large series of subjects with PNETs in order to assess the patient-reported outcomes (PROs), using different questionnaires capable of exploring the physical and mental aspects of their QoL as well as various psychological factors.

MATERIALS AND METHODS

Patients

Fifty-one consecutive patients with pathological confirmation of PNET who were admitted as outpatients to our Department from January to April 2009 were enrolled in the study. The histological specimens were obtained at surgery in 41 patients and at percutaneous biopsy using computed tomography/ultrasonography/endoscopic ultrasonography in the remaining 10 patients. The epidemiological and clinical data of the 51 patients studied are reported in Table 1. There were 21 males (41.2%) and 30 females (58.8%). The mean age of the patients was 61.0 ± 10.3 years (range, 34-86 years) and the duration of the disease was 5.8 ± 5.2 years (range, 1-28 years). Forty-one patients (80.4%) were married and 10 (19.6%) were single. Thirteen patients had finished elementary school (25.5%), 18 had finished middle school (35.3%), 13 had a high school diploma (25.5%) and seven had a university degree (13.7%). The majority of the patients were still working (38, 74.5%) while 13 were retired (25.5%). Twenty patients in the present series were drinkers (39.2%) while 22 (43.1%) were smokers. Thirty-five patients (68.6%) had one or more comorbidities (arterial hypertension in 21 patients, cardiac diseases in nine patients, chronic obstructive pulmonary diseases in fjour patients, gastrointestinal diseases such as peptic ulcers, gallstones and/or colon polyposis in eight patients, neuroendocrine diseases such as thyroid or hypophysis diseases in five patients, urogenital diseases in three patients, and other diseases in the remaining four patients) and 41 (80.4%) received drugs for causes not related to the PNET. Forty patients (78.4%) had undergone surgery at least once for causes unrelated to the neuroendocrine neoplasia (cholecystectomy in eight patients, appendectomy in 22 patients, inguinal hernioplasty in seven patients, other abdominal surgery in three patients, tonsillectomy and other otorhinolaryngological procedures in 17 patients, urological surgery in seven patients, gynecologic procedures in 10 patients and other surgical procedures in three patients). Forty-one of the 51 patients (80.4%) underwent surgery for their neuroendocrine pancreatic

Table 1 Demographic and clinical characteristics of the 44 patients who completed the questionnaires (mean ± SD)

	n (%)
Sex	
Males	18 (40.9)
Females	26 (59.1)
Age at interview (yr)	61.0 ± 9.8
Disease duration (yr)	5.8 ± 5.4
Body mass index (kg/m^2)	25.9 ± 4.3
Marital status	
Single/Widowed/Divorced	10 (22.7)
Married	34 (77.3)
Diploma	
Elementary school	11 (25.0)
Middle school	18 (40.9)
High school	10 (22.7)
University degree	5 (11.4)
Job	
Current workers	32 (72.7)
Managers	6
Employees	16
Industrial workers	3
Housewives	7
Retired	12 (27.3)
Alcohol habit	
Alcohol drinkers	18 (40.9)
Current drinkers	14
Drinking duration (yr)	40.2 ± 12.5
Smoking habit	
Smokers	20 (45.5)
Actual smokers	5
No. of cigarettes smoked per day	10.6 ± 5.9
Smoking duration (yr)	28.7 ± 13.3
Comorbidities	30 (68.2)
Non disease specific drugs	35 (79.5)
Previous surgery not due to neuroendocrine tumors	33 (77.0)
Surgery due to pancreatic neuroendocrine tumors	35 (79.5)
Pancreatic head resection	8
Distal pancreatectomy	20
Total pancreatectomy	2
Enucleation	5
Status of neuroendocrine tumors	
Disease free	25 (56.8)
Lymph node involvement/liver metastases	19 (43.2)
Specific drugs and treatment	
No drugs	30 (68.2)
Somatostatin analogues alone	9 (20.5)
Somatostatin analogues plus radiometabolic therapy	5 (11.4)
Pain in the month prior to complete the questionnaire	17 (38.6)
Dyspepsia	24 (54.5)
Diabetes	12 (27.3)

Data are reported as absolute and relative frequencies or mean ± SD.

disease: resective surgery was performed in 36 patients and enucleation of the cancer in the remaining 5 patients. At the time of the study, 29 patients were disease-free (56.9%) and the remaining 22 had advanced disease (lymph node involvement or liver metastases). Seventeen patients (33.3%) were currently being treated medically: 11 (21.6%) with somatostatin analogues alone, and 6 (11.8%) with somatostatin analogues and radiometabolic treatment. Nineteen patients (37.3%) had experienced pain in the month prior to the interview whereas 30 patients (58.8%) had dyspeptic symptoms evaluated according to the Rome III criteria[12]. Among the 30 patients with dyspeptic symptoms, the majority (25, 83.3%) had experienced post-

prandial distress syndrome and five had had heartburn (16.7%). Finally, 14 patients (27.5%) had diabetes secondary to pancreatic surgery.

The mean body mass index (BMI) was 25.7 ± 4.1 kg/m^2; in particular, according to the WHO criteria[13], 1 patient (2.0%) was underweight (BMI < 18.5 kg/m^2), 22 (43.1%) had normal weight (BMI between 18.5 and 24.9 kg/m^2), 21 (41.2%) were pre-obese (BMI between 25.0 and 30.0 kg/m^2) and the remaining 7 (13.7%) were obese (BMI > 30 kg/m^2).

Questionnaires

The Italian versions of the SF-12™ Health Survey (SF-12), State Trait Anxiety Inventory (STAI) Y-1 and Y-2, 12 items General Health Questionnaire (GHQ-12), and Beck Depression Inventory-Ⅱ (BDI-Ⅱ) were used for the purpose of the study.

The SF-12 questionnaires had previously been developed and tested on the Italian population in general[14]. It consists of 12 items which generate two summary scales capable of exploring the physical and mental components. High scale scores of the SF-12 physical (PCS) and mental (MCS) component summaries represent a good QoL. The normative population tested with the SF-12 questionnaire included 61 434 Italian subjects; these subjects were randomly sampled from the electoral lists, regardless of their health status[14]. The values of this group represent the average of the health-related QoL of the general Italian population. The stratified mean ± SD values of the PCS and the MCS referring to 44 sex- and age-matched Italian subjects of this population were used as a normative group.

The GHQ-12 is a measure of current mental health and since its development[15], it has been translated into the Italian language and has been used for the Italian population[16,17]. The GHQ-12 has become a commonly used instrument for detecting psychiatric disorders[18]. The scale asks whether the respondent has recently experienced a particular symptom or behavior. Each item is rated on a four-point scale (less than usual, no more than usual, slightly more than usual or much more than usual). The score ranges from 0 to 12[19]; subjects with a score from 0 to 4 have a > 80% probability of having non-psychotic psychiatric disorders and they are generally considered cases (i.e. those subjects who needed psychological support) while those subjects with a score > 4 should be considered not affected by non-psychotic psychiatric disorders[20].

Due to the fact that the GHQ-12 is a general questionnaire regarding the psychological aspects of daily life, we used the following two specific questionnaires: the STAI[21] and the BDI-Ⅱ[22] to better evaluate anxiety disorders and depressive syndrome.

Regarding the assessment of anxiety, both state and trait anxiety can be assessed by using the 40 items of the STAI-Y-1 and Y-2[21]. The state (Y-1) and the trait (Y-2) portions of the inventory each consist of 20 item Likert format statements. The STAI has been extensively validated and the Italian version has already been used[23]. The scores of the two 20 item scales range from 20 to 80 and high scores represent a high level of anxiety[21]. In brief, the state of anxiety can vary in intensity and fluctuate over time depending on the perceived threat. The trait of anxiety is a tendency to perceive a wide range of living conditions as threatening and to react to them with a high intensity; this trend remains latent until it is activated by stress associated with real or imagined dangers. The normative population tested with the STAI questionnaire included 2363 Italian working people sampled regardless of their health status[23]. The stratified mean ± SD values of the STAI Y-1 and the STAI Y-2 used with 44 sex- and age-matched Italian subjects of this population were used as a normative group.

The BDI-Ⅱ[22] is a 21-item self-report instrument which assesses the severity of depressive symptoms in adolescents and adults over the 2 wk prior to its use. Each item is rated on a 4-point scale (0-3) with total scores ranging from 0 to 63. For interpretation of the BDI-Ⅱ, Beck *et al*[22] present a table of scores indicative of: severe (> 28); moderate (score ranging from 20 to 28) and mild (score ranging from 14 to 19) depression. Scores of ≤ 13 suggest an absence of depression. These scores observed in an American population having depressive symptoms can also be used for the respective Italian population[24]. The Italian version of this questionnaire was used[24].

All patients included in our study were fluent in the Italian language and the questionnaires were administered according to the recommendations suggested by the user manuals[14,19,23,24].

Ethics

The study was approved by the Senior Staff Committee of the Department of Digestive Diseases and Internal Medicine of the University of Bologna and was carried out in accordance with the Helsinki Declaration of the World Medical Association. All study participants gave oral informed consent.

Statistical analysis

The descriptive statistics applied were: mean, SD and ranges as well as absolute and relative frequencies. Three-way ANOVA was applied in order to estimate the various effects related to the SF-12 and STAI scores by adjusting for age (increasing trend among the age categories) and gender (males *vs* females). The 95% confidence intervals (95% CIs) of the estimates were also calculated. One-way ANOVA, one-way linear term ANOVA, Pearson correlation, Fisher exact test, Pearson chi-squared and linear-by-linear association chi-square were also applied where appropriate.

All statistical evaluations were carried out by running the SPSS version 13.0 for Windows. Two-tailed *P* values less than 0.05 were considered statistically significant.

RESULTS

Forty-four (86.3%) of the 51 patients answered the questionnaires; the demographic and clinical characteristics of these patients are reported in Table 1. Seven patients

Table 2 Effects of neuroendocrine tumors of the pancreas on the SF-12 physical (PCS) and mental (MCS) component summaries estimated by means of three-way ANOVA adjusted for age (increasing trend among the age classes) and gender

	PCS		MCS	
	Effects (95% CI)	*P* value	Effects (95% CI)	*P* value
Overall effects of the disease (patients *vs* normative group)	-1.16 (-5.66 to 3.34)	0.610	-5.32 (-10.30 to -0.35)	0.036
Effects of the disease within males	-1.17 (-8.01 to 5.68)	0.735	-6.12 (-13.69 to 1.45)	0.112
Effects of the disease within females	-1.15 (-6.99 to 4.69)	0.697	-4.53 (-10.99 to 1.93)	0.167
Interaction between the effects of the disease and gender (males *vs* females)	-0.02 (-9.02 to 8.97)	0.996	-1.59 (-11.54 to 8.37)	0.752
Interaction between the effects of the disease and age	3.32 (-6.96 to 13.60)	0.522	9.54 (-1.84 to 20.92)	0.099
Interaction between the effects of the disease and age within males	4.10 (-11.88 to 20.09)	0.611	9.24 (-8.44 to 26.93)	0.301
Interaction between the effects of the disease and age within females	2.54 (-10.39 to 15.48)	0.697	9.84 (-4.47 to 24.15)	0.175
Interaction between the effects of the disease and age and gender	1.56 (-19.00 to 22.12)	0.880	-0.59 (-23.35 to 22.17)	0.959

95% CI: 95% confidence interval.

Table 3 Effects of neuroendocrine tumors of the pancreas on the STAI Y-1 (anxiety state) and Y-2 (anxiety trait) estimated by means of three-way ANOVA adjusted for age (increasing trend among the age classes) and gender

	STAI anxiety state (Y-1)		STAI anxiety trait (Y-2)	
	Effects (95% CI)	*P* value	Effects (95% CI)	*P* value
Overall effects of the disease (patients *vs* normative group)	-5.16 (-12.56 to 2.23)	0.169	0.77 (-5.84 to 7.39)	0.817
Effects of the disease within males	-4.66 (-16.98 to 7.67)	0.454	-0.48 (-11.50 to 10.54)	0.932
Effects of the disease within females	-5.66 (-13.84 to 2.51)	0.172	2.02 (-5.29 to 9.33)	0.584
Interaction between the effects of the disease and gender (males *vs* females)	1.00 (-13.79 to 15.79)	0.893	-2.50 (-15.72 to 10.73)	0.708
Interaction between the effects of the disease and age	7.69 (-7.10 to 22.48)	0.304	-2.58 (-15.81 to 10.64)	0.698
Interaction between the effects of the disease and age within males	5.30 (-19.35 to 29.95)	0.670	-0.05 (-22.09 to 21.99)	0.996
Interaction between the effects of the disease and age within females	10.07 (-6.28 to 26.42)	0.224	-5.12 (-19.74 to 9.51)	0.488
Interaction between the effects of the disease and age and gender	-4.77 (-34.35 to 24.81)	0.749	5.06 (-21.39 to 31.51)	0.704

(13.7%) refused to participate in the study: no significant differences among the demographic and clinical data were found between participants and those who refused to answer the questionnaire (data not shown for brevity).

Overall analysis of the SF-12 questionnaire in the 44 patients showed that the values of the PCS score are representative of a relatively good physical QoL and they were not significantly different from those of the normative population (PCS: 44.7 ± 11.0 *vs* 46.1 ± 9.9, *P* = 0.610). The MCS score was significantly lower in patients (42.4 ± 13.0) as compared to the norms (48.2 ± 9.8, *P* = 0.036). Moreover, a stratified analysis (Table 2) failed to show any significant interaction between sex and age and the effect of the disease on the MCS of PNET patients.

Regarding the GHQ-12 questionnaire, we identified 11 patients (25.0%) having non-psychotic psychiatric disorders. Shown in Table 3, the results of the STAI demonstrated that anxiety was similar in patients and the normative population.

Finally, in order to explore the depressive syndrome in detail, the BDI-Ⅱ identified eight patients (18.2%) with moderate depression, nine patients (20.5%) with mild depression and 27 patients (61.4%) with no depression.

We also explored the relationships between the results of the various questionnaire scores. The MCS was highly related (*P* < 0.001) to both anxiety state and trait (STAI Y-1 and Y-2, respectively) whereas the PCS was only significantly related to anxiety trait (*P* = 0.043) but not to the anxiety state (*P* = 0.222). As shown in Table 4, only the STAI scores were significantly associated with the presence of non-psychotic psychiatric disorders as

evaluated by the GHQ-12 while both the SF-12 and the STAI were significantly related to the depressive symptoms as assessed by the BDI-Ⅱ. In addition, a significant (*P* = 0.011) positive relationship was also found between the presence of non-psychotic psychiatric disorders and the depressive state (absence of depression: 4/27, 14.8%; mild depression: 2/9, 22.2%; moderate depression: 5/8, 62.5%).

Table 5 shows the possible relationships between the demographic and clinical characteristics of the PNET patients and the results of the questionnaires investigated. MCS significantly improved with age (*P* = 0.042), and anxiety state (STAI Y-1) significantly decreased with age (*P* = 0.038). Workers had an MCS (39.8 ± 13.1) significantly lower than retired people (49.2 ± 10.7, *P* = 0.032). The patients that did not receive non-specific disease drugs had a PCS score (51.7 ± 8.6, *P* = 0.032) significantly higher (42.9 ± 11.70) than those who were taking non-disease specific drugs; PCS was also significantly higher in patients who underwent surgery for PNET (46.4 ± 10.8) compared with those who were not operated on (38.3 ± 10.0, *P* = 0.049). Pain worsened both the STAI Y-1 (patients with pain had a score of 48.9 ± 12.7 and those without 41.5 ± 11.0, *P* = 0.046) and Y-2 (patients with pain had a score of 45.8 ± 12.0 and those without 39.1 ± 9.7, *P* = 0.049) scores. Finally patients with dyspeptic symptoms had a worse MCS (patients with dyspepsia had a score of 38.4 ± 13.2 and those without 47.1 ± 11.4, *P* = 0.025). The frequency of dyspepsia was 37.0% (10/27) in patients without depressive symptoms evaluated with the BDI-Ⅱ, 88.9%

Table 4 Relationships between SF-12 and STAI scores, and GHQ-12 and BDI-II scores in the 44 patients with neuroendocrine tumors of the pancreas (mean ± SD)

	SF-12		STAI	
	PCS	MCS	Anxiety state (Y-1)	Anxiety trait (Y-2)
GHQ-12[1]				
Subjects without non-psychotic psychiatric disorders (score ≤ 4, n = 33)	46.1 ± 9.8	44.4 ± 11.8	42.3 ± 10.9	39.1 ± 10.1
Subjects with psychotic psychiatric disorders (score > 4, n = 11)	40.6 ± 13.9	36.1 ± 15.0	50.7 ± 13.9	49.6 ± 10.3
P value	0.156	0.066	0.043	0.005
BDI-II[2]				
Absence of depression (score ≤ 13, n = 27)	48.2 ± 8.8	47.3 ± 10.3	39.1 ± 9.1	36.1 ± 8.1
Mild depression (score 14-19, n = 9)	40.0 ± 11.8	40.1 ± 14.8	44.2 ± 7.7	43.6 ± 4.6
Moderate depression (score 20-28, n = 8)	38.4 ± 13.3	28.2 ± 8.3	62.3 ± 7.2	58.8 ± 4.3
Severe depression (score > 28, n = 0)	-	-	-	-
P value	0.010	< 0.001	< 0.001	< 0.001

[1]One-way ANOVA; [2]ANOVA linear term.

Table 5 Relationship between demographic and clinical characteristics of the 44 patients who completed the questionnaires and the results of the questionnaires used (in bold the significant associations)

	P					
	SF-12 PCS	SF-12 MCS	GHQ-12	STAI Y-1	STAI Y-2	BDI-II
Gender (males *vs* females)	0.589[1]	0.630[1]	0.480[2]	0.317[1]	0.214[1]	0.387[3]
Age at interview	0.113[4]	**0.042[4] (r = 0.309)**	0.351[1]	**0.038[4] (r = -0.314)**	0.309[4]	0.525[5]
Disease duration	0.962[4]	0.751[4]	0.912[1]	0.669[4]	0.752[4]	0.856[5]
BMI	0.766[4]	0.185[4]	0.125[1]	0.241[4]	0.514[4]	0.723[5]
Marital status (single *vs* married)	0.181[1]	0.093[1]	0.237[2]	0.110[1]	0.556[1]	0.443[3]
Diploma (trend from elementary school to university degree)	0.185[5]	0.916[5]	0.648[3]	0.705[5]	0.373[5]	0.405[3]
Job (workers *vs* retired)	0.240[1]	**0.032[1] (-9.4 ± 4.23)**	0.240[2]	0.054[1]	0.057[1]	0.227[3]
Alcohol habit (drinkers *vs* non-drinkers)	0.407[1]	0.745[1]	0.480[2]	0.653[1]	0.724[1]	0.930[3]
Smoking habit (smokers *vs* non-smokers)	0.349[1]	0.465[1]	0.294[2]	0.124[1]	0.122[1]	0.601[3]
Comorbidities (present *vs* absent)	0.651[1]	0.923[1]	1.000[2]	0.435[1]	0.202[1]	0.402[3]
Non-disease specific drugs (yes *vs* no)	**0.032[1] (-8.7 ± 3.9)**	0.322[1]	0.085[2]	0.369[1]	0.152[1]	0.140[3]
Previous surgery not due to neuroendocrine tumors (yes *vs* no)	0.775[1]	0.238[1]	0.241[2]	0.439[1]	0.437[1]	0.321[3]
Surgery due to pancreatic neuroendocrine tumors (yes *vs* no)	**0.049[1] (8.1 ± 4.0)**	0.778[1]	0.669[2]	0.987[1]	0.880[1]	0.957[3]
Disease status (disease free patients *vs* patients having lymph node involvement or liver metastases)	0.875[1]	0.454[1]	1.000[2]	0.300[1]	0.169[1]	0.105[3]
Specific treatment (yes *vs* no)	0.816[1]	0.454[1]	0.722[2]	0.925[1]	0.733[1]	0.985[3]
Pain in the last month (present *vs* absent)	0.152[1]	0.371[1]	0.075[2]	**0.046[1] (7.4 ± 3.6)**	**0.049[1] (6.7 ± 3.3)**	0.190[3]
Dyspepsia (present *vs* absent)	0.115[1]	**0.025[1] (-8.7 ± 3.8)**	0.728[2]	0.199[1]	0.195[1]	**0.015[3,6]**
Diabetes (present *vs* absent)	0.908[1]	0.263[1]	0.457[2]	0.883[1]	0.581[1]	0.938[3]

[1]One-way ANOVA (effect estimates; mean ± SE); [2]Fisher exact test; [3]Liner-by-linear association chi-square (frequencies); [4]Pearson correlation (regression coefficient; r); [5]One-way linear term ANOVA; [6]Frequency of dyspepsia: according to BDI-II: absence of depression (score ≤ 13) 10/27 (37.0%); mild depression (score 14-19) 8/9 (88.9%); moderate depression (score 20-28) 6/8 (75.0%). In order to quantify the relationships, the various effect estimates - evaluated according to the statistical analysis applied - have been reported in parentheses.

