INTRODUCTION

The incidence of complications associated with living donor liver transplantation (LDLT) is known to be greater than that associated with deceased donor liver transplantation (DDLT)[1-4]. In the patients who undergo LDLT, the incidence of complications, including mild complications, during the perioperative period can be as high as 82.8%. In particular, the rate of development of biliary complications after LDLT is twice that after DDLT[1]. Moreover, patients who undergo LDLT often have inadequate liver function because of the limited volume of the graft. Therefore, the management of complications is very difficult, and the mortality rate in critical cases is high.

Compared to other abdominal surgery, pancreatic fistula is a quite rare complication after LDLT[1-5], but it is theoretically possible because LDLT involves surgery in the area surrounding the portal vein, the pancreas, and the spleen. Pancreatic fistula causes hemorrhage, abscess, etc., and may result in the death of the patient[6-8]. Only 1 previous study has reported 2 cases of leakage of pancreatic fluid after liver transplantation. In those cases, leakage of a mixture of pancreatic fluid and bile was observed at the anastomosis site of the bile duct after DDLT[9]. However, there is no clear consensus on the management of pancreatic fistula after liver transplantation. Generally, conservative therapy is the first line of treatment, and surgery is performed only when the patient does not respond to conservative therapy[6-8]. Recently, however, endoscopic treatment has attracted more attention because it is less invasive than surgical treatment. We present 2 cases in which endoscopic treatment was effective against refractory pancreatic fistulas that developed after LDLT.

CASE REPORT

Case 1

A 61-year-old woman underwent LDLT for primary biliary cirrhosis; the graft was obtained from the left liver lobe of her son. Abdominal computed tomography (CT) performed on postoperative day (POD) 7 revealed a portal vein thrombus; therefore, urgent exploratory laparotomy was performed. The thrombus was believed to have been induced by reduced portal blood flow, which was caused by splenorenal steal by an artificial shunt. After removing the thrombus, portal vein reconstruction was performed by using the right external iliac vein, and this procedure was followed by splenectomy. To increase the portal blood flow, a splenorenal shunt was ligated. The main pancreatic duct on the dorsal side of the pancreas was injured at the time of hemostatic manipulation; however, this injury was not identified immediately. CT performed on POD 13 revealed a hematoma at the lower edge of the pancreas. CT on POD 21 revealed that the hematoma under the pancreas had decreased in size (Figure 1). The amylase level of the drainage fluid was 22 690 IU/L; therefore, the hematoma at the inferior edge of the pancreas was considered to have ruptured because of a pancreatic leak. Another CT examination revealed fluid collection in the mesentery on the ventral side of the upper pole of the left kidney; therefore, open drainage was performed. To this end, a drain was placed at the tail of the pancreas and administration of octreotide was started. However, the leakage of pancreatic fluid from the drain did not stop. After several days, the patient's general status stabilized, but surgical treatment for pancreatic fistula was still unsafe because of inadequate liver function. Therefore, the patient was discharged on POD 128 with the drain in place and was followed up. The patient was in a stable state at discharge. However, on POD 318, she was readmitted to our department because of fever. Examinations revealed that the drain at the tail of the pancreas had deviated and that the patient had developed liver necrosis, supposedly because of contact with the drain. On POD 320, we repositioned the drain by using a fluoroscope. A contrast test performed at that time revealed that the main pancreatic duct was completely disrupted (Figure 2). The patient was diagnosed with refractory pancreatic fistula, and an endoscopic naso-pancreatic drainage (ENPD) tube was inserted to the proximal side of the leakage on POD 331 (Figure 3); this procedure resulted in a remarkable decrease in drain output (Figures 4 and 5). The ENPD tube was removed on POD 368, and the drains at the tail of the pancreas were removed on POD 371. The patient was discharged on POD 375 without abnormal fluid collection around the pancreas (Figure 6). The patient is well without the recurrence of pancreatic fistula up to this time.

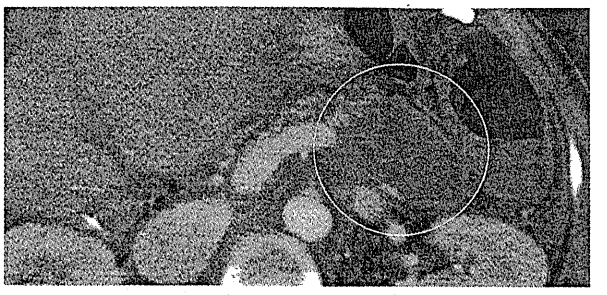

Figure 1 Computed tomography performed on postoperative day 13 showing fluid collection (circle) at the lower edge of the pancreas. The hematoma was considered to be ruptured.

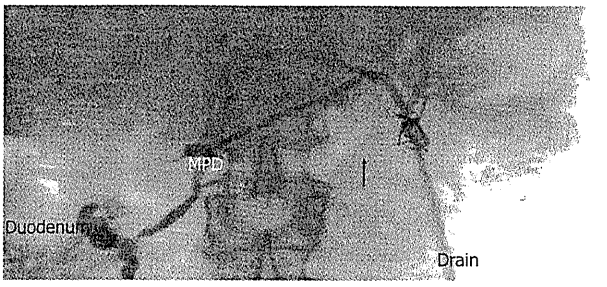

Figure 2 Pancreatographic examination of the drain at the tail of the pancreas (Drain) in case 1 reveals the disrupted (arrow) main pancreatic duct with flow of contrast into the duodenum. MPD: Main pancreatic duct.