(8/9) in those with mild depressive symptoms, and 75% (6/8) in those with moderate depressive symptoms (*P* = 0.015).

DISCUSSION

The correct management of neuroendocrine tumors of the pancreas includes diagnosis, management of the functional hormonal syndrome when present and management of the potentially malignant tumor. Control of the hormonal syndrome, when present, is achieved preoperatively in order to stabilize patient status for the operation[25] whereas, in the case of recurrence and in the case of a non-surgical approach to these tumors,

medical treatment is the main option[26].

In the present study, approximately 57% of the patients were disease-free at the time of the interview whereas, as previously reported[2], patients with advanced disease were treated medically. Thus, especially in this latter group of subjects, PRO assessment seems to be important for evaluating the impact of this chronic disease[27] in order to understand how biology interacts with cultural, social, interpersonal and psychological aspects. In fact, QoL also plays a central role in how the variety of symptoms and the medical management of disease are perceived by those affected. However, there are a limited number of studies evaluating the patient point of view regarding his own disease[3-10,28]. All

these studies evaluated the QoL in patients with various neuroendocrine tumors of the gastrointestinal tract and almost all[3-6,8-10,28] used the European Organisation for Research and Treatment of Cancer Quality of life Questionnaire-C30 (EORTC) questionnaire for quantifying the QoL of the patients; only one study explored the PROs using a different questionnaire such as the SF-36[7]. Only two studies compared the EORTC results of neuroendocrine tumor patients with a normative population[6,9], but the patients enrolled had carcinoid tumors which represent only a part of the neuroendocrine tumors of the gastrointestinal tract. Thus, we focused our attention on the PROs in a well defined group of patients, i.e. those with a diagnosis of PNET. For this purpose, we utilized the SF-12 questionnaire. The choice of this questionnaire in evaluating the PROs was based on the following assumptions: the simplicity of this questionnaire (it is based on 12 questions only whereas the EORTC contains 30 questions); the two SF-12 component summaries have a high level of reliability in evaluating the QoL similar to that of the domains/scores of the EORTC questionnaire[29]; it is also possible to compare the data of patients with a nationwide normative population (there is no Italian normative population for EORTC) and, finally, SF-12 has been already tested in patients with neuroendocrine tumors of the ileum[30]. Thus, from a practical point of view, the SF-12 questionnaire is more reliable and easier to use in routine clinical practice than the EORTC. Only a few studies have also explored psychological aspects of the disease, mainly using the Hospital Anxiety and Depression Scale (HADS) for evaluating this topic[3,6,7,9,10]. Thus, we planned the present study in order to evaluate not only the presence of generic psychological distress by using the GHQ-12 questionnaire but also to determine whether the psychological distress eventually present is related to anxiety or depressive syndrome, and to compare these psychological aspects to those of the normative Italian population. For this purpose, we utilized two specific questionnaires, the STAI and the BDI-II, which are largely used in this setting.

The values of the SF-12 summary scores as compared to the norms showed that the 44 patients in the present study seemed to perceive their physical QoL as relatively good even if they had a tumor, and this finding agreed with previous reports[4,5,9,10] whereas mental aspects were significantly impaired as compared to the norms. This seems to be due to the disease itself and is not related to the effects of gender and age. In fact, we carried out a stratified analysis which showed no significant interaction between age and sex, and the effect of the disease. We also attempted to identify the factors capable of modifying the mental component of QoL of patients with PNET. Surprisingly, we found that our patients were not affected by anxiety, but mild and moderate depressive symptoms were present in about 40% of the patients studied. Our data differed from those previously published which showed that patients

with neuroendocrine tumors had anxiety and depression in varying proportions from low, as reported by Larsson et al[4], to high, as reported by Fröjd et al[10]. These results may be due to the fact that we compared the data to the general population. In fact, when we analyzed the questionnaire score within the group of patients without comparison to the normative population, we found that the MCS and the anxiety state were better in older patients, and that workers had an MCS significantly lower than retired people. PCS was significantly higher in patients that did not receive non-disease specific drugs as compared to those who were taking non-disease specific drugs; this score was also significantly higher in patients who underwent surgery for PNET as compared to those who were not operated on. Furthermore, we also found that pain worsened both the anxiety state and trait (STAI Y-1 and Y-2) scores. Finally, patients with dyspeptic symptoms had worse MCS scores and they also presented with more depressive symptoms than those without. In clinical practice, these red flags should also be taken into consideration; even if the tumor does not affect physical condition (only 2% of patients were underweight), the workers were worried about their condition which could limit their daily activity and they probably needed psychological support.

Furthermore, these patients needed more intensive medical treatment for alleviating the pain flares and the dyspeptic symptoms.

Finally, the seven patients who refused to participate in the study were similar in demographic and clinical variables to the 44 subjects who completed the questionnaires; thus, we can assume that the results obtained can be extended to the entire Italian PNET population, at least under our experimental conditions.

In conclusion, knowledge of the patient's reported outcome in patients with PNET may help in decision-making providing important information about the long-term effects of depressive symptoms in cancer survivors, and help to identify potential adjustment problems. Current workers and especially those with dyspeptic symptoms are those patients who need specific and intensive medical and psychological support because of the presence of depressive symptoms.

COMMENTS

Background
Pancreatic neuroendocrine tumors (PNETs) are generally slow growing and patients may have prolonged survival. There are no studies specifically focusing on the localization site of neuroendocrine tumors such as PNETs.

Research frontiers
patient-reported outcome (PRO) assessment is important for evaluating the impact of this chronic disease in order to understand how biology interacts with the cultural, social, interpersonal and psychological aspects. In fact, quality of life (QoL) also plays a central role in how the variety of symptoms and the medical management of the disease are perceived by those affected.

Innovations and breakthroughs
Of the patients affected by PNETs, current workers and especially those with dyspeptic symptoms are those subjects who need a specific and intensive medical and psychological support because of the presence of depressive symptoms.

Applications

Knowledge of the patient's reported outcome in patients with PNETs may help in decision-making providing important information about the long-term effects of depressive symptoms in cancer survivors, and help to identify potential adjustment problems.

Terminology

Patient-reported outcomes provide a means of gaining an insight into the way patients perceive their health and the impact that treatments or adjustments to lifestyle have on their QoL.

Peer review

The paper by Pezzilli *et al* assessed the physical and mental status of patients with PNETs. They conclude that patients had a comparable physical but a lower mental score than these 44 individuals. The idea is rather innovative and the results are interesting.

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BRIEF ARTICLE

High circulating N-terminal pro-brain natriuretic peptide and tumor necrosis factor-α in mixed cryoglobulinemia

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Abstract

AIM: To evaluate serum levels of N-terminal pro-brain natriuretic peptide (NTproBNP) and tumor necrosis factor α (TNF-α) in a large series of patients with hepatitis C associated with mixed cryoglobulinemia (MC+HCV).

METHODS: Serum NTproBNP and TNF-α levels were assayed in 50 patients with MC+HCV, and in 50 sex- and age-matched controls.

RESULTS: Cryoglobulinemic patients showed significantly higher mean NTproBNP and TNF-α levels than controls ($P < 0.001$; Mann-Whitney U test). By defining high NTproBNP level as a value higher than 125 pg/mL (the single cut-off point for outpatients under 75 years of age), 30% of MC+HCV and 6% of controls had high NTproBNP (χ^2, $P < 0.01$). With a cut-off point of 300 pg/mL (used to rule out heart failure (HF) in patients under 75 years of age), 8% of MC+HCV and 0 controls had high NTproBNP (χ^2, $P < 0.04$). With a cut-off point of 900 pg/mL (used for ruling in HF in patients aged 50-75 years; such as the patients of our study), 6% of MC+HCV and 0 controls had high NTproBNP (χ^2, $P = 0.08$).

CONCLUSION: The study demonstrates high levels of circulating NTproBNP and TNF-α in MC+HCV patients. The increase of NTproBNP may indicate the presence of a subclinical cardiac dysfunction.

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Key words: NTProBNP; Tumor necrosis factor α; Hepatitis C; Mixed cryoglobulinemia; Heart failure

INTRODUCTION

The most common clinical features of hepatitis C associated with mixed cryoglobulinemia (MC+HCV) are correlated with vasculitis in the various organs and sometimes with increased viscosity of the plasma[1,2]. Signs and symptoms include purpura, ulcers of the extremities, arthralgia, proteinuria, hepatic damage, abdominal pain, mental confusion, oligo-anuria, hemorrhagic diathesis, and sometimes congestive heart failure (HF)[1-4]. Furthermore, HF has been described as heralding the clinical onset of essential mixed cryoglobulinemia (MC)[5]. Moreover, many MC+HCV patients experience symptoms such as fatigue, dyspnea and reduced physical activity. However, in many patients, these symptoms are not proportional to the liver involvement and resemble symptoms of HF.

Several studies have shown that plasma levels of brain natriuretic peptide (BNP) and N-terminal proBNP (NTproBNP) are reliable diagnostic and prognostic

markers for cardiac disease[6,7] that correlate with symptoms of HF and the severity of systolic and diastolic dysfunction[8]. Some authors have recently stated that NTproBNP appears superior to BNP for the evaluation of suspected acute HF in patients with preserved left ventricular ejection fraction[9,10]. In the study by O'Donoghue et al[9], NTproBNP seems to correlate with HF severity better than BNP and is more sensitive.

Cytokines play an important role in chronic HF, and it has been shown that TNF-α and NTproBNP are independent predictors of long-term risk of death[11,12] for HF. Circulating TNF-α has been recently shown to be high in patients with MC[13].

However, to our knowledge, until now no study has evaluated circulating NTproBNP together with TNF-α levels, as possible markers of HF, in MC+HCV patients affected by cryoglobulinemic vasculitis.

The aim of this study was to evaluate serum levels of NTproBNP in a series of MC+HCV patients, and to correlate this parameter with the clinical features of the disease, and with the circulating levels of TNF-α.

MATERIALS AND METHODS

Patients

Fifty MC+HCV patients (37 females and 13 males; mean age 57 ± 9 years; mean disease duration 10 ± 11 years), consecutively referred to our Rheumatology Unit, were recruited for the study between 2001 and 2006. The diagnosis of MC+HCV was based on the presence of serum mixed (IgG-IgM) cryoglobulins and the classical clinical triad, purpura, weakness, arthralgias, and on the exclusion of other well-known systemic disorders, such as immuno-rheumatic and neoplastic diseases[1,2,14-16].

The study included only patients with MC+HCV, without liver cirrhosis or hepatocellular carcinoma (assessed by histology, laboratory evidence of liver failure and/or ultrasound-proven portal hypertension)[17,18]. None of the patients had evident signs of HF, organic renal disease (patients with serum creatinine > 1.2 mg/dL and/or proteinuria > 0.5 g/24 h were excluded), thyroid disease, diabetes, cancer or any other major diseases. All patients had normal cardiac physical examinations and normal blood pressure.

Thirty eight out of 55 (76%) MC+HCV patients underwent liver biopsy for diagnostic purposes; liver histology activity index (grade) or stage of liver fibrosis were evaluated according to Ishak et al[19]. The mean activity index (grade) in MC+HCV patients was 5.0 ± 1.2, and the mean stage was 2.0 ± 0.9. Main demographic and clinico-serological features of MC+HCV patients are reported in Table 1.

Among the patients, 17 had been previously treated with interferon-alpha (IFN-α) for an average of 7 mo (range, 1-13 mo), at a mean dosage of 10.4 MU/wk; the time elapsed from the last course of IFN-α treatment ranged from 6 to 69 mo (mean 40 ± 22 mo). No statistically significant differences were observed in the main demographic and clinico-serological features of MC+HCV patients treated or untreated with IFN-α.

Table 1 Demographic and clinico-serological features of 50 MC+HCV patients

Age (yr)	57 ± 9
Male/female	13/37
Disease duration with MC (yr)	10 ± 11
Purpura	82%
Active vasculitis	31%
Weakness	91%
Arthralgias	83%
Arthritis	14%
Raynaud's phenomenon	51%
Sjogren's syndrome	45%
Peripheral neuropathy	59%
Aminotransferase elevation and/or histologic activity[1]	71%
Cryocrit (%)	4.2 ± 8.9
CH50 (normal: 160-220 U)	111 ± 36
C3 (normal: 60-130 mg/dL)	81 ± 36
C4 (normal: 20-55 mg/dL)	14 ± 18
Autoantibodies[2]	25%

[1]Increase of the liver enzyme (alanine aminotransferase) and/or liver histological alterations. [2]Presence of anti-nuclear and/or anti-mitochondrial and/or anti-smooth muscle and/or anti-extractable nuclear antigen autoantibodies. MC+HCV: Hepatitis C associated with mixed cryoglobulinemia.

At the time of study, 35 MC+HCV patients were taking low doses of corticosteroids, 8 had previously been on corticosteroids and 7 had never been treated with corticosteroids. No MC+HCV patient had had plasma exchange treatment in the last year before the study. In both patients and controls, a careful medical history was collected, in particular with regard to family history of thyroid disease, smoking habits, and drugs. The presence of Raynaud's phenomenon, Sjogren's syndrome, skin ulcers, peripheral neuropathy, and renal and liver involvement in MC+HCV patients was evaluated as previously described[16]. Routine blood chemistry was carried out by standard methods.

Controls

Each of the 50 MC+HCV patients eligible for the study was matched, by sex and age, one-to-one with a control group of healthy subjects of the general population from the same geographic area (North-West Tuscany). This control group was extracted from a larger sample of 1640 subjects taking part in a population-based survey of thyroid disorders; only HCV-negative subjects, without clinical and laboratory evidence of thyroid and liver disorders or autoimmune diseases and not treated with immunomodulators were included.

None of the controls had signs of HF, organic renal disease, thyroid disease, diabetes, cancer or any other major diseases. All patients had normal cardiac physical examination results and normal blood pressure.

Extraction of the control group from the original population was performed by finding the closest age match (± 2 years) to each case within either gender. When more than one age-match was available per case, the choice was made at random.

The study protocol was approved by the local Ethics Committee. All subjects gave their informed consent to enter the study.

Figure 1 Plasma NTproBNP circulating levels (were significantly higher in MC+HCV patients than in controls; Mann Whitney *U* test). [a]$P < 0.05$ *vs* control.

Immunological studies

Cryocrit was measured as the percentage of packed cryoglobulins after cold centrifugation of the serum; cryoglobulin composition was determined by the incorporation in cryoprecipitates of monoclonal or polyclonal IgM-rheumatoid factor (i.e. MC type II or MC type III); hemolytic complement C3-C4 fractions were measured as previously described[16], anti-nuclear, anti-smooth muscle, and anti-mitochondrial autoantibodies were detected by current techniques[16]. Sera with a titre > 1:40 were considered positive. Anti-extractable nuclear antigen antibodies, including anti-Scl70, -Sm, -RNP, -SSA/SSB, -PCNA, -SL and -Jo1 specificities, were detected by counter-immunoelectrophoresis according to the methods described by Bunn *et al*[20].

Virological studies

Antibodies against HCV (anti-HCV) and HCV-RNA were determined in serum clotted and centrifuged at 37℃ and stored at -70℃. Anti-HCV and HCV-RNA levels [assayed by polymerase chain reaction (PCR) technique] in the serum were investigated as previously described[21,22].

Cytokines, chemokines and analytical assays

Blood samples for analysis of plasma NTproBNP were collected, centrifuged and plasma was stored at -80℃ until analysis. Plasma concentrations of NTproBNP were measured by a sandwich immunoassay on an Elecsys 2010 (Roche Diagnostics, Mannheim, Germany).

Serum TNF-α concentrations were measured using commercially available kits (R&D Systems, Minneapolis, MN). The mean minimum detectable dose was 0.12 pg/mL for TNF-α; the intra- and inter-assay coefficients of variation were 5.8% and 10.2%. Samples were assayed in duplicate. Quality control pools of low, normal, or high concentration for all parameters were included in each assay. Alanine aminotransferase (ALT) was assayed by conventional methods[18].

Statistical analysis

Values are given as mean ± SD for normally distributed variables, or as median ± IQR for not normally distributed variables (NTproBNP, TNF-α). Group values were compared by univariate ANOVA, for normally distributed variables; or by Kruskal-Wallis ($\geq$ 3 groups) or Mann-Whitney *U* (2 groups) tests. Proportions were compared by the chi-square test. *Post-hoc* comparisons on normally distributed variables were carried out using the Bonferroni-Dunn test. Univariate analysis was performed by simple regression. A multivariate logistic regression analysis considering age, gender, ALT, and presence or absence of active vasculitis as independent variables and presence or absence of high levels of NTproBNP or TNF-α as dependent variables was performed in MC+HCV patients.

RESULTS

Plasma NTproBNP concentrations were significantly ($P < 0.001$; Mann-Whitney *U* test) higher in MC+HCV patients (mean 123 ± 112 pg/mL; median 36 pg/mL, range 8-1547 pg/mL) than in controls (mean 11 ± 12 pg/mL; median 3.1 pg/mL range 2-145 pg/mL) (Figure 1).

By defining high NTproBNP level as a value higher than 125 pg/mL (the single cut-off point for outpatients under 75 years of age[23]) 15/35 MC+HCV and 3/47 controls had high NTproBNP (χ^2, $P < 0.002$).

With a cut-off point of 300 pg/mL (used to rule out HF in patients under 75 years of age[23]) 4/46 MC+HCV and 0/50 controls had high NTproBNP (χ^2, $P < 0.041$).

With a cut-off point of 900 pg/mL (used for ruling in HF in patients with age 50-75; such as the patients of our study[23]) 3/47 MC+HCV and 0/50 controls had high NTproBNP (χ^2, $P = 0.08$).

In order to better define the role of increased serum NTproBNP in MC+HCV, mean levels of this chemokine were separately evaluated (by Mann Whitney *U* test) among MC+HCV patient subgroups defined according to main demographic and clinical features (age > 55 years; gender; disease duration > 10 years; presence or absence of purpura, active vasculitis, weakness, arthralgias, arthritis, Raynaud's phenomenon, Sjogren's syndrome, peripheral neuropathy, aminotransferase elevation and/or histologic activity in the liver), but no significance was found. No significant correlations were observed between NTproBNP levels and serological findings of MC+HCV (levels of cryocrit and complement, presence/absence of autoantibodies) or previous/ongoing treatments.

Serum TNF-α was detectable in 85% of controls and in all MC+HCV patients; mean levels were significantly ($P < 0.01$; Mann Whitney *U* test) higher in MC+HCV patients (mean 26 ± 65 pg/mL; median 8.3 pg/mL, range 1.3-269 pg/mL) than in controls (mean 1.6 ± 1.1 pg/mL; median 1.2 pg/mL, range 0.6-3.4 pg/mL). No correlation was found between serum TNF-α and any of the following; ALT level, liver histology activity index, stage of liver fibrosis, the presence of active vasculitis, or the other demographic, serological and clinical features of MC.

MC+HCV patients had, obviously, raised ALT enzymes ($P < 0.0001$), in comparison with controls.

No association was observed between NTproBNP or TNF-α levels and ALT levels in the MC+HCV patients.

DISCUSSION

Our study demonstrates the presence of significantly high serum levels of NTproBNP in patients with MC+HCV compared to healthy controls. Furthermore, our study confirms significantly high serum levels of TNF-α in patients with MC+HCV compared to healthy controls.

Some authors have recently stated that NTproBNP appears superior to BNP for the evaluation of suspected acute HF in patients with preserved left ventricular ejection fraction[9,10]. Furthermore[9], NTproBNP seems to correlate with HF severity better than BNP and appears more sensitive. The International Collaborative for NTproBNP Study (ICON) helped in defining appropriate cut-off points for NTproBNP in the emergency department[23]: 300 pg/mL should be used to rule out HF, while 450 pg/mL, 900 pg/mL, or 1800 pg/mL, depending on age (< 50, 50-75, or > 75 years; respectively), should be applied for ruling in HF. The age stratifications do offer significant positive predictive value. For outpatient evaluation the manufacturer suggested a single cut-off point of 125 pg/mL for patients under 75 years of age and 450 pg/mL for patients above 75 years of age[23].

The levels of NTproBNP found in MC+HCV are included in a gray zone (125-900 pg/mL) that is not necessarily associated with HF, however in 3/50 (6%) of MC+HCV patients the NTproBNP levels were higher than 900 pg/mL, which is the cut-off value for ruling in HF for patients of age 50-75 years, such as the patients of our study. Since NTproBNP level seems to correlate with HF severity, the values in the gray zone may be suggestive of a subclinical cardiac impairment. The exclusion of patients with renal failure from the study suggests that the NTproBNP increase is not related to any kidney involvement in the MC+HCV patients.

In a previous work, Matsumori *et al*[24] showed there was a significantly higher NTproBNP level in HCV patients and observed that the presence of anti-HCV antibodies in sera was more prevalent in patients with myocarditis and HF than in the general population. Future studies comparing NTproBNP levels between HCV and MC+HCV patients will be needed to evaluate the specific influence of the cryoglobulinemic vasculitis.

The findings of the present study may have important implications for patients with MC+HCV. Most patients complain about fatigue, dyspnea and reduced physical capacity. The pathogenesis of these symptoms is not well understood and sometimes attributed to the liver injury. However, it seems possible that these patients experience cardiac impairment which could at least contribute to these symptoms. Furthermore, among signs and symptoms of MC+HCV, sometimes congestive HF may be found, which in some cases has been described as heralding the clinical onset of essential MC[3-5].

Testing of NTproBNP level may serve as a screening marker for cardiac insufficiency in the differential diagnosis of fatigue and dyspnea and may aid the decision for further diagnostic testing of cardiac function as has been described for other groups of patients[25-28]. Besides diagnostic consequences, evaluation of NTproBNP may have therapeutic consequences for patients with MC+HCV. In patients with known congestive HF, elevated plasma BNP concentrations could be reduced by treatment with ACE inhibitors[29] or angiotensin II receptor antagonists[30] as well as treatment with diuretics and vasodilators[31]. As a consequence, plasma NTproBNP concentration may guide the intensity of pharmacotherapy as some interventional studies have suggested[32,33].

Cytokines play an important role in chronic HF, and it has been shown that TNF-α and NTproBNP are independent predictors of long-term risk of death[11,12] for patients with HF.

Our study confirms a high serum TNF-α level in HCV+ve patients, as previously demonstrated in other studies of HCV+ve patients[34-36]. The increase of TNF-α in MC+HCV patients is unlikely to be due to a more aggressive liver disease; in fact, no correlation was found between TNF-α levels and ALT levels, or with degree of liver inflammation in our study[13]. Other studies have shown an increased production of TNF-α by lymphocytes in MC+HCV patients[37,38], suggesting that the increase of TNF-α may be due to activation of lymphoid cells.

The fact that TNF-α and NTproBNP are independent predictors of long-term risk[11] is in agreement with the results of our study; in fact, no relationship has been observed between TNF-α and NTproBNP.

It has been shown that combining measurements of pro-inflammatory cytokine TNF-α and NTproBNP seems a promising tool in the prognostic assessment of HF patients[12]. However, even if we have shown that NTproBNP and TNF-α are both high in the circulation of MC+HCV patients, we cannot exclude that different pathogenetic pathways may be differentially implicated in the increase of each of these factors.

The interest in finding reliable markers of cardiac dysfunction is intensified by studies that strongly suggest an association between HCV chronic infection and atherosclerotic disease in the carotid or coronary artery[39-41].

In conclusion, our study shows elevated levels of NTproBNP in patients with MC+HCV, in association with TNF-α. This may indicate the presence of cardiac dysfunction and explain, at least in part, some of the clinical symptoms of patients with MC+HCV. Further larger, possibly multicenter prospective studies quantifying symptoms and correlating these with echocardiographic parameters are needed to confirm this association.

COMMENTS

Background
The most common clinical features of hepatitis C associated with mixed

cryoglobulinemia (MC+HCV) are correlated with vasculitis in various organs and heart failure (HF) has been described as heralding the clinical onset of essential mixed cryoglobulinemia (MC). To our knowledge, until now no study has evaluated circulating NTproBNP together with TNF-α levels as possible markers of HF, in MC+HCV patients affected by cryoglobulinemic vasculitis.

Innovations and breakthroughs

This study demonstrates elevated serum levels of NTproBNP in patients with MC+HCV, in association with high TNF-α levels. This may indicate the presence of cardiac dysfunction and explain, at least in part, some of the clinical symptoms of patients with MC+HCV who have no evident signs of HF. Further larger, possibly multicenter prospective studies quantifying symptoms and correlating these with echocardiographic parameters are needed to confirm this association.

Applications

NTproBNP evaluation may serve as a screening marker for cardiac insufficiency in the differential diagnosis of fatigue and dyspnea, may aid the decision for further diagnostic testing of cardiac function and may have therapeutic and diagnostic consequences for patients with MC+HCV. In patients with known congestive HF, elevated plasma BNP concentrations could be reduced by treatment with ACE inhibitors, angiotensin II receptor antagonists and treatment with diuretics and vasodilators. As a consequence, plasma NTproBNP concentration may guide the intensity of pharmacotherapy as some interventional studies have suggested.

Terminology

Plasma levels of BNP and NTproBNP are reliable diagnostic and prognostic markers for cardiac disease which correlate with symptoms of HF and the severity of systolic and diastolic dysfunction. TNF-α is a cytokine involved in systemic inflammation and is a member of a group of cytokines that stimulate the acute phase reaction. Circulating TNF-α has been recently shown to be high in patients with MC.

Peer review

The study by authors has evaluated the circulating levels of NTproBNP and TNF-α in patients with HCV-related cryoglobulinemia. They found higher levels of both proteins in these patients as compared with normal controls and conclude that they may have a potential role in cardiac dysfunction of cryoglobulinemic patients. Though crude, these data are interesting.

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BRIEF ARTICLE

Hepatitis C virus genotype 3a infection and hepatocellular carcinoma: Pakistan experience

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Abstract

AIM: To assess the association between chronic hepatitis C virus (HCV) infection and hepatocellular carcinoma (HCC) in Pakistan, and the genotype distribution among these HCC patients.

METHODS: One hundred and sixty-one subjects with HCC were included in this study. Liver biopsy was performed on 145 of the patients; sixteen were excluded because they failed to fulfill the inclusion criteria. Qualitative polymerase chain reaction (PCR) was performed for hepatitis B virus and HCV. Samples positive for HCV RNA were genotyped using genotype-specific PCR and confirmed by HCV 5' noncoding region sequencing analysis.

RESULTS: Chronic HCV infection was identified a major risk factor (63.44% of tested HCC patients) for the development of HCC. The time from HCV infection to appearance of cancer was 10-50 years. In the HCC patient population, broader distributions of genotypes were present with genotype 3a as the predominant genotype. Using the type-specific genotyping method, we found HCV genotype 3a in 40.96%, 3b in 15.66%, 1a in 9.63%, and 1b in 2.40% of HCC tissue samples. About 28% of cases were found with mixed genotypes. Two cases were unable to be genotyped because of low viral load. Sixty-six percent of treated patients with cirrhosis had an end of treatment response, but unfortunately they relapsed quickly when the treatment was discontinued, and HCC developed during a median 3.8 years.

CONCLUSION: There was a strong association between chronic HCV infection and HCC in Pakistan, and between HCV genotype 3a and HCC.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the commonest cancers in the world, with an estimated incidence of 5 000 000 to 10 000 000 new cases every year[1]. Hepatitis C virus (HCV) infection, hepatitis B virus (HBV) infection, alcoholic liver disease, and non-alcoholic fatty liver disease are the major causes of cirrhosis in patients with HCC[2,3].

Chronic HCV infection frequently leads to liver cirrhosis and is associated with an elevated risk for progression into HCC[4,5]. Epidemiological surveys have identified HCV in 10%-80% of HCC patients reported in different populations[5,6]. HCV has also been reported to be the major cause of HCC in Japan[7], Italy[6] and Spain[8], but is less important in South Africa[9] and Taiwan[10]. Association of HCV infection with HCC has also been well documented in the United States[11].

Etiology, clinical features, and survival of HCC vary considerably in different populations[12]. In Pakistan, HCC is a leading cause of death and accounts for 60%-90% of all primary liver malignancies[13]. Some studies have shown hepatitis B surface antigen (HBsAg) positivity in 60% of patients with HCC[14,15]. However, some other studies have reported positivity for HCV infection in up to 80% of patients with HCC[16]. It is believed that HCV infection is a major etiological factor for HCC[17], however, not all patients with HCV infection develop HCC. A number of host factors such as male sex, older age at infection, long disease duration, excessive alcohol consumption, and high liver iron overload have been reported to influence disease progression[18,19]. Several additional studies have noted variables such as chronic co-infection with HBV and human immunodeficiency virus (HIV)[20], obesity and steatosis[21], type 2 diabetes[22], and asymptomatic cryoglobulinemia[17,18,23]. In addition to these host factors, several viral factors such as genotype and peripheral viral load have also been reported to influence disease progression[24]. Some studies have identified that cirrhotic patients infected with HCV type 1b carry a significantly higher risk of developing HCC compared to those infected with other HCV types[25,26]. However, the results of other studies[27,28] are in disagreement with these studies, and demonstrate no association of a particular HCV genotype with the development of HCC.

No such studies on the association or otherwise of HCV genotype with the development of HCC are available from Pakistan. Therefore, this study was performed to: (1) study various risk factors for the development of HCC; (2) investigate the prevalence of HCV in patients with HCC; and (3) evaluate if there is any association between particular HCV genotypes and HCC.

MATERIALS AND METHODS

Patients
For initial examination, 161 subjects with chronic hepatitis managed as end-stage liver disease patients at various hospitals of Punjab and North West Frontier Province of Pakistan were enrolled. All these patients underwent ultrasound-guided liver biopsy. Of these 161 subjects, 145 satisfied the inclusion criteria such as: HCC was confirmed by liver imaging (ultrasonography and computed tomography); histologically confirmed HCC; chronic liver disease of any etiology, with ascites and encephalopathy. Sixteen patients were excluded from the study because they failed to fulfill the study

criteria (14 subjects) or were unwilling to participate in the study (two subjects). The study was started in March 2001 and ended in April 2009. The clinical records of these patients were examined to identify the etiology of HCC. Documentation of the histology of liver tissue surrounding the cancer, together with possible sources of transmission and duration of blood-borne infectious hepatitis, was made. The time of transmission of HCV infection was calculated from the time of first major/minor surgery or first blood transfusion; only these patients were used to calculate the range/median duration of infection. Serum samples were collected and stored at -20℃, at the time of diagnosis of HCC. All the liver biopsies were transported in liquid nitrogen and stored at -70℃. Liver function tests such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase and bilirubin levels of all the samples were estimated using an auto-analyzer (Hitachi, Tokyo, Japan). Serum α-fetoprotein (AFP) concentration was determined by solid-phase, two-site chemiluminescent immunometric commercial diagnostic assay, using an Immulite-100 automated immunoassay system (Diagnostic Products, Los Angeles, CA, USA). From all the subjects, written informed consent was obtained. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee and Institutional Review Board of the Centre.

ELISAs for HBsAg, anti-HBsAg, anti-hepatitis B core antigen (HBc), anti-HCV and anti-HIV 1 and 2
All the patients were screened for HBsAg, anti-HBsAg, anti-HBc, anti-HCV and anti-HIV 1 and 2 using third-generation ELISA kits (DRG Instruments, Germany) as described by the manufacturer.

HBV qualitative polymerase chain reaction (PCR)
Hepatitis B viral DNA was extracted from 200 μL of stored serum and 2-5 mg of liver tissue of each of the patients using Gentra DNA Isolation Kit (PUREGENE, USA). Qualitative detection of serum HBV DNA was done by PCR amplification of the surface antigen gene using specific forward (5'AGAACATCGCATCAGGAC TC-3'; nt: 159-178) and reverse (5'CATAGGTATCTTG CGAAAGC-3'; 642-623) primers. One microliter of the first-round products was re-amplified using nested PCR with internal forward (5'AGGACCCCTGCTCGTGTT AC-3'; 181-200) and reverse (5'AGATGATGGGATG-GGAATAC-3'; nt: 619-600) primers. The amplified products were detected on agarose gel electrophoresis after staining with ethidium bromide and visualization on a UV transilluminator.

Qualitative and quantitative detection of HCV RNA
HCV qualitative RT-PCR was carried out as described previously[29]. HCV RNA was quantified using a SmartCycler II Real-time PCR (Cepheid, USA), using HCV RNA quantitative kits (Sacace Biotechnologies, Italy) according to the kit protocol.

Table 1 Characteristics, biochemistry and etiology of HCC patients (n = 145) n (%)

Risk factor	Value
Age ± SD (yr)	58 ± 11
Male	107 (73.79)
ALT	61 (42.1)
AST	61 (42.1)
Alkaline phosphatase	145 (100)
Bilirubin	145 (100)
AFP elevation	125 (86.2)
Cirrhosis present	98 (67.58)
HBsAg-positive (alone)	18 (12.41)
Anti-HBc-positive (alone)	2 (1.37)
Anti-HBs-positive	10 (6.89)
HBV-DNA PCR-positive (alone)	26 (17.93)
Anti-HCV-positive (alone)	92 (63.44)
HCV-RNA PCR-positive	83 (57.24)
HBV- and HCV-positive	19 (13.10)
No known etiology	6 (4.13)

HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; AST: Aminotransferase; AFP: α-fetoprotein; HCV: Hepatitis C virus; HBV: Hepatitis B virus.

HCV genotyping

Core HCV genotyping were performed as described previously[30] for all HCV-RNA-positive sera and tissues. Genotypes were confirmed by HCV 5' noncoding region (5' NCR) sequencing using ABI PRISM 3100 Genetic Analyzer (Applied Biosystem Inc., Foster City, CA, USA) in both directions. Sequences of isolates were aligned with representative sequences for each major genotype and subtype selected from the GenBank database with the help of the Multalign program. The phylogenetic analysis of HCV isolates was performed with MEGA 3.0 software[31], and phylogenetic trees were constructed by the neighbor-joining method, using the bootstrap-resampling test from the MEGA program (1000 bootstrap replications).

HCV treatment

The medical records of the HCV-related HCC patients showed that a total 21 patients had been treated previously for HCV infection. These treated patients had received 3 MU recombinant interferon-α three times weekly, subcutaneously, and ribavirin (10 mg/kg per day) for a total of 24 wk.

Statistical analysis

The data were analyzed and summary statistical analysis was carried out using SPSS for Windows version 10.0. The results for all variables were given in the form of averages (SD). The χ^2/Fisher's exact test and independent sample t test were used for categorical/continuous variables.

RESULTS

Characteristics and biochemistry of HCC patients

Patient demographics and biochemical and clinical data are shown in Table 1. HCC patients were older (58 ± 11 years), were predominantly male (73.8%), and had no history of chronic alcoholism. Data collection was incomplete for one aspect, namely, exact duration of illness. The time of HCV transmission was calculated from the time of first major/minor surgery or first blood transfusion, which might not have been the exact date of virus acquisition. All the patients with HCC had raised levels of serum bilirubin (> 1.0 mg/dL) and alkaline phosphatase (> 300 U/L). ALT and AST levels were abnormal (ALT > 40 IU/mL, AST > 35 IU/mL) only in 42% of patients. AFP level was elevated (> 15 IU/mL) in 86.2% of patients with HCC. Cirrhosis was present in 67.6% of HCC patients. All the patients were found to be negative for anti-HIV.

Etiology of HCC

Out of the 145 patients with HCC, HCV antibodies were present in 92 (63.4%) serum samples. Two patients were found to be tissue-positive by PCR but no anti-HCV antibodies were present. Eighty-one patients were found to be tissue-positive by PCR out of 92 anti-HCV-positive patients (88.04%). Of these patients with HCC caused by HCV, 68 were male and 13 were female. The mean age was 55 ± 10 years for HCV-related HCC. HCV RNA was detected in the serum of all these 81 tissue-positive patients. All the patients with HCV-related HCC had a history of chronic HCV infection. The peripheral HCV RNA loads were as low as 10 000 copies to as high as 3.7×10^8 copies/mL. No significance difference was found between the viral loads in serum and tissues of the same patients. Twenty-eight cases were caused by HBV, of whom 18 (19.31%) also had markers for current HBV infection (HBsAg-positive), and two patients (1.37%) had markers for past infection (HBsAg-negative; anti-HBsAg-positive; anti-HBc positive). The age was 65 ± 12 years for HBV-associated HCC patients. Nineteen (13.1%) of the HCC cases had markers for HCV and HBV. Out of these 19 cases with dual infection, two were HBV-DNA-positive and HBsAg-negative. In 6 (4.13%) cases, the etiology of liver cancer could not be determined from the medical records or serology. All these six HCC patients with unknown etiology were younger than the HCV-related HCC patients (45-50 years).

Distribution of HCV genotypes in HCC patients

Table 2 shows the results of HCV genotyping. A total of 83 tissue samples (81 positive for HCV RNA and anti-HCV, and two positive for HCV RNA and negative for anti-HCV) were used for HCV genotyping. Using the type-specific genotyping method, we found HCV genotype 3a in 40.96%, 3b in 15.66%, 1a in 9.63%, and 1b in 2.40% of HCC tissue samples. Twenty-four tissues (28.91%) were found with mixed genotypes. Of the 24 mixed genotypes, 10 were infected with genotypes 3a and 3b, eight with 1a and 3a, and six with 1a and 3b. Two tissue samples were found to be untypable as no genotype was detected. Both of the untypable patients had no cirrhosis and had a low viral load (< 10^4 IU/mL). The genotyping results for all single genotypes were confirmed by sequencing. The sequence data of the sequences were submitted to GenBank. The Accession

Table 2 Results of HCV genotype determination in HCC patients (*n* = 83)[1]		
HCV genotype	No. of HCC cases	Percentage
1a	8	9.63
1b	2	2.40
3a	34	40.96
3b	13	15.66
3a + 3b	10	12.48
1a + 3a	8	9.63
1a + 3b	6	7.22
NT	2	2.40

[1]Eighty-one were positive for tissue/serum RNA by PCR and positive for serum anti-HCV, and two were positive for tissue/serum RNA by PCR but negative for serum anti-HCV. Eleven patients with HCC caused by HCV were not genotyped, as these were anti-HCV-positive by ELISA, but were HCV-RNA-negative, thus they could not be genotyped utilizing the molecular genotyping method. NT: Not typed.

Numbers provided for our nucleotide sequences by GenBank are EF173955-EF174011.

Anti-viral treatment history

The medical records of the patients showed that 21 of 94 patients with HCV-associated HCC had received previous standard interferon therapy for a total of 24 wk. Of these treated patients, 13 were male and eight were female. Cirrhosis was present in all of these 21 treated patients. Twenty of these patients had genotype 3a (12 male and eight female) and one 3b (male). Fourteen (66.7%) of these patients (eight male and six female; all with genotype 3a) had an end of treatment response but relapsed after discontinuation of treatment, with no sustained viral response.

DISCUSSION

Several viral and host factors have been studied extensively since the identification of HCV infection as a major risk factor for the development of HCC[4,9,17]. Among the viral factors, the presence of some HCV genotypes adds to the list of risk factors for HCC. In the present study, the etiology of 145 patients with HCC was assessed with special emphasis on HCV genotype. More than 73% of the enrolled patients with HCC were male. It has been reported already that men have a higher liver cancer rate than women, with a ratio between 2:1 and 4:1[32]. The reasons for the higher proportion of male patients with HCC might be the possibility that more men are infected with HBV and HCV, consume alcohol, smoke, have increased iron stores, higher body mass index, and a possible involvement of male sex hormones in the onset of HCC[33]. Most patients (96.5%) in the current study were elderly and their ages ranged from 58 to 68 years. They were possibly infected on receiving injections or major/minor surgery at a median time of 20 years previously. Our observation of late onset of HCC is in agreement with earlier reports from other parts of the world where the transition from acute infection to cirrhosis and detection of HCC took 20-30 years[34]. It is important to mention here that, in recent years, with the increasing incidence of HCC, the age of patients with HCC has been decreasing among persons aged 45-60 years[2]. AFP elevation was observed in the present study in about 86% of patients with HCC. Cirrhosis was present in > 67% of HCC cases studied. Previous studies have shown that cirrhosis underlies HCC in > 80% of affected individuals[35,36]. Therefore, any agent that leads to cirrhosis should be seen as a risk factor for the development of HCC. It has also been reported that the risk among those with cirrhosis increases in parallel with the impairment of liver function, and in subjects with increased AFP concentration[35].

Thirty-two percent of our patients with HCC were without liver cirrhosis, which showed that infection with HCV and HBV could be correlated with the emergence of HCC, even in the absence of liver cirrhosis. It has been established that the mechanism for the development of HCC in HBV-related cases is associated with the integration of HBV DNA into hepatocytes[37]. However, such a mechanism has not been established for HCV, because to date, integration of HCV RNA into cellular DNA has not been reported, even when there has been evidence for the direct involvement of HCV in oncogenicity. According to recent reports, the possible risks are involvement of various viral proteins such as core, NS3 and NS4 in the induction of liver cell proliferation, by interfering directly with the major cellular transduction networks[38,39].

Several major findings have emerged from the current study. The first finding is the identification of chronic HCV infection as a major risk factor for the development of HCC in Pakistan, because anti-HCV was observed in > 63% of patients with HCC. The overall anti-HCV prevalence rate is 14%-15% and HBV carrier rate is 2%-3% in the general population of Pakistan[29,40]. Overall, our data are consistent with the results of studies already reported from high-risk areas for HCC such as Japan, Italy and Spain, where majority of reported HCC cases are HCV-related[6-8]. It is clear from the present study and from others that the greatest proportional increases have occurred recently in HCV-related HCC worldwide, and that HBV-related HCC has been stable and at its lowest rate[16]. The rate of HCV-related HCC is likely to continue to increase, and it is estimated that this increase will peak around the year 2010, not only in North America and Europe[41], but also in the rest of the world including Pakistan. Presently, the annual incidence of HCV-related HCC ranges between 2% and 8%[38].

The second major finding of the current study is the evidence that links HBV with HCC, with about 19% of cases caused by HBV. This link was expected and is unquestionable, as has been reported previously[40]. Co-infection with HBV was also found as an additional etiological factor for HCC in the current study, which supports other published studies[42,43]. In two HCV-RNA-positive patients, HBV DNA was detected even in the absence of serological markers for HBV in serum. Previously, it has also been reported that the rate of

occult infection in such patients can be as high as 63%[44]. It has been reported that the implementation of HBV vaccination has resulted in a significant decrease in the incidence of HBV-related HCC[45]. In 4.13% cases in the present study, the etiology of liver cancer could not be determined from medical records or from serology and molecular biology. All these patients were non-drinking males, but were chain smokers.

Another more interesting and somewhat surprising finding in the present study was the observation that HCV genotype 3a was the predominant genotype in 41% of HCC cases. This suggests that genotype 3a is a major risk factor associated with the development of HCC compared with other HCV genotypes. However, the question whether HCV genotype plays a role in the development of liver cirrhosis and HCC is still debatable. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for HCC[25,26]. However, some other studies have revealed no preferential role of individual HCV genotypes in HCC[27,28]. Although in our study HCV genotype 3a was predominant in HCV-related HCC, this genotype has been reported previously to induce a high sustained response, and has been less responsible for severe disease as compared to genotypes 1a, 1b and 4[46]. It seems that the high percentage of HCC in patients with HCV genotype 3a might result from the fact that genotype 3a can equally cause increased oncogenicity, as can other genotypes such as 1a and 1b. Finally, patients with cirrhosis had no sustained response rates and treatment did not reduce the incidence of HCC. However, more studies with a large number of cirrhotic patients, along with adequate controls, are required to confirm this observation of the current study.

In conclusion, HCC was found mostly in patients with chronic HCV infection and with liver cirrhosis in Pakistan. There also seemed to be a strong association between chronic HCV infection with genotype 3a and HCC, as the high prevalence of genotype 3a in the HCC population reflected increased oncogenicity. Treatment did not stop the development of HCC. However, studies with larger numbers of patients could confirm that HCV genotypes vary in their propensity to produce clinically significant liver disease.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is currently one of the fastest growing causes of cancer-related deaths worldwide. The geographical prevalence varies considerably from country to country and Pakistan is a high-risk area for the disease. A strong association has been established between chronic hepatitis C virus (HCV) infection and hepatocarcinogenesis. A specific HCV genotype could play a role in the development of HCC.

Research frontiers

In Pakistan, HCC is a leading cause of death and accounts for 60%-90% of all primary liver malignancies. Positivity for HCV infection is up to 80% in HCC. Are HCV genotypes playing a role in the development of liver cirrhosis and HCC? This question remains debatable. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for the development of HCC. However, some other studies have revealed no preferential role of individual HCV genotypes in HCC. Therefore, the current study was carried out to assess if there was any association between chronic hepatitis infection with various HCV genotypes and HCC.

Innovations and breakthroughs

In the current study, the authors identified various risk factors for the development of HCC and particularly investigated the prevalence of HCV in patients with HCC. They further assessed the association between chronic hepatitis infection with various HCV genotypes and HCC, and found a strong association between chronic HCV infection with genotype 3a and HCC. Previously, the effect of HCV genotype 1b has been scrutinized as a risk factor for HCC.

Peer review

This paper describes the relationship between HCV genotype 3a infection and HCC development. Although there have been many studies on the difference among HCV genotypes in hepatocarcinogenesis, there has not been a sufficient number of reports on genotype 3a. Therefore, this paper deals with an interesting issue.

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BRIEF ARTICLE

Gastric leptomeningeal carcinomatosis: Multi-center retrospective analysis of 54 cases

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Abstract

AIM: To identify the clinical features and outcomes of infrequently reported leptomeningeal carcinomatosis (LMC) of gastric cancer.

METHODS: We analyzed 54 cases of cytologically confirmed gastric LMC at four institutions from 1994 to 2007.

RESULTS: The male-to-female ratio was 32:22, and the patients ranged in age from 28 to 78 years (median, 48.5 years). The majority of patients had advanced disease at initial diagnosis of gastric cancer. The clinical or pathologic tumor, node and metastasis stage of the primary gastric cancer was IV in 38 patients (70%). The median interval from diagnosis of the primary malignancy to the diagnosis of LMC was 6.3 mo, ranging between 0 and 73.1 mo. Of the initial endoscopic findings for the 45 available patients, 23 (51%) of the patients were Bormann type III and 15 (33%) patients were Bormann type IV. Pathologically, 94% of cases proved to be poorly differentiated adenocarcinomas. Signet ring cell component was also observed in 40% of patients. Headache (85%) and nausea/vomiting (58%) were the most common presenting symptoms of LMC. A gadolinium-enhanced magnetic resonance imaging was conducted in 51 patients. Leptomeningeal enhancement was noted in 45 cases (82%). Intrathecal (IT) chemotherapy was administered to 36 patients-primarily methotrexate alone (61%), but also in combination with hydrocortisone/± Ara-C (39%). The median number of IT treatments was 7 (range, 1-18). Concomitant radiotherapy was administered to 18 patients, and concomitant chemotherapy to seven patients. Seventeen patients (46%) achieved cytological negative conversion. Median overall survival duration from the diagnosis of LMC was 6.7 wk (95% CI: 4.3-9.1 wk). In the univariate analysis of survival duration, hemoglobin, IT chemotherapy, and cytological negative conversion showed superior survival duration ($P = 0.038$, $P = 0.010$, and $P = 0.002$, respectively). However, in our multivariate analysis, only cytological negative conversion was predictive of relatively longer survival duration (3.6, 6.7 and 14.6 wk, $P = 0.030$, RR: 0.415, 95% CI: 0.188-0.918).

CONCLUSION: Although these patients had a fatal clinical course, cytologic negative conversion by IT chemotherapy may improve survival.

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INTRODUCTION

Leptomeningeal carcinomatosis (LMC) is defined as malignant infiltration of the pia mater and the arachnoid membrane. LMC is one of the most serious complications that can occur in cancer patients[1]. According to the results of a large autopsy study, the incidence of LMC was 5%-8% in cancer patients[2]. As a significant proportion of these patients have asymptomatic microscopic disease, the clinical diagnosis of LMC has been established in 2%-4% of patients during the course of their malignancy[1]. LMC is frequently detected in patients with leukemia, breast cancer, lymphoma, and lung cancer[3]. Among solid tumors, LMC is observed more frequently in cases of disseminated and progressive disease. Although a subset of patients, particularly those with lymphoma or breast cancer, may survive for more than 12 mo with a reasonable quality of life, leptomeningeal metastasis from solid tumors is associated with a poor overall prognosis. The treatment of LMC is palliative and unsatisfactory. No evidence demonstrating the superiority of intrathecal (IT) treatment compared to best palliative care is currently available from clinical trials.

Furthermore, the development of LMC from a gastric cancer is a very rare occurrence. Some articles have reported that the incidence of LMC in patients with gastric cancer was responsible for 0.16% of all cases of gastric cancer[4]. Due to its rarity, the clinical features and prognostic factors of LMC as a metastasis from gastric carcinoma have yet to be clearly characterized. The benefits of IT chemotherapy are also currently a matter of some contention.

Gastric cancer is the most common malignancy in Korea[5]. Because of the high prevalence of gastric cancer in Korea, we took the opportunity to study gastric cancer patients with LMC. The principal objective of this study was to review our experience with LMC associated with gastric cancer, and to evaluate its clinical features and the efficacy of a variety of treatment modalities in terms of neurological status and overall survival (OS).

MATERIALS AND METHODS

Patients

From 1995 to 2007, 22 154 patients were diagnosed with gastric cancer at four independent institutions. Among them, 54 patients who were diagnosed with leptomeningeal seeding metastasis of gastric cancer were analyzed. Although it is not representative of the cohort of patients, the prevalence of LMC was 0.24%.

Eligibility for this study included: (1) patients with histologically confirmed adenocarcinoma of the stomach; (2) cytologically confirmed malignancy on cerebrospinal fluid (CSF) analysis, patients with suspected LMC by magnetic resonance imaging (MRI) and negative cytology were excluded; (3) no history of any other malignancies.

We retrospectively analyzed the patients' medical records including the patients' characteristics, clinical symptoms, laboratory and radiologic findings, treatment modality and outcomes, final follow-up, and survival duration.

Statistical analysis

Comparisons of categorical variables among groups were conducted using the chi-square test and Fisher's exact test. OS was calculated from the cytological confirmation of LMC and plotted *via* the Kaplan-Meier method. Comparison of survival according to prognostic factors was evaluated *via* a log-rank test, and forward stepwise Cox proportional hazard models were employed to evaluate the joint effect of predictive variables. $P < 0.05$ was considered significant. Analysis of the data was conducted using SPSS for Windows V. 15.0 (SPSS Inc., Chicago, IL, USA) statistical software.

RESULTS

Patients' characteristics

We analyzed 54 cases of cytologically confirmed gastric LMC at four institutions from 1994 to 2007. The clinical characteristics of these patients are summarized in Table 1. The male-to-female ratio was 32:22, and patients ranged in age from 28 to 78 years (median, 48.5 years). The majority of patients had advanced disease at initial diagnosis of gastric cancer. The clinical or pathologic tumor, node and metastasis stage of the primary gastric cancer was IV in 47 patients (87%). Stage I -III patients received curative operation. Among the stage IV patients, 13 patients had T4N1-2 or N3 (No. of nodes > 15) by pathologic features through curative operation. M1 node positive patients were counted as palliative surgery. Of the initial endoscopic findings in the available 45 patients, Bormann type III and IV were reported for 23 (51%) and 15 (33%) patients, respectively. Pathologically, 94% of cases proved to be poorly differentiated adenocarcinomas. Signet ring cell component was also observed in 40% of patients.

LMC patterns

The median interval from diagnosis of the primary gastric cancer to the diagnosis of LMC was 6.3 mo, ranging from 0 to 73.1 mo. Five patients presented with initial LMC. The majority of patients (59.3%) initially presented with metastatic gastric cancer without LMC, and then progressed to LMC. One-third of the patients presented with curable disease at the initial diagnosis of gastric cancer (Table 2).

Clinical symptoms

The most frequently observed presenting symptoms of LMC were nonspecific symptoms such as headache (85%) and nausea/vomiting (58%). In addition, various neurological clinical signs and symptoms were noted

Table 1 Patients' characteristics (_n_ = 54)

No. of patients	_n_ (%)
Gender	
Male/female	32 (59.3)/22 (40.7)
Age (yr)	
Median (range)	48 (28-78)
⩾ 60/< 60	15 (27.8)/39 (72.2)
Initial stage	
I - II	2 (3.7)
III	2 (3.7)
IV	47 (87.0)
Not available	3 (5.6)
Operation	
Curative	17 (31.5)
Palliative	15 (27.8)
Inoperable	18 (33.3)
Not available	4 (7.5)
Initial endoscopic finding (_n_ = 47)	
Site	
Cardia	1 (1.9)
Fundus	1 (1.9)
Body	20 (37.0)
Antrum, pylorus	16 (29.7)
Diffuse whole stomach	9 (19.1)
Borrmann type (_n_ = 45)	
Early gastric cancer	2 (4.4)
I (polypoid)	1 (2.2)
II (ulcerative)	4 (8.9)
III (ucero-infiltrative)	23 (51.2)
IV (diffuse infiltrative)	15 (33.3)
Differentiation (_n_ = 47)	
Moderate	3 (6.4)
Poor	25 (53.2)
Poor with signet ring cell	19 (40.4)

Table 2 Patterns of leptomeningeal carcinomatosis (_n_ = 54)

	_n_ (%)
Time to LMC (mo)	
Median (range)	6.3 (0-73.1)
LMC presentation	
Curative/recurred/progression	7 (13.0)
Curative/recurred LMC	10 (18.5)
Metastatic/progression	32 (59.3)
Initially LMC	5 (9.3)

LMC: Leptomeningeal carcinomatosis.

including altered mental status, seizure, motor weakness, sensory change, diplopia, hearing loss, and facial palsy (Table 3).

CSF analysis and image findings

Lumbar puncture and analysis of CSF is a crucial laboratory test in the diagnosis of LMC. All the patients presented with malignant cells on cytological analysis _via_ the inclusion criteria. An elevated opening pressure on lumbar puncture was noted in 58% of the subjects. The mean CSF pressure in the patients was 222.1 mm CSF. 78.8% and 53.8% of patients had elevated white blood cells and protein in CSF, respectively (Table 4).

Brain computed tomography was assessed in eight patients and leptomeningeal enhancement was observed only in one patient. A gadolinium-enhanced MRI was

Table 3 Symptoms of leptomeningeal carcinomatosis (_n_ = 54)

	_n_ (%)
Cerebral symptom	
Headache	46 (85.1)
Nausea & vomiting	32 (59.2)
Dizziness	13 (24.0)
Mental change	12 (22.2)
Seizure	10 (18.5)
Gait difficulty	2 (3.7)
Dysarthria	2 (3.7)
Psychosis	1 (1.9)
Cranial symptom	
Diplopia	3 (5.6)
Hearing loss	2 (3.7)
Facial palsy	1 (1.9)
Ptosis	1 (1.9)
Spinal symptom	
Weakness	5 (11.1)
Paresthesia	2 (3.7)
Back pain	1 (1.9)

Table 4 CSF finding of leptomeningeal carcinomatosis

CSF	No. of > WNL[1]	mean ± SD
Pressure (mm CSF)	29/50 (58.0%)	222.1 ± 158.4
WBC (_n_/mm³)	41/52 (78.8%)	36.7 ± 59.0
Protein (mg/dL)	28/52 (53.8%)	129.5 ± 250.8

[1]CSF pressure > 160 mm; CSF protein > 50 mg/dL; Cell count > 5/mm³.
CSF: Cerebrospinal fluid; WNL: Within normal limit; WBC: White blood cell.

conducted in 51 patients. Leptomeningeal enhancement was noted in 45 cases (82%).

Treatment modalities and outcomes

IT chemotherapy was administered to 36 patients, principally with methotrexate (MTx) alone (61%) or in combination with hydrocortisone/± Ara-C (41%). The median number of IT treatments was 7 (range, 1-18). Seventeen patients (46%) achieved cytological negative conversion (Table 5).

Thirteen patients were treated with whole brain irradiation coupled with IT chemotherapy. Six patients received radiation treatment alone.

Additional systemic chemotherapy was given to 10 patients. Three patients were treated with the orally available 5-fluorouracil (5-FU) drugs - capecitabine, S-1, and tegafur-uracil. Irinotecan/leucovorin/5-FU, 5-FU/cisplatin, and paclitaxel/cisplatin were administered to four, two and one patients, respectively. Seven patients were treated with chemotherapy plus IT chemotherapy. Three patients received chemotherapy alone. The median number of cycles administered was 2 (range, 1-6). Among the treated patients, only one exhibited a detectable response to treatment.

Survival and prognostic factors

Median OS duration from diagnosis of LMC was 6.7 wk (95% CI: 4.3-9.1 wk) (Figure 1). In the univariate analysis of survival duration, hemoglobin, IT chemotherapy, and cytologic negative conversion showed superior

Table 5 Treatment with intrathecal chemotherapy (*n* = 36)

	n (%)
Regimen	
MTx	22 (61.1)
MTx + steroid	4 (11.1)
MTx + Ara-C + steroid	10 (27.8)
Concurrent/sequential	
+ alone	16 (44.4)
+ radiotherapy	13 (36.1)
+ chemotherapy	2 (5.6)
+ chemoradiotherapy	5 (13.9)
No. of cycles	
Median (range)	7 (1-18)
Cytological response	
(-) conversion	17 (47.2)

MTx: Methotrexate; Ara-C: Cytarabine.

Figure 1 Median overall survival (OS) duration from diagnosis of leptomeningeal carcinomatosis. Median OS was 6.7 wk (95% CI: 4.3-9.1 wk).

Figure 2 Cytologically negative conversion proved predictive of relatively longer survival duration (*P* = 0.030, RR: 0.415, 95% CI: 0.188-0.918).

survival duration ($P = 0.038$, $P = 0.010$, and $P = 0.002$, respectively). However, in the multivariate analysis, only cytologic negative conversion was predictive of relatively longer survival duration (3.6, 6.7 and 14.6 wk, $P = 0.030$, RR: 0.415, 95% CI: 0.188-0.918) (Table 6, Figure 2).

DISCUSSION

Adenocarcinoma is the predominant histological type in LMC of solid tumors[6]. Among patients diagnosed with

Table 6 Survival duration and prognostic factors

Factors	Median OS (mo)	Univariate	Multivariate
Gender			
Male	7.1	0.491	-
Female	6.7		
Age (yr)			
> 60	12.4	0.214	-
≤ 60	6.4		
PS (LMC)			
0, 1	11.7	0.975	-
≥ 2	6.4		
Hb (LMC)			
> 11	12.4	0.038	NS
≤ 11	5.7		
MRI enhance			
Negative	5.7	0.316	-
Positive	7.1		
CSF pressure			
> 120	6.4	0.163	NS
≤ 120	8.1		
CSF protein			
> 40	6.0	0.539	-
≤ 40	11.7		
IT chemotherapy			
No	3.6	0.010	NS
Yes	11.7		
Radiotherapy			
No	4.6	0.516	-
Yes	6.9		
Cytologic response			
No	5.1	0.002	0.030
Yes	14.6		(HR: 0.415, 95% CI: 0.188-0.918)

OS: Overall survival; PS (LMC): Performance status scale by Eastern Cooperative Oncology Group at leptomeningeal carcinomatosis; Hb (LMC): Hemoglobin at leptomeningeal carcinomatosis; MRI: Magnetic resonance imaging; CSF: Cerebrospinal fluid; IT: Intrathecal; HR: Hazard ration; CI: Confidence interval.

LMC, the most frequently encountered solid tumors are breast (12%-34%), lung (14%-29%), and melanoma (17%-25%)[3]. Unlike Western reports, gastric cancer is the principal etiology of LMC in solid tumors in Korea[7].

CNS metastasis is a very rare complication of gastric cancer, and occurs in 0.16%-0.69% of gastric cancer patients in general, including Korean reports[4,8,9]. Although all the included patients demonstrated CSF cytologically confirmed malignancy, the prevalence of LMC in this study was 0.24% in all gastric cancer patients.

Consistent with other studies, the majority of patients had Bormann type Ⅲ or Ⅳ advanced gastric cancer of poorly differentiated or signet-ring cell histopathology, which increased the tendency for distant metastasis and poor prognosis[4,10,11]. Similar to the results of a previous study, LMC patients presented with an advanced stage and Bormann type Ⅲ or Ⅳ advanced gastric cancer of poorly differentiated or signet-ring cell histopathology.

LMC is an ultimately fatal disease[12-15]. A minority of patients, usually those with breast cancer or lymphoid malignancies, may achieve disease-free survival of a year or more, however, the median OS for patients with LMC is only 4-6 wk if untreated and 2-4 mo with therapy[6,12,15]. In our study, the median survival duration was just

6.7 wk. Although LMC patients tend to have a poor performance status, approximately two-thirds of patients who receive IT chemotherapy and 47.2% patients who responded to therapy achieved longer survival duration. The independent prognostic factor for survival was cytologic negative conversion by IT chemotherapy. Although the small sample size and inherent selection bias of the retrospective design of this study makes any conclusions regarding the outcomes of treatment somewhat difficult, the findings of our study indicate that cytologic negative conversion by IT chemotherapy may improve survival by arresting neurologic progression in selected patients.

MTx remains the most frequently utilized drug for IT administration, despite its limited success and serious complications[16,17]. Combination IT chemotherapy with MTx, arabinoside and hydrocortisone has been reported to be more effective than MTx alone in solid tumor LMC[18]. However, approximately 10% of gastric cancer patients were enrolled in this study, and the efficacy of arabinoside against gastric cancer is questionable.

Craniospinal irradiation may be a one-treatment modality. However, the additional or sequential role of radiation has been controversial[19]. In our study, additional effects of radiotherapy were not observed. As response to radiation is associated with the sensitivity or resistance of primary tumors and malignant cells circulating in the CSF space, radiation is occasionally not feasible for palliative treatment.

Systemic chemotherapy was also administered to a limited number of patients who had better performance status[6,15,20]. In our study, patients who were treated with systemic chemotherapy showed the best median OS duration (21.6 wk, 95% CI: 3.2-40 wk). However, all the treatments were administered sequentially after IT chemotherapy and the patients responded to treatment.

COMMENTS

Background

Leptomeningeal carcinomatosis (LMC) occurs in approximately 5% of cancer patients. The most common cancers involving the leptomeninges are breast and lung cancer. However, gastric adenocarcinoma has been infrequently reported in conjunction with LMC. This retrospective analysis was performed to identify the clinical features and outcomes of infrequently reported LMC of gastric cancer.

Research frontiers

This is the first large scale study on gastric LMC. LMC is a rare component in the clinical manifestation of gastric cancer. LMC usually presents at a relatively young age, at an advanced stage, and is of a poorly differentiated pathologic type.

Innovations and breakthroughs

Although gastric LMC had a fatal clinical course, the findings of our study suggest that cytologic negative conversion by intrathecal (IT) chemotherapy may improve survival by arresting neurologic progression in selected patients.

Applications

These results could provide basic clinical data on gastric LMC for physicians and demonstrate the role of IT chemotherapy.

Terminology

Leptomeninges (literally thin meninges) is a term referring to the pia mater and arachnoid mater. LMC is a condition in which a tumor diffusely spreads to the leptomeninges. Intrathecal chemotherapy involves anticancer drugs injected into the fluid-filled space between the thin layers of tissue that cover the brain and spinal cord.

Peer review

The results are interesting and suggest that cytologic negative conversion by IT chemotherapy may improve survival by arresting neurologic progression in selected patients.

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BRIEF ARTICLE

Mitigation of indomethacin-induced gastric mucosal lesions by a potent specific type V phosphodiesterase inhibitor

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Abstract

AIM: To investigate the gastroprotective effect of vardenafil against indomethacin-induced gastric damage.

METHODS: Forty-eight female Wistar albino rats were randomly divided into 6 groups. Group 1 received saline only. Group 2 (indomethacin) received indomethacin. Rats in group 3 and 4 were pretreated with different doses of famotidine. Group 5 and 6 were pretreated with different doses of vardenafil. Rats in groups 3 to 6 received 25 mg/kg indomethacin 30 min after pretreatment. The animals were sacrificed 6 h later and their stomachs were opened. Gastric lesions were counted and measured. The stomach of each animal was divided in two parts for histopathological examinations and nitric oxide (NO) and malondialdehyde (MDA) assays, respectively.

RESULTS: There were no gastric mucosal lesion in the saline group but all rats in the indomethacin group had gastric mucosal ulcerations (ulcer count; 6.25 ± 3.49, and mean ulcer area; 21.00 ± 12.35). Ulcer counts were

diminished with famotidine 5 mg/kg (4.12 ± 2.47, $P > 0.05$), 20 mg/kg (2.37 ± 4.43, $P < 0.05$), vardenafil 2 mg/kg (4.37 ± 3.06), and vardenafil 10 mg/kg (1.25 ± 1.38, $P < 0.05$) compared to the indomethacin group. Gastric mucosal lesion areas were diminished with famotidine 5 mg/kg (8.62 ± 2.97, $P < 0.001$), famotidine 20 mg/kg (0.94 ± 2.06, $P < 0.001$), vardenafil 2 mg/kg (6.62 ± 5.87, $P < 0.001$), and vardenafil 10 mg/kg (0.75 ± 0.88, $P < 0.001$) compared to the indomethacin group. MDA levels were significantly higher in indomethacin group (28.48 ± 14.51), compared to the famotidine 5 mg/kg ($6,21 \pm 1.88$, $P < 0.05$), famotidine 20 mg/kg (5.88 ± 1.60. $P < 0.05$), vardenafil 2 mg/kg (15.87 ± 3.93, $P < 0.05$), and vardenafil 10 mg/kg (10.97 ± 4.50, $P < 0.05$). NO concentration in gastric tissues of the famotidine groups were significantly increased ($P < 0.05$), but the NO increases in the vardenafil groups were not statistically significant. Histopathology revealed diminished gastric damage for pretreatment groups compared to the indomethacin group ($P < 0.05$).

CONCLUSION: Vardenafil affords a significant dose-dependent protection against indomethacin induced gastric mucosal lesions in rats.

INTRODUCTION

Gastric mucosal damage is a common disorder of the gastrointestinal system. The pathogenesis of gastric ulcers is based on complex interactions between aggressive and protective factors. Nonsteroidal anti-inflammatory drugs

(NSAID) are known to be aggressive agents for gastric ulcer development. People of advancing age need many drugs, including NSAIDs, for the treatment of pain and inflammation due to rheumatological disturbances.

Vardenafil is a phosphodiesterase (PDE) V inhibitor that has been used for the treatment of erectile dysfunction[1] and, more recently, for pulmonary hypertension[2,3]. Recent laboratory studies demonstrated successful effects of PDE V inhibitors on cardioprotection after ischemia reperfusion injury[4,5], as well as in ischemic injury of other organs, such as the colon, liver, and brain[6,7]. Deibert et al[8] revealed that vardenafil had increased portal flow and lowered portal pressure in patients with cirrhotic livers.

Diminished mucosal circulation has been blamed as one of the etiological factors in gastric ulcer formation. Like prostaglandins, the L-Arginine/nitric oxide (NO) pathway is a major protective system in gastric mucosa[9] via relaxation of the arterial smooth muscles. Inhibition of nitric oxide synthase aggravates the injury in animal models of gastric ulcers[10].

In this study, we have studied the effects of vardenafil on the acute gastric injury caused by administration of indomethacin.

MATERIALS AND METHODS

The study was approved by the Zonguldak Karaelmas University (ZKU) Animal Experiments Local Ethic Committee. The study was carried out on 48 female Wistar albino rats weighing 200-250 g, obtained from the Experimental Animal Laboratory of Medical Faculty of ZKU. The rats were kept under standard conditions (temperature; 22-24℃, and 12:12 h light/dark). The experimental procedures were carried out in accordance with international guidelines for the use and care of laboratory animals. All animals were fed with pellet food produced especially for experimental animals. Water was available ad libitum. All experiments were performed at the same time of the day to avoid diurnal variations of putative regulators of gastric functions.

Famotidine and vardenafil were dissolved in distilled water. All drug solutions and suspensions were freshly prepared. Gastric ulcers were inflicted by oral administration of indomethacin 16-18 h after starvation.

Animals were randomly divided into six groups. In Group 1 (n:8) rats received only 8 mL/kg of saline by gavage. Rats in Group 2 (n:8) received 25 mg/kg indomethacin in a volume of 8 mL/kg of saline. The rats in group 3 and 4 were pretreated with famotidine, 5 mg/kg and 20 mg/kg, respectively. Rats in groups 5 and 6 were pretreated with 2 mg/kg and 10 mg/kg vardenafil, respectively. After 30 min, 25 mg/kg indomethacin in a volume of 8 mL/kg of saline were administered by gavage. Six hours after oral administration of indomethacin, all rat groups were anesthetized with an intramuscular injection of 100 mg/kg Ketamine (Ketalar®, Parke Davis-Eczacıbaşı, Istanbul, Turkey). A midline abdominal incision was then performed. All rat groups were sacrificed via cardiac puncture, and their stomachs rapidly removed, opened by an incision along the lesser curvature, and rinsed in ice-cold distilled water[11]. Gastric tissues were pinned out on a wax platform. Macroscopic damage to the gastric mucosa was assessed. Hemorrhagic and ulcerative lesions were counted and their lengths were measured on square millimeter paper. Gastric mucosal lesions were expressed as the sum of the lengths (mm) of all lesions for each stomach, which was used as the ulcer index (UI)[12,13]. Gastric lesions were judged by two independent researchers who were blinded to the protocol. The average score of the two independent observers were taken into account, and the sum of the total scores was divided by the number of animals to obtain the mean UI for each group.

The stomach of each animal was divided into two parts. One part of the stomach was excised, immersed in saline, and immediately stored at -40℃ for measurement of NO and MDA levels.

Gastric tissues were homogenized in ten volumes of 150 mmol/L ice-cold KCl using a glass teflon homogenizer (Ultra Turrax IKA T18 Basic) after cutting the tissues into small pieces with scissors (for 2 min at 5000 r/min). The homogenate was then centrifuged at $5000 \times g$ for 15 min. The supernatant was used for analysis. High-performance liquid chromatographic analysis was performed using a Shimadzu HPLC system (Kyoto, Japan) with an MDA kit (Immundiagnostik AG, Bensheim, Germany). Spectrophotometric measurements of total antioxidant status (TAS) (Randox, Crumlin, UK) was performed using a Shimadzu UV-1601 (Kyoto, Japan) spectrophotometer. Serum nitric oxide levels (nitrite + nitrate) were measured, after conversion of nitrate to nitrite by copperized cadmium granules, by a spectrophotometer at 545 nm (Shimadzu, Tokyo, Japan)[14]. Protein assays were measured on an Advia 2400 chemistry analyzer (Bayer Healthcare Instruments, Tarrytown, NY, USA). Results were expressed as μmol/g protein for NO and nmol/g protein for MDA.

The other part of the stomach was fixed in 10% neutral formalin, embedded in paraffin, and cut into 5 μm sections. The sections were stained with hematoxylin eosin (HE) and examined under the light microscope by a blinded pathologist for histological changes.

The results obtained from vardenafil groups were evaluated by comparing them with those of sham, indomethacin, and famotidine groups.

Statistical analysis

The statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 12.0 for Windows. All data are expressed as the mean ± SD. Mann-Whitney U and χ^2 tests were used for statistical analysis of data among all groups. $P < 0.05$ was considered as statistically significant.

RESULTS

Macroscopic analysis showed that there was no gastric mucosal lesion in the sham group. There were gastric mucosal lesions in all stomachs of the indomethacin 25 mg/kg treated group. The mean ulcer area was 21.00 ± 12.35 in the indomethacin group. Gastric mucosal damage was significantly reduced by famotidine 20 mg/kg and vardenafil 10 mg/kg pretreatments. In both groups, the mean count of ulceration and the mean count of

Table 1 Macroscopic evaluation of gastric mucosa

Groups	*n*	Weight (gr)	GML count	GML area mm²
Sham	8	223.50 ± 18.40 (200-249)	0	0
Indomethacin	8	225.00 ± 13.77 (201-248)	6.25 ± 3.49 (1-11)[a]	21.00 ± 12.35 (1-36)[b]
Famotidine 5 (F5)	8	223.25 ± 13.13 (203-236)	4.12 ± 2.47 (2-8)	8.62 ± 2.97 (3-12)
Famotidine 20 (F20)	8	224.75 ± 14.78 (200-247)	2.37 ± 4.43 (0-13)	0.94 ± 2,06 (0-6)[d]
Vardenafil 2 (V2)	8	221.12 ± 13.27 (204-242)	4.37 ± 3.06 (0-8)	6.62 ± 5.87 (0-16)[e]
Vardenafil 10 (V10)	8	224.12 ± 15.16 (202-250)	1.25 ± 1.38 (0-3)[c,g]	0.75 ± 0.88 (0-2)[d,g]

GML: Gastric mucosal lesion. The values are presented as mean ± SD, (min-max). [a]$P < 0.05$, [b]$P < 0.001$ *vs* all other groups; [c]$P < 0.05$, [d]$P < 0.001$ *vs* group F5; [e]$P < 0.05$ *vs* group F20; [g]$P < 0.05$ *vs* group V2.

Table 2 MDA and NO levels in gastric tissues in each group

Group	*n*	MDA (nmol/g protein)	NO (μmol/g protein)
Sham	8	9.4 ± 4.47 (3.6-14.3)	35.67 ± 5.69 (30.21-46.63)
Indomethacin	8	28.48 ± 14.51 (7.1-45)[a]	27.20 ± 6.25 (20.0-38.78)
Famotidine 5 (F5)	8	6.21 ± 1.88 (3.4-9.5)	40.82 ± 9.42 (31.08-59.92)[c]
Famotidine20 (F20)	8	5.88 ± 1.60 (3.3-7.7)	51.22 ± 15.27 (34.24-77.50)[c]
Vardenafil 2 (V2)	8	15.87 ± 3.93 (11.6-23.8)[e]	31.01 ± 20.27 (21-55.15)[e]
Vardenafil 10 (V10)	8	10.97 ± 4.50 (5.4-19.9)[g,h]	33.55 ± 9.29 (22.16-48.51)[g]

The values are presented as mean ± SD, (min-max). [a]$P < 0.05$ *vs* the other group; [c]$P < 0.05$ *vs* indomethacin group; [e]$P < 0.05$ *vs* famotidine groups (F5 and F20); [g]$P < 0.05$ *vs* group F20; [h]$P < 0.01$ *vs* group F20.

ulcer area were significantly lower than the control group. Gastric mucosal lesion areas were significantly diminished in rats pretreated with famotidine 5 mg/kg and vardenafil 2 mg/kg, when compared with the control group, but this did not reach statistical significance in respect to ulcer count. The mean ulcer area in the vardenafil 2 mg/kg group and vardenafil 10 mg/kg group were 6.62 ± 5.87 and 0.75 ± 0.88, respectively. Macroscopic evaluation of gastric mucosal lesion counts and gastric mucosal lesion areas for each group are presented in Table 1. In damaged stomachs, mucosal lesions of various sizes and forms were dispersed to all stomach surfaces. Those lesions consisted of elongated bands parallel to the long axis of the stomach. Lesions of the gastric mucosa in each group are shown in Figure 1. Tissue MDA and NO levels are presented in Table 2 for each group.

On histopathological examination, erosion, inflammation, hemorrhage, and necrosis were abundant in the indomethacin group. Those lesions were encountered with increasing frequency in the Famotidine 20 mg/kg, vardenafil 10 mg/kg, famotidine 5 mg/kg, and vardenafil 2 mg/kg groups. There were statistically significant ($P < 0.05$) differences between the indomethacin group and pretreatment groups. Famotidine 20 mg/kg pretreatment had the most efficient protective effect against indomethacin-induced gastric mucosal lesions. Minimal hemorrhage, minimal focal necrosis, and superficial erosions were observed in 25% of the rats given 2 mg/kg vardenafil. A high dose (10 mg/kg) of vardenafil had a potent protective effect against indomethacin-induced gastric mucosal lesions, but vardenafil in low doses (2 mg/kg) did protect the gastric mucosa against the harmful effects of indomethacin, similarly famotidine 5 mg/kg did. Microscopic views of the normal and damaged gastric mucosa are shown in Figure 2. In our study, vardenafil has gastroprotective effects against indomethacin-induced gastric mucosal damage. The potency of this effect is stronger in high doses than low doses.

DISCUSSION

Despite the great progress in the treatment protocols, peptic ulcers are still a major ongoing heath problem. The gastric barrier protects the mucosa against damage of its deeper structures by noxious substances. Mucosal microcirculation of the stomach has an important role in gastric mucosal protection[15]. Prostaglandins and NO are the main factors that regulate gastric blood flow. NSAIDs cause gastric mucosal damage by inhibiting endogenous prostaglandins due to inhibition of COX-1 and COX-2[16,17]. Prospective data from the Arthritis, Rheumatism, and Aging Medical Information System (ARAMIS) states that 13 of every 100 patients with rheumatoid arthritis treated with NSAID for one year suffer from serious gastrointestinal complications related to the NSAIDs[18]. Indomethacin administration increases aggressive factors but decreases protective factors[19,20].

There is no doubt on the protective effect of H2 blockers. The gastroprotective effects of H2 blockers are significantly greater when given in high doses than in low doses. The results in our experiment, either in low or in high doses of H2 blockers, were in accordance with the literature. Widespread use of H2 blockers could not prevent peptic ulcer related disorders. Therefore, the search for new alternatives with novel mechanisms of action is ongoing.

PDE type-5 inhibitors, which were developed as cardioprotective drugs, are commonly used in the treatment of erectile dysfunction. Sildenafil citrate

Figure 1 Gross appearance of the opened stomachs in the experimental groups. A: Appearance of normal mucosa of the stomach (Saline); B: Severe mucosal injury (Indomethacin); C: Diminished mucosal injury (Group F5); D: Gastric mucosa without any lesion (Group F20); E: Partially protected gastric mucosa against the harmful effect of indomethacin (Group V2); F: Lesion free gastric mucosa (Group V10).

Figure 2 Normal rat gastric mucosa in the saline group and Group V10 (A and C, HE; × 100); gastric mucosal hemorrhage and necrosis in indomethacin group and Group V2 (B and D, HE; × 200, × 100) are shown.

has shown gastroprotective effects in experimental studies[21-23], and its gastroprotective effect was dose-dependent. Vardenafil is more potent than sildenafil. The gastroprotective effect of vardenafil has not yet been

studied. In our study, the antiulcer activity of vardenafil was investigated against indomethacin-induced gastric mucosal damage. Vardenafil decreased indomethacin-induced gastric mucosal lesions significantly at high doses (10 mg/kg). Macroscopically, vardenafil at a dose of 2 mg/kg has a protective effect on the gastric mucosa similar to famotidine at 5 mg/kg.

Macroscopic evaluations of gastric tissues revealed that vardenafil given in 2 mg/kg protects gastric mucosa better than famotidine at a dose of 5 mg/kg. Vardenafil has clinically important gastroprotective effects at high doses (10 mg/kg). Thus, the gastroprotective effect of vardenafil was dose dependent. In the stomach tissue of rats given indomethacin, the level of the lipid peroxidation product, MDA, increased significantly compared to the sham operated group. Tissues exposed to oxidative stress include large amounts of toxic oxygen radicals, which induce lipid peroxidation leading to MDA formation[24,25]. The lowest MDA values were detected in the famotidine groups. The mean MDA values in the vardenafil groups were similar to the sham group (Table 2). Thus, vardenafil pretreatment has inhibited MDA production in indomethacin treated rats.

Possible mechanisms of gastroprotection of PDE V inhibitors are increased production of tissue NO[23,26-28] or increased tissue cGMP level without modifying NO content[22,25,29,30]. The NO levels are slightly elevated in vardenafil pretreated rats in our study; however, the level of NO in either of vardenafil groups did not surpass the level of NO determined in the sham group. Some studies revealed gastroprotective effects of some agents without significant alterations in NO or MDA levels[31]. Determination of tissue cGMP level was not included in our study design. This is a short armcoming of our study design. PDE V inhibitors might prevent indomethacin-induced gastric mucosal damage in either mechanism.

In conclusion, vardenafil reduced gastric mucosal damage significantly at a high dose. Patients treated with PDE type-5 inhibitors might benefit from the additional gastroprotective advantages of these drugs, especially in high doses.

COMMENTS

Background

Peptic ulcer is a common disorder of the gastrointestinal system. The increase in non-steroid anti inflammatory drug (NSAID) ingestion in the treatment of inflammation, fever, and pain is one of the major etiological factors for peptic ulcers. Despite the many drug treatment protocols used to date, peptic ulcer still remains a major public health problem.

Research frontiers

Vardenafil has being used in the treatment of functional impotence; however, its effects on gastric mucosa have not yet been investigated. The research's aim was to determine its effectiveness in gastroprotection against NSAID-induced gastric lesions in comparison with famotidine.

Innovations and breakthroughs

Multiple agents have been used to prevent NSAID-induced peptic ulcer. This work is the first experimental study that show the beneficial effects of Vardenafil (a phosphodiesterase type V inhibitor) on NSAID-induced gastric ulcer. The gastroprotective effect of vardenafil against NSAID-induced peptic ulcer is dose dependent.

Applications

The study results suggest that vardenafil might be used as a potential therapeutic drug to prevent NSAID-induced gastric ulcer formation.

Peer review

This work might provide the first experimental data that directly shows the beneficial effect of a phosphodiesterase V inhibitor on gastric ulc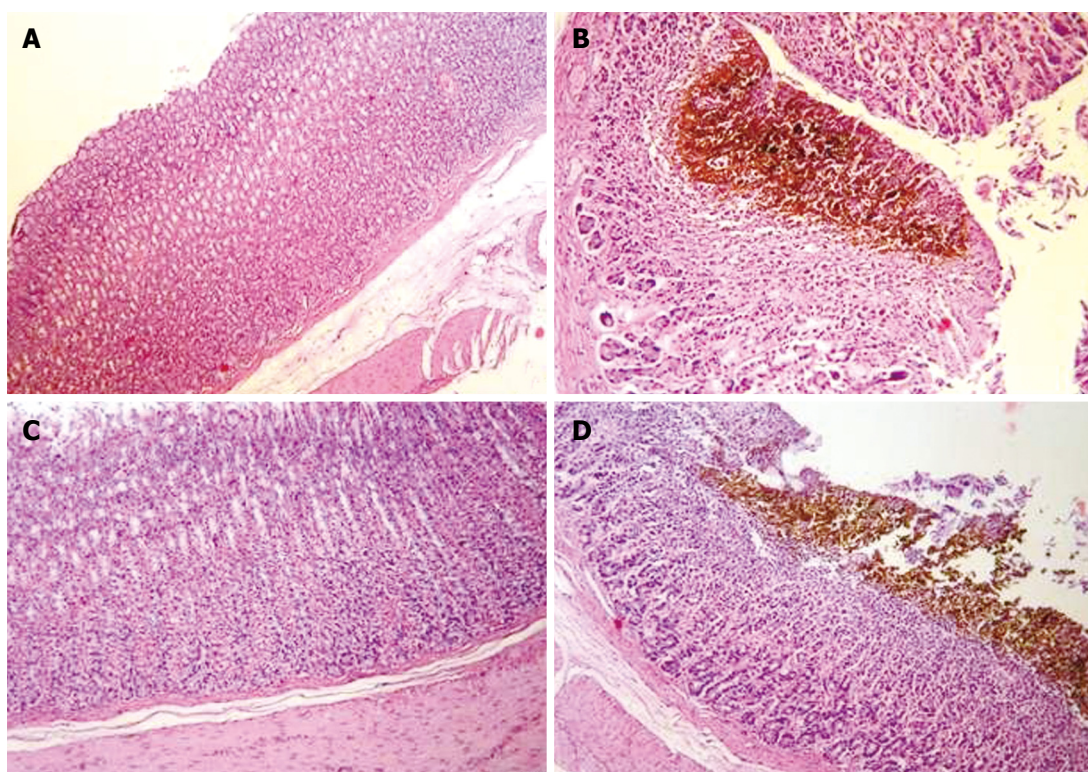ers. In this manuscript, Karakaya et al report that administration of a phosphodiesterase V inhibitor (Vardenafil), dose-dependently suppresses indomethacin-induced gastric ulcers in rats. For comparison purposes, famotidine was used. There is only limited information suggesting the potential beneficial effect of vardenafil on ulcer healing, the data presented would be considered to provide attractive clinical information, although a clear mechanistic insight is not provided.

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BRIEF ARTICLE

Efficacy and safety of rabeprazole in non-steroidal anti-inflammatory drug-induced ulcer in Japan

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Abstract

AIM: To investigate the efficacy and safety of rabeprazole under continuous non-steroidal anti-inflammatory drug (NSAID) administration for NSAID-induced ulcer in Japan.

METHODS: Subjects comprised patients undergoing NSAID treatment in whom upper gastrointestinal endoscopy revealed an ulcerous lesion (open ulcer) with diameter $\geq$ 3 mm, who required continuous NSAID treatment. Endoscopies were performed at the start of treatment, during the treatment period, and at the conclusion (or discontinuation) of treatment. Findings were evaluated as size (maximum diameter) and stage based on the Sakita-Miwa classification. An ulcer was regarded as cured when the "white coating" was seen to have disappeared under endoscopy. As criteria for evaluating safety, all medically untoward symptoms and signs (adverse events, laboratory abnormalities, accidental symptoms, *etc*.) occurring after the start of rabeprazole treatment were handled as adverse events.

RESULTS: Endoscopic cure rate in 38 patients in the efficacy analysis (endoscopic evaluation) was 71.1% (27/38). Among those 38 patients, 35 had gastric ulcer with a cure rate of 71.4% (25/35), and 3 had duodenal ulcer with a cure rate of 66.7% (2/3). Three adverse drug reactions were reported from 64 patients in the safety analysis (interstitial pneumonia, low white blood cell count and pruritus); thus, the incidence rate for adverse drug reactions was 4.7% (3/64).

CONCLUSION: The treatment efficacy of rabeprazole for NSAID-induced ulcer under continuous NSAID administration was confirmed.

INTRODUCTION

In clinical practice, non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed for arthralgia and rheumatoid arthritis (RA)[1,2]. NSAIDs exert potent anti-inflammatory and analgesic effects but are clinically problematic in that gastric mucosal injury can be induced as an adverse reaction[3,4]. For example, up to 25% of patients using NSAIDs develop peptic ulcer[5,6]. A United States study reported that the number of people taking NSAIDs has reached 13 million per year, with approximately 100 000 requiring hospital treatment for upper gastrointestinal injury and 17 000 reported deaths annually[7]. The medical cost exceeds $4 billion per year in the USA[8].

In Japan, the Japan Rheumatism Foundation has reported the results of an epidemiological survey of the incidence of NSAID-induced gastric mucosal lesions in arthritis patients[9]. According to that report, of the 1008 patients with arthritis who took NSAIDs for $\geq$ 3 mo, upper gastrointestinal tract lesions were observed in 62.2%, including 15.5% with gastric ulcer and 1.9% with duodenal ulcer. This suggests that NSAID-induced gastric ulcers occur at a higher rate than the incidence of gastric ulcer found by physical examination (2.2%-4.1%). Moreover, more than 40% of patients were asymptomatic despite the presence of a lesion. In Japan, which faces an increasingly aging society, chronic diseases requiring long-term treatment with NSAIDs (e.g. RA, low back pain and arthralgia) are expected to increase. Devising methods for coping with the expected associated increases in gastric mucosal lesions will thus become increasingly important.

Preparations like proton pump inhibitors (PPIs) and misoprostol are known for curing NSAID-induced gastrointestinal mucosal damage[10-13]. However, misoprostol often induces some adverse reactions like diarrhea[14].

Given this background, the "Evidence-Based Guidelines for Gastric Ulcer" were issued in 2003 (2nd edition in 2007) proposing policies for the treatment and prevention of NSAID-induced ulcers[15]. The guidelines recommend the following therapies: (1) if possible, discontinuation of NSAIDs and initiation of conventional ulcer treatment[16]; and (2) if NSAID treatment cannot be discontinued, initiation of treatment with a PPI or prostaglandin preparation. However, the evidence (clinical results) put forth in the guidelines was based entirely on foreign reports, with no evidence of use from Japanese studies[15].

Rabeprazole, a newer PPI, provides reliable control of gastric acid secretion, with more potent antisecretory activity than that of other PPIs such as omeprazole and lansoprazole, a more rapid rise in intragastric pH, and less effect of CYP2C19 on its metabolism[17-20].

Based on our belief in the importance of investigating the efficacy and safety of the PPI rabeprazole on NSAID-induced ulcers in Japanese individuals, we conducted this survey of 38 medical departments (primarily departments of gastroenterology and internal medicine) at 38 facilities nationwide. This survey was conducted between 1 August 2004 and 31 January 2006, in accordance with good post-marketing surveillance practice.

MATERIALS AND METHODS

Subjects

Subjects included in this survey were patients undergoing NSAID treatment in whom upper gastrointestinal endoscopy revealed an ulcerous lesion (open ulcer) with diameter $\geq$ 3 mm, who required continuous NSAID treatment. Patients taking low-dose aspirin to prevent clot/thrombus formation were excluded as subjects. Patients meeting any of the following criteria were also excluded: (1) history of hypersensitivity to rabeprazole components; (2) exposed blood vessels in the ulcer base; or (3) judged by an investigator as unsuitable for participation in this survey.

Methods

A central registration system was adopted. The registration form was mailed within 1 wk of the registration date. Rabeprazole were to be administered in accordance with the following dose: once daily oral administration of 10 mg of rabeprazole sodium, which can be increased to once daily oral administration of 20 mg, depending on the symptoms (Standard dosage for adults): Standard treatment duration is 8 wk in the case of gastric or anastomotic ulcer, and 6 wk in the case of duodenal ulcer. The Japanese standard dosages of the three most used NSAIDs in this study are as follows; diclofenac sodium, 25-100 mg/d; loxoprofen, 60-180 mg/d; lornoxicam, 12-18 mg/d.

Figure 1 Patient disposition. [1]Patients treated for longer than the allowed treatment period. The allowed treatment period was defined in the protocol as 8 wk for patients with gastric ulcer and 6 wk for patients with duodenal ulcer, + 2 wk for each (i.e. 10 wk for gastric ulcer and 8 wk for duodenal ulcer); [2]Patients for whom endoscopic evaluation was performed prior to 31 d before the start of rabeprazole treatment or more than 31 d after the conclusion of rabeprazole treatment.

Evaluation

Endoscopic findings: Endoscopies were performed at the start of treatment, during the treatment period, and at the conclusion (or discontinuation) of treatment. Findings were evaluated as size (maximum diameter) and stage based on the Sakita-Miwa classification. When multiple ulcers existed in a single subject, only the largest one was recorded. An ulcer was regarded as cured when the "white coating" was seen to have disappeared under endoscopy.

Adverse events: As criteria for evaluating safety, all medically untoward symptoms and signs (adverse events, laboratory abnormalities, accidental symptoms, etc.) occurring after the start of rabeprazole treatment were handled as adverse events.

RESULTS

Patient disposition

One hundred three patients were enrolled in the present study. Due to violation of enrollment and patients meeting exclusion criteria like no concomitant use of NSAIDs, 38 patients were put in the efficacy analysis and 64 patients in the safety analysis (Figure 1).

Efficacy

Demographic and baseline characteristics: Demographic and baseline characteristics of the 38 patients in the efficacy analysis (endoscopic evaluation) are shown in Table 1. Well-used NSAIDs by patients were diclofenac sodium in 36.8% (14/38), loxoprofen 36.8% in (14/38) and lornoxicam in 13.2% (5/38). The main reasons for use of NSAIDs observed were RA in 39.5% (15/38) and low back pain in 36.8% (14/38).

Among 25 subjects undergoing examination for Helicobacter pylori (H pylori) infection, 64.0% (16/25) were positive, and 36.0% (9/25) were negative.

Table 1 Demographic & baseline characteristics [Efficacy analysis (endoscopic evaluation: n = 38)]

Item		Subjects	
		n	%
Age at baseline (yr)	20-39	0	0.0
	40-64	16	42.1
	$\geqslant$ 65	22	57.9
Sex	Male	14	36.8
	Female	24	63.2
Diagnosis	Gastric ulcer	33	86.8
	Duodenal ulcer	3	7.9
	Gastric/duodenal ulcer	2	5.3
Ulcer history	Initial occurrence	15	39.5
	Reoccurrence	13	34.2
	Unknown	10	26.3
Prior history	No	28	73.7
	Yes	10	26.3
History of allergies	No	31	81.6
	Yes	5	13.2
	Unknown	2	5.3
Type of NSAID (duplicates included)	Diclofenac sodium	14	36.8
	Loxoprofen sodium	14	36.8
	Other	15	39.5
Anti-ulcer treatment before rabeprazole treatment (within 1 mo)	No	12	31.6
	Yes	26	68.4
Concomitant medication (not including NSAIDs)	No	5	13.2
	Yes	33	86.8
Endoscopic findings before start of treatment (ulcer size)	3 $\leqslant$ size < 10 mm	21	55.3
	10 $\leqslant$ size < 20 mm	13	34.2
	$\geqslant$ 20 mm	4	10.5
Rabeprazole treatment duration	$\leqslant$ 56 d	20	52.6
	> 56 d	18	47.4
Rabeprazole dosage	10 mg	24	63.2
	20 mg	12	31.6
	10 mg→20 mg	1	2.6
	20 mg→10 mg	1	2.6

Table 2 Endoscopic cure rate

	10 mg group	20 mg group	Modified dose group[2]	Total
Gastric ulcer[1]	71.4% (15/21)	75.0% (9/12)	50.0% (1/2)	71.4% (25/35)
Duodenal ulcer	66.7% (2/3)	-	-	66.7% (2/3)
Total	70.8% (17/24)	75.0% (9/12)	50.0% (1/2)	71.1% (27/38)

[1]The 2 patients with "gastric/duodenal ulcer" were included in tabulations for gastric ulcer; [2]Modified dose group consisted of 1 patient changing from 10 mg to 20 mg and 1 patient changing from 20 mg to 10 mg.

Endoscopic cure rate: Endoscopic cure rate for the 38 patients in the efficacy analysis (endoscopic evaluation) was 71.1% (27/38) (Table 2). According to diagnosis, the cure rate was 71.4% (25/35) for gastric ulcer and 66.7% (2/3) for duodenal ulcer. In addition, the cure rate was lower in the gastric antrum (55.6%; 10/18) than in the gastric corpus (91.7%; 11/12).

Safety
Demographic and baseline characteristics: Demo-

Table 3 Demographic & baseline characteristics (Safety analysis: n = 64)

Item		Subjects	
		n	%
Age at baseline (yr)	20-39	1	1.6
	40-64	34	53.1
	$\geqslant$ 65	29	45.3
Sex	Male	27	42.2
	Female	37	57.8
Diagnosis	Gastric ulcer	57	89.1
	Duodenal ulcer	4	6.3
	Gastric/duodenal ulcer	2	3.1
	Anastomotic ulcer	1	1.6
Ulcer history	Initial occurrence	29	45.3
	Reoccurrence	20	31.3
	Unknown	15	23.4
Prior history	No	47	73.4
	Yes	17	26.6
History of allergies	No	55	85.9
	Yes	6	9.4
	Unknown	3	4.7
Type of NSAID (duplicates included)	Diclofenac sodium	34	53.1
	Loxoprofen sodium	20	31.3
	Other	27	42.2
Anti-ulcer treatment before rabeprazole treatment (within 1 mo)	No	19	29.7
	Yes	45	70.3
Concomitant medication (not including NSAIDs)	No	7	10.9
	Yes	57	89.1
Endoscopic findings before start of treatment (ulcer size)	3 $\leqslant$ size < 10 mm	31	48.4
	10 $\leqslant$ size < 20 mm	25	39.1
	$\geqslant$ 20 mm	5	7.8
	Unknown	3	4.7
Rabeprazole treatment duration (gastric ulcer, gastric/duodenal ulcer, duodenal ulcer, anastomotic ulcer)	$\leqslant$ 56 d	27	42.2
	> 56 d	37	57.8
Rabeprazole dosage	10 mg	39	60.9
	20 mg	19	29.7
	10 mg→20 mg	1	1.6
	20 mg→10 mg	5	7.8

graphic and baseline characteristics of 64 patients in the safety analysis are shown in Table 3. Well-used NSAIDs by patients were diclofenac sodium in 53.1% (34/64), loxoprofen in 31.3% (20/64) and lornoxicam in 10.9% (7/64). The main reasons for using NSAIDs were RA in 40.6% (26/64), low back pain in 28.1% (18/64) and osteoarthritis in 15.6% (10/64). Among 42 subjects undergoing examination for *H pylori* infection, 59.5% (25/42) were positive, and 40.5% (17/42) were negative.

Incidence of adverse drug reactions: Among the 64 patients in the safety analysis, 3 adverse drug reactions were observed in 3 patients. The incidence rate for adverse drug reactions was thus 4.7% (3/64). Adverse reactions comprised 1 case of "interstitial pneumonia" (serious; outcome: recovering) (1.6%); 1 case of "low white blood cell count" (non-serious; outcome: recovered) (1.6%); and 1 case of "pruritus" (non-serious; outcome: recovered) (1.6%).

DISCUSSION

Endoscopic cure rate was 71.1% (27/38). By disease, the endoscopic cure rate in gastric ulcer patients after 8 wk of treatment was 71.4% (25/35) and the endoscopic cure rate in duodenal ulcer patients after 6 wk of treatment was 66.7% (2/3). In other clinical studies of rabeprazole, reported cure rates have been 93.5% (72/77 patients)[21] and 96.4% (27/28) for gastric ulcer, and 100% (23/23) for duodenal ulcer[22]. Endoscopic cure rate in this survey, at 71.1%, did not reach the general cure rates obtained for chronic gastric and duodenal ulcers. These results strongly suggest that continuous administration of NSAIDs may act to delay ulcer healing.

Conversely, in studies similar to this study relating to the healing of gastric and duodenal ulcers in the presence of NSAID treatment, according to the results of Shiokawa et al[23] in the first such study in Japan, cure rates for gastric and duodenal ulcer were 70% (35/50) and 83.3% (5/6), respectively, when a prostaglandin was used at 800 μg/d. With regard to the healing effect of PPI use, only results from foreign studies are available[24-27]. Hawkey et al[26] conducted a comparative examination of the results of studies on the effects of 20 mg and 40 mg of omeprazole, and 800 μg of misoprostol. After 8 wk of administration, the gastric ulcer cure rate was 87.2% (102/117) with 20 mg of omeprazole and 79.5% (105/132) with 40 mg. The cure rate in the group administered 20 mg of omeprazole was significantly higher than the 72.8% (91/125 patients) in the control group administered 800 μg of misoprostol. In a study of lansoprazole, Agrawal et al[27] compared the results of 15 mg and 30 mg of lansoprazole versus 300 mg of ranitidine hydrochloride. Gastric ulcer cure rates after 8 wk of treatment were 68.6% (81/118) with 15 mg lansoprazole and 72.6% (85/117) with 30 mg. Cure rates in both lansoprazole groups were significantly higher than the 53.0% (61/115 patients) obtained using ranitidine hydrochloride. The present survey found no significant difference between rabeprazole doses with respect to cure rates for NSAID-induced ulcer, with a cure rate of 70.8% (17/24) using a dose of 10 mg and 75.0% (9/12) at 20 mg. The cure rates observed here did not differ significantly from those obtained using the other PPIs described above.

Conversely, how *H pylori* infection affects healing of NSAID-induced ulcers remains controversial and is an important topic that should be considered in future studies, and a recently published guideline addressed this point[28]. This survey investigated the dependency of cure rate on *H pylori* infection, but no significant difference was observed. The cure rate in *H pylori*-positive patients was 81.3% (13/16), compared to 77.8% (7/9) in *H pylori*-negative patients.

Symptoms and signs, and the conditions of occurrence of these presentations, are commonly considered to differ between NSAID-induced ulcer patients and patients with ordinary ulcers. Saigenji et al reported that the pretreatment incidence of epigastralgia was 100% (72/72 patients) in patients with gastric ulcer[21]. However, the

Table 4 Clinical characteristics of NSAIDs-induced ulcer (safety analysis: n = 64)

Site of ulcer	n	%	Ulcer form	n	%	No. of ulcers	n	%
Ulcer condition								
Corpus	19	29.7	Round	27	42.2	Single	42	65.6
Angle	7	10.9	Elliptical	23	35.9	Multiple	21	32.8
Antrum	31	48.4	Irregular	11	17.2			
Bulb	4	6.3	Other	2	3.1			
Efferent loop	1	1.6						

pretreatment incidence rate for epigastralgia was 70.2% (33/41) in the present survey, which was comparatively lower than the incidence observed with ordinary gastric ulcer. In general, incidences for symptoms and signs are lower with NSAID-induced ulcers[29], and the present results support this.

Data on the site, shape and number of NSAID-induced ulcers were also tabulated in this survey. For the 90 patients with NSAID-induced ulcer (not including the 9 patients in the dataset of patients analyzed who did not display NSAID-induced ulcer), the original ulcer site was most frequent at the gastric antrum (43.3%) and the most common shapes were round (35.6%) and elliptical (37.8%), although irregularly shaped ulcers were also observed (23.3%). In terms of the number of ulcers, multiple ulcers were present in 33.3% (30/90) of cases. The clinical characteristics of NSAID-induced ulcers that were confirmed in the 64 patients in the safety analysis were also tabulated. The gastric antrum was the site of the ulcer in 48.4% (31/64) of patients; the shape was round in 42.2% (27/64) and elliptical in 35.9% (23/64) while 32.8% (21/64) of cases displayed multiple ulcers (Table 4).

NSAID-induced ulcer in gastric antrum, the most common in the present study, seemed to be harder to cure compared to NSAID-induced ulcers in the gastric corpus or gastric angle, as reported by Mizokami et al[30].

Measures for responding to NSAID-induced lesions found in normal medical examinations will become increasingly important as society continues to age. This survey, conducted in accordance with the guidelines, proposes one direction for treatment.

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COMMENTS

Background

A non-steroidal anti-inflammatory drug (NSAID) is widely prescribed for patients with rheumatoid arthritis, low back pain, *etc*. However, NSAID damages gastrointestinal mucosa and induces peptic ulcers. Use of a proton pump inhibitor (PPI) is recommended to cure those peptic ulcers.

Research frontiers

This multicenter study all over Japan revealed the safety profile and efficacy of rabeprazole, a type of PPI, for NSAID-induced ulcer.

Innovations and breakthroughs

This is one of the studies to investigate the safety profile and efficacy of rabeprazole for NSAID-induced ulcer.

Applications

Safety and efficacy of rabeprazole was comparable to other types of PPIs, which means more diversity of treatment options for patients suffering from NSAID-induced ulcer, and this is important since the number of patients using NSAIDs is expected to increase in the aging society in Japan.

Peer review

The manuscript by Mizokami provides the results of studies on the efficacy and safety of rabeprazole for ulcerous lesions in patients undergoing NSAID treatment. This is a reasonable study and the data are well presented.

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CASE REPORT

Unexplained liver laceration after metastasis radiofrequency ablation

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Abstract

Many studies have established the role of radiofrequency (RF) ablation as a minimally invasive treatment for liver metastases. Although relatively safe, several complications have been reported with the increased use of RF ablation. We describe here a case of unexplained liver laceration after a RF procedure. A woman who presented a solitary metachronous liver metastasis underwent RF ablation treatment for this lesion. Six hours later the patient displayed fatigue and pallor. Emergency blood tests showed a haemoglobin level of < 7 g/dL and markedly elevated transaminase levels. A computed tomography examination revealed two areas of liver laceration with haematoma, one of them following the path of the needle and the other leading away from the first. Following a blood transfusion, the patient was haemodynamically stable and completely recovered 24 h later. The patient remained in bed for 1 wk. No surgical intervention was required, and she was discharged 1 wk later.

Key words: Colon cancer; Liver haemorrhage; Liver laceration; Liver metastases; Radiofrequency ablation

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INTRODUCTION

Many studies have demonstrated the benefits of radiofrequency (RF) ablation as a minimally invasive treatment for hepatic metastases from colon carcinoma[1-3]. Good outcomes with low morbidity and mortality have been identified. Therefore, the use of RF ablation is increasing in the field of oncology[4,5]. Although a wide spectrum of complications has been described, all of these complications occur at low frequencies. Haemorrhage is the most common complication, mainly related to mechanical injury of the blood vessels in patients with cirrhosis, and it is probably exacerbated by a coagulation deficit and the rich blood supply of the tumour[6].

We present here a case of multiple liver haematomas occurring as a secondary response to RF ablation of a solitary metastasis.

CASE REPORT

A 72-year-old woman underwent RF ablation for the treatment of a metachronous solitary liver metastasis from rectal adenocarcinoma, which had been treated 18 mo before with preoperative chemoradiotherapy and anterior resection. The lesion was located in segment VIII.

The procedure went well and the patient was initially haemodynamically stable. One hour later her condition suddenly worsened. She was clinically dizzy and had fatigue and pallor. We detected hypotension and tachycardia. Emergency blood tests showed a haemoglobin level of < 7 g/dL and markedly elevated transaminase levels. A computed tomography examination revealed an accumulation of intraperitoneal fluid in the pelvis and two liver haematomas. The images showed two liver lacerations, one following the path of the needle (Figure 1A-C) and another, unexplained laceration leading away from the first (Figure 2). The patient had no serious coughing or hiccupping after the RF treatment, or any other complications that might have caused increased abdominal pressure and tumour rupture.
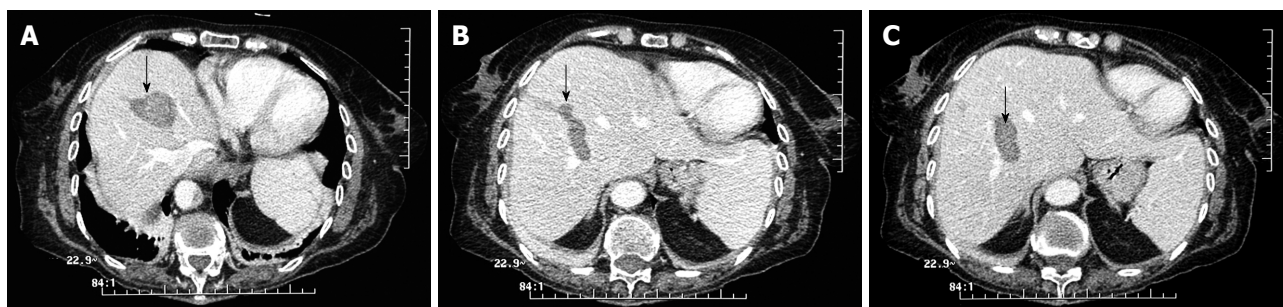
Figure 1 Secondary lacerations resulting from a radiofrequency ablation procedure to treat a liver metastasis from colorectal adenocarcinoma. A: Image of liver laceration grade Ⅲ in a central location (arrow); B: Image showing the first laceration following the path of the RF needle (arrow); C: Image of the first laceration in a lower liver tomography cut (arrow).

Figure 2 The second and unexplained liver laceration located in segments Ⅴ-Ⅵ (arrow).

Subsequently, the patient received a blood transfusion and close monitoring. She was haemodynamically stable after the second blood transfusion and completely recovered 24 h later. The patient remained in bed for 1 wk. No surgical intervention was required.

DISCUSSION

RF ablation is a minimally invasive treatment of hepatic metastases from colon carcinoma, and can achieve good outcomes with low morbidity and mortality rates. Therefore, the use of RF ablation is increasing in the field of oncology[1,2]. Although some studies which included large numbers of patients have found low rates of complications after RF ablation of hepatic tumors (ranging from 2.4% to 8.9%), the rate of intraperitoneal haemorrhage is low (0.46%-1.6%) but relevant because this technique is increasingly used with few selection criteria for patients[6]. The etiology of this potentially grave complication is variable.

The reported reasons for haemorrhage are usually related to mechanical injuries to the liver blood vessels and occur most often in patients with cirrhosis, and are probably due to a coagulation deficit and the rich blood supply of the tumour[4,6]. Other cases have been attributed to serious coughing or hiccups after the RF treatment[7]. These complications might cause increased abdominal pressure and tumour rupture, particularly if the tumour location is near the capsule[6].

Liver laceration has rarely been described as a cause of haemorrhage. Although this latter complication is very infrequent, it has been reported, and has been associated with inappropriate electrode positioning or mechanical injury of the soft liver during the procedure[8]. Such mechanical injury can be induced by coughing or position changes, causing increasing abdominal pressure, and possibly displacing the electrode slightly[8].

In our case the procedure went well, without incident, and the patient did not present any of the complications described above, such as coughing or hiccups. The tumour was not located near the capsule but at a depth within segment Ⅷ.

We suggest that these two lacerations might have resulted from direct mechanical injury due to penetration by the electrode into liver tissues that were soft and damaged as a result of prior treatment with chemotherapy. The second laceration may have occurred due to difficulty in positioning the electrode to access such a profound lesion, causing liver disruption away from the first laceration.

Most haemorrhages require blood transfusions or surgical intervention[6]. In our case the patient recovered spontaneously after blood transfusion and bed rest without the necessity of surgical intervention.

It is absolutely essential to minimise complications associated with RF ablation treatments, and to correctly deal with complications which do arise[6]. The close observation of patients after RF ablation treatments, and early intervention to minimise the damage and severity of complications, are warranted.

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CASE REPORT

Gastric choriocarcinoma admixed with an α-fetoprotein-producing adenocarcinoma and separated adenocarcinoma

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Abstract

We report a case of gastric choriocarcinoma admixed with an α-fetoprotein (AFP)-producing adenocarcinoma. A 70-year-old man was hospitalized for gastric cancer that was detected during screening by esophagogastroduodenoscopy (EGD). Initial laboratory data showed the increased serum level of AFP and EGD revealed a 5-cm ulcerofungating mass in the greater curvature of the gastric antrum. The patient underwent radical subtotal gastrectomy with D2 lymph node dissection and Billroth Ⅱ gastrojejunostomy. Histopathological evaluation confirmed double primary gastric cancer: gastric choriocarcinoma admixed with an AFP-producing adenocarcinoma and separated adenocarcinoma. At 2 wk postoperatively, his human chorionic gonadotropin and AFP levels had reduced and six cycles of adjuvant chemotherapy were initiated. No recurrence or distant metastasis was observed at 4 years postoperatively.

Key words: α-fetoproteins; Adenocarcinoma; Choriocarcinoma; Stomach neoplasms

Eom BW, Jung SY, Yoon H, Kook MC, Ryu KW, Lee JH, Kim YW. Gastric choriocarcinoma admixed with an α-fetoprotein-producing adenocarcinoma and separated adenocarcinoma. *World J Gastroenterol* 2009; 15(40): 5106-5108 Available from: URL: http://www.wjgnet.com/1007-9327/15/5106.asp DOI: http://dx.doi.org/10.3748/wjg.15.5106

INTRODUCTION

Choriocarcinoma is a rapidly growing, highly invasive, widely metastasizing neoplasm that derives from either trophoblastic or totipotential germ cells. It often arises in the uterus in association with pregnancy, and its most common extragonadal sites are the mediastinum, ovary and testis[1].

Primary gastric choriocarcinoma (PGC) is a rare neoplasm that constitutes less than 1% of all gastric cancers[2]. It was first described by Davidsohn in 1905 and more than 140 cases have been reported in the international medical literature. Several studies have indicated that the pathogenesis of PGC can be explained by the dedifferentiation of malignant adenocarcinoma tissue to the level of the embryonal ectoderm, and the retention of an ability to form trophoblasts[3]. However, the clinicopathological and prognostic factors of PGC are unreliable because of the small numbers of PGC cases reported.

α-fetoprotein (AFP) is a fetal serum protein that is produced by fetal liver and yolk sac cells, and some fetal gastrointestinal cells. AFP levels decrease gradually after birth and reach adult levels at 8-12 mo. However, AFP levels are elevated in patients with hepatocellular carcinoma and in those with non-cancerous liver disease associated with liver regeneration. AFP-producing tumors have been reported in several different organs, and commonly in the stomach[4-8]. The proportion of gastric cancers that secrete AFP has been reported to be 2.7%-8.0%[9]. AFP-producing gastric carcinomas have high proliferative activity and are associated with low levels of apoptosis and rich neovascularization. They are divided into three subtypes: hepatoid, yolk sac tumor-like, and fetal gastrointestinal[10]. Here, we report the case of a 70-year-old man with gastric choriocarcinoma admixed with AFP-producing adenocarcinoma.

CASE REPORT

A 70-year-old man was referred to our hospital for

Figure 1 Gross pathology showed a 5.8 cm × 3.2 cm ulcerofungating mass in the antrum, with extensive hemorrhage and light gray fibrosis, and a 2.5 cm × 2.0 cm ulcerative lesion.
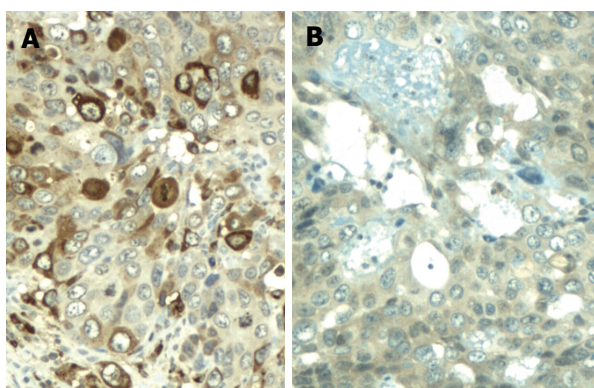

Figure 3 Immunohistochemical staining showed positive immunoreactivity for β-human chorionic gonadotropin (A) and focal positivity for α-fetoprotein (B).

Figure 2 Microscopically, massive numbers of pleomorphic, bizarre tumor cells with hemorrhage were revealed: syncytiotrophoblasts and cytotrophoblasts (HE, A × 40, B × 100).

gastric cancer that was detected during screening by esophagogastroduodenoscopy (EGD). No significant medical history was identified, except dysuria caused by bladder contraction. Initial laboratory data showed a serum level of AFP of 32.3 ng/mL (normal range: 0-15 ng/mL), but no other abnormality, which included other tumor markers, such as, carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9). EGD revealed a 5-cm ulcerofungating mass that was comprised of three septate ulcers in the greater curvature of the gastric antrum. A pathological examination of endoscopic biopsy tissues confirmed the presence of moderately differentiated tubular adenocarcinoma. Subsequent abdominopelvic computed tomography visualized a gastric mass with deep ulceration in the gastric antrum with perigastric lymph node enlargement. No metastatic lesions were observed in the liver, lung or peritoneum, and chest radiography showed no significant findings.

Radical subtotal gastrectomy with D2 lymph node dissection and Billroth Ⅱ gastrojejunostomy were performed. Grossly, the resected specimen contained double lesions: the first was a 5.8 cm × 3.2 cm ulcerofungating mass in the antrum, with extensive hemorrhage and light gray fibrosis; and the second was a nearby 2.5 cm × 2.0 cm ulcerative lesion (Figure 1). Microscopically, massive numbers of pleomorphic,

bizarre tumor cells with hemorrhage (syncytiotrophoblasts and cytotrophoblasts) were observed in the first lesion. Hematoxylin and eosin (HE)-stained tissues revealed a bubbly purple cytoplasm and giant nuclei at a magnification of 40 × (Figure 2A) and 100 × (Figure 2B). The tumor involved the proper muscle layer (T2a) and metastasis was found in four of 56 regional lymph nodes (N1). Immunohistochemical staining showed positive immunoreactivity for β-human chorionic gonadotropin (HCG) (Figure 3A) and focal positivity for AFP (Figure 3B). These findings confirmed the presence of gastric choriocarcinoma that contained small foci of an AFP-producing adenocarcinoma. The second lesion was moderately differentiated tubular adenocarcinoma, which extended to the submucosal layer (T1b). It was close to, but distinct from the first lesion, which was negative by immunohistochemical staining for β-HCG and AFP.

The patient had an uneventful postoperative course and was discharged on postoperative day 9. Two weeks later, his HCG level was 176 mIU/mL (normal range: 0-10 mIU/mL) and his AFP level was 10.0 ng/mL. Six cycles of adjuvant chemotherapy with capecitabine (Xeloda; Hoffmann-La Roche Inc., Nutley, NJ, USA) was started at 2500 mg/m² per day for 14 d/cycle. After two cycles, his β-HCG level had declined to < 3 mIU/mL, and has since remained at this level. No recurrence or distant metastasis had occurred at his 4-year postoperative follow-up.

DISCUSSION

Several theories have been proposed to explain the histopathogenesis of primary choriocarcinoma of the stomach. These hypotheses include an origin from a gonadal angle displaced in the abdomen[11], a histological resemblance to choriocarcinoma[1], an origin from an underlying gastric teratoma[12], and the dedifferentiation or opisthoplatia of carcinoma cells to the level of the embryonal ectoderm with an ability to form trophoblasts[13]. Of these, the dedifferentiation theory, proposed by Pick in 1926, is accepted most widely. Based on the observation that many cases of PGC have been found with coexistent adenocarcinoma, Pick proposed that choriocarcinoma could arise by overgrowth

and elimination of the original adenocarcinoma[4]. Furthermore, the findings of a comparative genomic hybridization and fluorescence *in situ* hybridization study by Liu *et al*[14] support this theory; they have concluded that PGC possesses genetic characteristics of adenocarcinoma and gestational choriocarcinoma.

Applying the dedifferentiation theory proposed by Pick to the present case, we are led to consider that β-HCG-producing choriocarcinomas is dedifferentiated from AFP-producing adenocarcinomas. However, this is supposition and further studies on the pathogenesis of choriocarcinoma are required.

Of the three subtypes of AFP-producing gastric cancers, the described case was of the fetal gastrointestinal type that appeared to develop as a result of fetal gastrointestinal epithelium recapitulation in a tubular adenocarcinoma. The clinical implications of the three subtypes have not been evaluated, although the best-characterized hepatoid type, which is the most common, is known to have a high malignant potential and to be associated with poor survival[15].

The prognosis of PGC is poor because of its high metastatic potential, especially to the liver, lung and regional lymph nodes, and most PGC patients succumb within a year of operation. Therefore, the treatment of choice for PGC is controversial, especially palliative gastric resection. However, in several cases, curative resection and adjuvant chemotherapy (such as 5-fluorouracil and cisplatin combination) have been found to promote long-term survival[1].

Furthermore, survival for AFP-producing gastric cancer is also dismal because of the high risk of liver metastasis[16]. A high proportion of patients with AFP-producing early gastric cancer that underwent curable resection died from liver metastasis[17]. In the described case, the patient survived recurrence free without distant metastasis for more than 3 years after surgery and adjuvant chemotherapy.

In conclusion, the described case suggests that gastric choriocarcinoma admixed with an AFP-producing adenocarcinoma has a good prognosis. Curative resection, appropriate chemotherapy, and the absence of synchronous liver metastasis are considered favorable prognostic factors in PGC. Further evaluations of its pathogenesis and of the cause of its good prognosis are necessary.

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CASE REPORT

A case of intussuscepted Meckel's diverticulum

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Abstract

We report colonoscopic features of an intussuscepted Meckel's diverticulum, presenting with hematochezia. A 35-year-old woman presented to the emergency room with acute onset, transient, sharp, severe epigastric pain that began 6 h earlier. Colonoscopy revealed a reddish, soft, fist-sized polypoid lesion in the terminal ileum. The lesion was misinterpreted as a hematoma by an inexperienced endoscopist. The patient began to complain of intermittent, severe periumbilical pain following the colonoscopic examination. Subsequent computed tomography showed an enteric intussusception. An exploratory laparotomy revealed an intussuscepted Meckel's diverticulum, with transmural infarction. Colonoscopy was of little use in assessing the intussusception. However, colonoscopic examination may be performed initially, especially in an intussuscepted Meckel's diverticulum presenting with hematochezia. Endoscopists should note the endoscopic features of an intussuscepted Meckel's diverticulum.

INTRODUCTION

Abdominal computed tomography (CT) is currently considered the most sensitive radiological method for confirming intussusception[1,2]. However, it is quite possible for colonoscopy to be selected as the initial diagnostic method when intussusception presents primarily as hematochezia. Colonoscopy may be useful for confirming the presence of intussusception, localizing the disease, and demonstrating the underlying organic lesion serving as the lead point. Intussusception might be misinterpreted as another lesion, such as a hematoma or polyp, on a colonoscopic examination, especially by an inexperienced endoscopist. Here, we report a colonoscopic feature of an intussuscepted Meckel's diverticulum, presenting with hematochezia, which was initially misinterpreted as a hematoma on colonoscopic examination.

CASE REPORT

A 35-year-old woman presented to the emergency room with acute onset hematochezia that occurred over a weekend. She first noticed the hematochezia accompanied by transient, sharp, severe epigastric pain 6 h earlier. On physical examination, she was a lean young woman with stable vital signs. Her abdomen was soft with no tenderness or muscle guarding. Bowel sounds were hypoactive with a normal pitch. Digital rectal examination showed bright red stools on the glove. Laboratory tests revealed normal peripheral blood cell counts and blood chemistry, except for leukocytosis with a white blood count of $145\,000/mm^3$. Because she was admitted at a weekend, endoscopy was performed by an inexperienced endoscopist who had trained at

Figure 1 A polypoid lesion in the terminal ileum.

Figure 2 Contrast-enhanced abdominal CT showed massive invagination of long segmental ileal loops.

Figure 3 Macroscopically, a diverticular sac was noted along the antimesenteric border. The entire wall of both the diverticulum and intestine was affected by hemorrhagic infarction and the luminal surfaces were covered with a necrotic exudate.

Figure 4 Microscopically, the diverticular sac was totally involved in transmural infarction. Its wall had a continuous proper muscle layer and heterotopic gastric mucosa (Inset). (HE, × 40; inset × 200).

our endoscopic center for 2 years. Gastroscopy was negative. Colonoscopy revealed blood and mucus throughout the colon. At colonoscopy, a reddish, soft, fist-sized polypoid was found 30 cm into the terminal ileum (Figure 1). Forcing forceps against the lesion depressed the lesion. The endoscopist tried to remove the lesion using various endoscopic accessories, because it was misdiagnosed as a hematoma. The patient began to complain of intermittent, severe periumbilical pain following the colonoscopic examination. Subsequent CT showed a typical inhomogeneous target-shaped soft-tissue mass with a layering effect, suggestive of enteric intussusception (Figure 2). An exploratory laparotomy revealed enteric intussusception, with gangrene. Segmental resection of the small intestine was performed. The pathology revealed an intussuscepted Meckel's diverticulum with transmural infarction along the antimesenteric border (Figure 3). Microscopic examination disclosed an ectopic gastric mucosa and a complete proper muscle layer in the diverticular wall (Figure 4). The patient had an uneventful postoperative course and was discharged on the ninth post-operative day.

DISCUSSION

Adult intussusception constitutes 5% of all cases of intussusception and accounts for only 1%-5% of intestinal obstructions in adults[3]. Almost 90% of the cases of intussusception in adults are secondary to a pathological condition that serves as a lead point, such as carcinomas, polyps, Meckel's diverticulum, colonic diverticulum, and strictures or benign tumors, which are usually discovered intraoperatively[4-6]. A Meckel's diverticulum is a remnant of the omphalomesenteric duct, which is normally obliterated by the 5th-8th wk of gestation. It is seen in 2% of the population. The lifetime risk of complications in patients with a Meckel's diverticulum is only 4%[7]. Thus, an intussuscepted Meckel's diverticulum is very rare, despite the well-known association of enteric intussusception and Meckel's diverticulum.

Abdominal CT can help to confirm the presence of intussusception and distinguish between lead point and non-lead point intussusception and can potentially eliminate unnecessary surgery[7]. Colonoscopy is of little use for assessing intussusception. There are few reports on the colonoscopic features of an intussuscepted Meckel's diverticulum[8,9]. An inexperienced endoscopist might be unfamiliar with these features, which appear to depend on the severity, chronicity, and etiology of the intussusception. A spring-shaped polypoid mass with surface erosion or a large number of blood vessels has been seen at colonoscopy in intussuscepted Meckel's diverticulum without complications[8,9]. Incidental snare polypectomy might be performed in patients with

chronic intussusception that presents as a polypoid mass on a barium or endoscopic examination[10]. This poses a high risk of perforation in a background of chronic tissue ischemia and possible necrosis of the intussuscepted bowel segment wall. An intussuscepted Meckel's diverticulum, with gangrene, such as in our case, might be misdiagnosed as a hematoma at colonoscopy, because of the severe hyperemic and edematous mucosal changes. In conclusion, endoscopists should be aware of the endoscopic features of an intussuscepted Meckel's diverticulum, because a colonoscopic examination can be performed initially, especially in an intussuscepted Meckel's diverticulum presenting with hematochezia.

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CASE REPORT

Liver transplantation for polycystic liver with massive hepatomegaly: A case report

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Abstract

A previous study has shown that liver or combined liver-kidney transplantation can be a valuable surgical technique for the treatment of polycystic liver disease. Herein, we present the case of a 35-year-old woman with polycystic liver disease, who underwent orthotopic liver transplantation (OLT) on November 11, 2008. The whole-size graft was taken from a deceased donor (a 51-year-old man who died of a heart attack). Resection in a patient with massive hepatomegaly is very difficult. Thus, after intercepting the portal hepatic vein, left hepatectomy was performed, then the vena cava was intercepted, the second and third porta hepatic isolated, and finally, right hepatectomy was performed. OLT was performed successfully. The recipient did well after transplantation. This case suggested that OLT is an effective therapeutic option for polycystic liver disease and left hepatectomy can be performed first during OLT if the liver is over enlarged.

Key words: Hepatectomy; Liver transplantation; Polycystic liver

INTRODUCTION

A previous study has shown that liver or combined liver-kidney transplantation can be a valuable surgical technique for the treatment of polycystic liver disease[1]. Here, we present the case of a 35-year-old woman with polycystic liver disease, who underwent orthotopic liver transplantation (OLT) on November 11, 2008.

CASE REPORT

In October 2008, a 35-year-old woman with a body weight of 52 kg was admitted to the Liver Transplantation Center, the First Affiliated Hospital of Nanjing Medical University to undergo liver transplantation. This patient had a significant family history of polycystic disease, and her mother had died of dyscrasia that resulted from polycystic liver and kidney diseases. She had normal liver function [alanine aminotransferase (ALT) 35.7 U/L, aspartate aminotransferase (AST) 21.7 U/L, total bilirubin 16.5 μmol/L, and prothrombin time 13.8 s], and normal renal function (urea 4.33 mmol/L, creatinine 77 μmol/L, glomerular filtration rate 62.4 mL/min), but had dyspnea, anorexia, abdominal pain, hypertension, increased abdominal girth, mild ascites, and a liver span of 20 cm below the left costal margin and 25 cm below the right costal margin before transplantation. OLT was performed on November 11, 2008, and the whole-size graft was taken from a 51-year-old man who had died of a heart attack. The blood phenotypes of the donor and recipient were type A, RH (+). OLT was performed, but resection of the massive hepatomegaly was very difficult, because the enlarged polycystic liver occupied nearly the whole epigastric zone and mid abdomen (Figure 1A), which resulted in a small surgical space, especially for
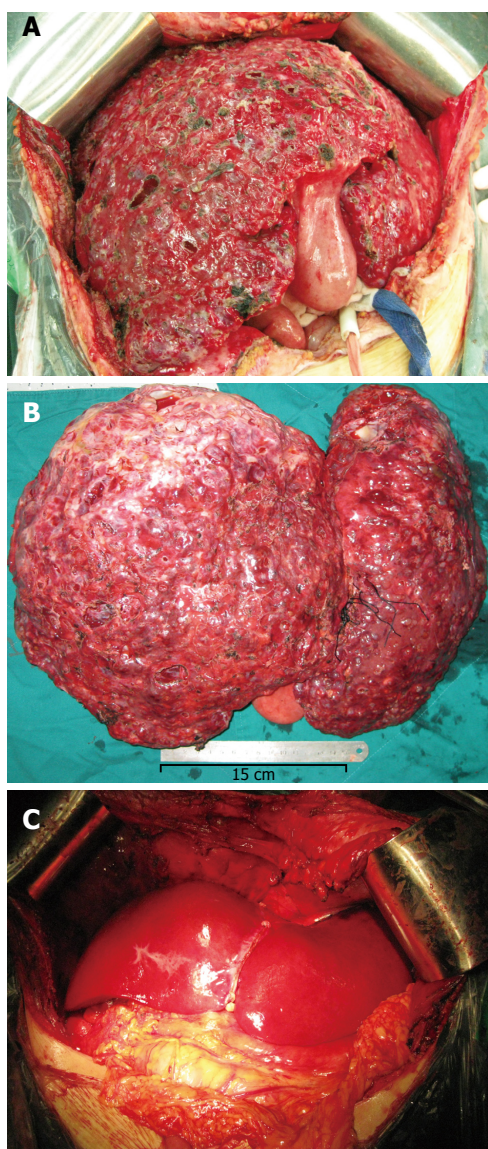
Figure 1 The liver before and after liver transplantation. A: Polycystic liver after cyst fenestration. The liver was still very large; B: The excised polycystic liver contained innumerable cysts; C: After OLT, the donor liver was much smaller than the recipient liver.

manipulation of hepatic blood vessels behind the liver. Thus, after intercepting the portal vein and vena cava, left hepatectomy was performed, followed by isolation of the hepatic veins and short hepatic veins at the posterior of the liver, and finally, right hepatectomy. OLT was then performed successfully (Figure 1C). The excised liver weighed 10.2 kg (approximate 20% of the body weight), which contained innumerable cysts and a large amount of cystic fluid (Figure 1B). The recipient did well after transplantation, and the hospitalization period was 21 d.

DISCUSSION

Polycystic liver disease is a rare, benign disorder[2].

Symptoms of polycystic liver disease are related mainly to the size of the liver. Patients with massive hepatomegaly may suffer from abdominal pain, vena caval obstruction, hypertension, hemorrhage, cyst infections, dyspnea, increased abdominal girth, and poor quality of life[3-6]. Cyst fenestration can be used to ameliorate the symptoms of massive hepatomegaly, but symptom relief is only temporary. Previous studies have shown that cyst aspiration, liver or combined liver-kidney transplantation may be a valuable surgical technique for the treatment of polycystic liver disease[1,7]. The first patient to undergo liver transplantation for polycystic liver-kidney disease was in 1988, however, the patient died intraoperatively from intractable bleeding[1].

In the present case, resection of massive hepatomegaly was very difficult, because the enlarged polycystic liver occupied nearly the whole epigastric zone and mid abdomen, therefore, the surgical space was very small, especially for manipulation of the hepatic veins and short hepatic veins at the posterior of the liver. During transplantation, cyst fenestration was used, but the volume of the liver could not be reduced significantly. Thus, after intercepting the portal vein and the vena cava, left hepatectomy was performed, followed by isolation of the hepatic veins and short hepatic veins, and finally, right hepatectomy. OLT was performed successfully. Left liver resection may be an effective treatment of choice, if the space is too small for manipulation of liver blood vessels behind the liver.

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Association of hepatitis C virus infection and diabetes

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Abstract

Epidemiologic studies have suggested a relation between hepatitis C virus (HCV) infection and diabetes mellitus. HCV infection is emerging as a metabolic disease, and diabetes mellitus as a risk factor for HCV infection. However, some data on the prevalence of antibodies to HCV in patients with diabetes are conflicting. These seroprevalence data should be interpreted with caution. Some potential bias may occur in those clinic-based studies that target a specific disease group. In this letter we explain some reasons for these conflicting studies.

Key words: Prevalence; Hepatitis C; Diabetes mellitus

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TO THE EDITOR

We read with great interest the article of Kaabia and collaborators[1] and we congratulate them for the originality of this interesting study in the Maghreb area. In this study, Kaabia and colleagues performed hepatitis C virus (HCV) screening in 1269 diabetic patients and 1315 non-diabetic patients, attending in the same health centers in Sousse, Tunisia. Authors found that the frequency of HCV antibodies was low in diabetic patients and in the control group, with no significant difference between the groups (1.3% *vs* 0.6%, $P = 0.057$).

The results of this study do not match those of several studies performed in numerous areas in the world, which found a higher prevalence of hepatitis C in diabetic patients[2,3]. Moreover, in Kaabia's study, anti-HCV seroprevalence was significantly higher in type 2 diabetes sub-group than in the control group (1.4% *vs* 0.6%, $P = 0.04$). Anti-HCV seropositivity was detected only in one patient of 121 patients with type 1 diabetes, which was lower than in type 2 diabetes group, but the difference was not statistically significant (0.82% *vs* 1.4%, $P = 0.50$).

Kaabia's study has several deficiencies; their results must be interpreted with precaution. The diabetic patients were more aged than non-diabetic patients, it will be more interesting if the authors have compared the HCV sero-prevalence between the two groups with age adjustment. Another bias of selection was introduced in this study by using the prevalence and not the incidence of HCV infection. Moreover, diabetes is an independent co-factor of fibrosis in chronic hepatitis C[4], and in patients with cirrhosis the survival rate is reduced in case of associated diabetes[5]. The hepatitis C prevalence is underestimated than in diabetic patients. Classification errors of diabetes could be made in the group of non-diabetics. Indeed the manner in which the diagnosis of diabetes was eliminated in this group was not specified in the article. Starting from the assumption that 50% of diabetics are not diagnosed, we wonder in which group the new cases of screening diabetes were classified ? It is also impossible to establish a chronological relation between the diabetes and hepatitis C in this transversal study and it is possible that the infection by virus C precedes the occurring of diabetics. The authors have not compared the risk factors of hepatitis C in infected diabetic patients and in non-infected diabetic patients. Several studies found that diabetic patients infected with HCV had the same frequency in drug-addiction past and in blood transfusion than diabetic patients not infected with HCV. And these factors when they exist are present before the occurring of diabetes[6].

Our group presented in 2006 in the ALFEDIAM Congress in Paris a study suggesting that the prevalence of hepatitis C is higher in Algerian diabetic patients[7]. In

this retrospective study, we investigated hepatitis C virus markers in 739 patients attending internal medicine department of university hospital center of Batna (Algeria) from January to December 2005. One hundred and fifty nine patients (73 men and 86 women) with diabetes mellitus diagnosed by conventional criteria[8] were studied. Their mean age was 60 years. Type 2 diabetes was present in 90% of patients. The control group consisted of 580 non-diabetic patients (229 men and 351 women). Their mean age was 50 years. Anti-HCV serology was determined in both groups using the third-generation micro-particle enzyme immunoassay. Anti-HCV sero-positivity was 17.5% in diabetic patients and 8.4% in non-diabetic patients ($P < 0.01$). Despite our small size sample, we found a statistically significant higher prevalence of hepatitis C among diabetic patients. However, after adjustment for age, this difference is statistically significant only in patients aged between 40 and 65 years (22.2% *vs* 9.3%, $P = 0.024$).

Is diabetes mellitus a risk factor for HCV infection; or is this later a risk factor for type 2 diabetes mellitus? That is the question. Further studies are required to elucidate the mechanism of this interesting association.

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Meetings

Events Calendar 2009

January 12-15, 2009
Hyatt Regency San Francisco, San Francisco, CA
Mouse Models of Cancer

January 21-24, 2009
Westin San Diego Hotel, San Diego, CA
Advances in Prostate Cancer Research

February 3-6, 2009
Carefree Resort and Villas, Carefree, AZ (Greater Phoenix Area)
Second AACR Conference
The Science of Cancer Health Disparities in Racial/Ethnic Minorities and the Medically Underserved

February 7-10, 2009
Hyatt Regency Boston, Boston, MA
Translation of the Cancer Genome

February 8-11, 2009
Westin New Orleans Canal Place, New Orleans, LA
Chemistry in Cancer Research: A Vital Partnership in Cancer Drug Discovery and Development

February 13-16, 2009
Hong Kong Convention and Exhibition Centre, Hong Kong, China
19th Conference of the APASL
http://www.apasl2009hongkong.org/en/home.aspx

February 27-28, 2009
Orlando, Florida
AGAI/AASLD/ASGE/ACG Training Directors' Workshop

February 27-Mar 1, 2009
Vienna, Austria
EASL/AASLD Monothematic: Nuclear Receptors and Liver Disease
www.easl.ch/vienna2009

March 13-14, 2009
Phoenix, Arizona
AGAI/AASLD Academic Skills Workshop

March 20-24, 2009
Marriott Wardman Park Hotel
Washington, DC
13th International Symposium on Viral Hepatitis and Liver Disease

March 23-26, 2009
Glasgow, Scotland
British Society of Gastroenterology (BSG) Annual Meeting
Email: bsg@mailbox.ulcc.ac.uk

April 8-9, 2009
Silver Spring, Maryland
2009 Hepatotoxicity Special Interest Group Meeting

April 18-22, 2009
Colorado Convention Center, Denver, CO
AACR 100th Annual Meeting 2009

April 22-26, 2009
Copenhagen, Denmark
the 44th Annual Meeting of the European Association for the Study of the Liver (EASL)
http://www.easl.ch/

May 17-20, 2009
Denver, Colorado, USA
Digestive Disease Week 2009

May 29-June 2, 2009
Orange County Convention Center
Orlando, Florida
45th ASCO Annual Meeting
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May 30, 2009
Chicago, Illinois
Endpoints Workshop: NASH

May 30-June 4, 2009
McCormick Place, Chicago, IL
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June 17-19, 2009
North Bethesda, MD
Accelerating Anticancer Agent Development

June 20-26, 2009
Flims, Switzerland
Methods in Clinical Cancer Research (Europe)

June 24-27 2009
Barcelona, Spain
ESMO Conference: 11th World Congress on Gastrointestinal Cancer
www.worldgicancer.com

June 25-28, 2009
Beijing International Convention Center (BICC), Beijing, China
World Conference on Interventional Oncology
http://www.chinamed.com.cn/wcio2009/

July 5-12, 2009
Snowmass, CO, United States
Pathobiology of Cancer: The Edward A. Smuckler Memorial Workshop

July 17-24, 2009
Aspen, CO, United States
Molecular Biology in Clinical Oncology

August 1-7, 2009
Vail Marriott Mountain Resort, Vail, CO, United States
Methods in Clinical Cancer Research

August 14-16, 2009
Bell Harbor Conference Center, Seattle, Washington, United States
Practical Solutions for Successful Management
http://www.asge.org/index.aspx?id=5040

September 23-26, 2009
Beijing International Convention Center (BICC), Beijing, China
19th World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists (IASGO)
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September 27-30, 2009
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Frontiers in Basic Cancer Research

October 13-16, 2009
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Advances in Breast Cancer Research: Genetics, Biology, and Clinical Applications

October 20-24, 2009
Versailles, France
Fifth International Conference on Tumor Microenvironment: Progression, Therapy, and Prevention

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AACR-NCI-EORTC Molecular Targets and Cancer Therapeutics

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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.

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15   Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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