Case 2

A 58-year-old man underwent LDLT for cirrhosis C; the graft was obtained from the right liver lobe of his daughter. Because the portal vein was occluded by a thrombus,

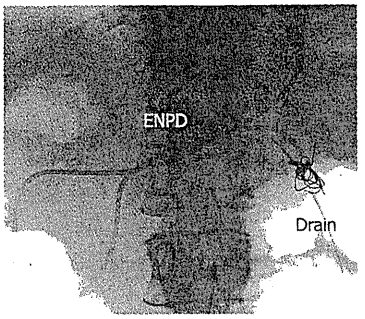

Figure 3 A radiograph showing postprocedure endoscopic naso-pancreatic drainage in case 1. Excellent drainage of the pancreatic duct is noted. ENPD: Endoscopic naso-pancreatic drainage.

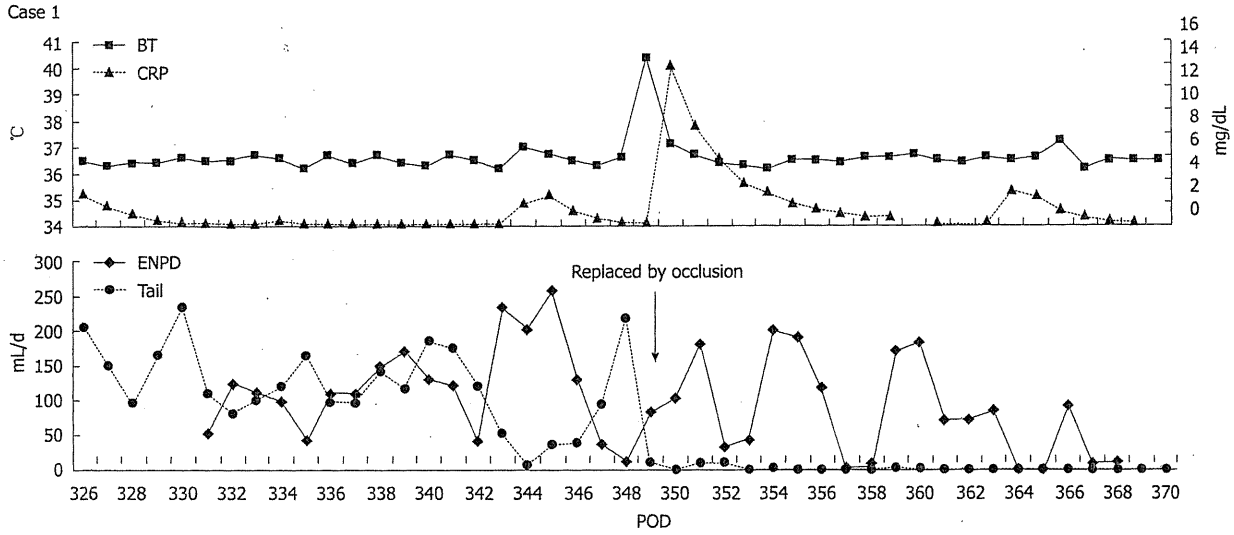

Figure 4 Upper chart shows the body temperature and serum C-reactive protein level. Lower one shows daily output of the endoscopic naso-pancreatic drainage tube and the drain at the tail of the pancreas (Tail) in case 1. The patient had an episode of fever caused by the occlusion of the endoscopic naso-pancreatic drainage (ENPD) tube. After the tube was replaced, the pancreatic fistula healed completely. BT: Body temperature; CRP: C-reactive protein; POD: Postoperative day.

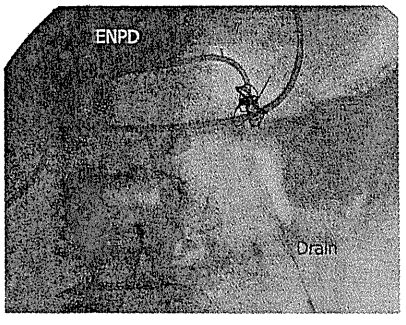

Figure 5 Contrast examination from the drain at the tail of the pancreas (Drain) in case 1 on postoperative day 363 reveals the closure of fistula. ENPD: Endoscopic naso-pancreatic drainage.

Figure 6 Computed tomography performed 2 d after removal of the drain in case 1 showing no fluid collection around the pancreas.

the portal and splenic veins were stripped off from the surrounding tissue and were exposed in order to remove the thrombus. However, the upper edge of the pancreatic head was injured during this process. The amylase level measured at the upper edge of the pancreatic drain was high on POD 1; therefore, the patient received octreotide on POD 2. On POD 5, the patient showed high fever and acute peritonitis. Therefore, an emergency exploratory laparotomy was performed. Because fluid collection was observed around the pancreas, drains were placed at both the right and left edges of the pancreas. On POD 21, the total output from the drain was 460 mL/d, and the amylase level of the drainage fluid was 166 700 IU/L. The patient was

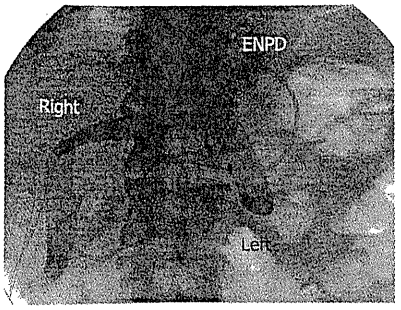

Figure 7 A radiograph showing postprocedure endoscopic naso-pancreatic drainage in case 2. The image shows drains placed at both the right and left edges of the pancreas (Right and Left) and endoscopic naso-pancreatic drainage (ENPD).

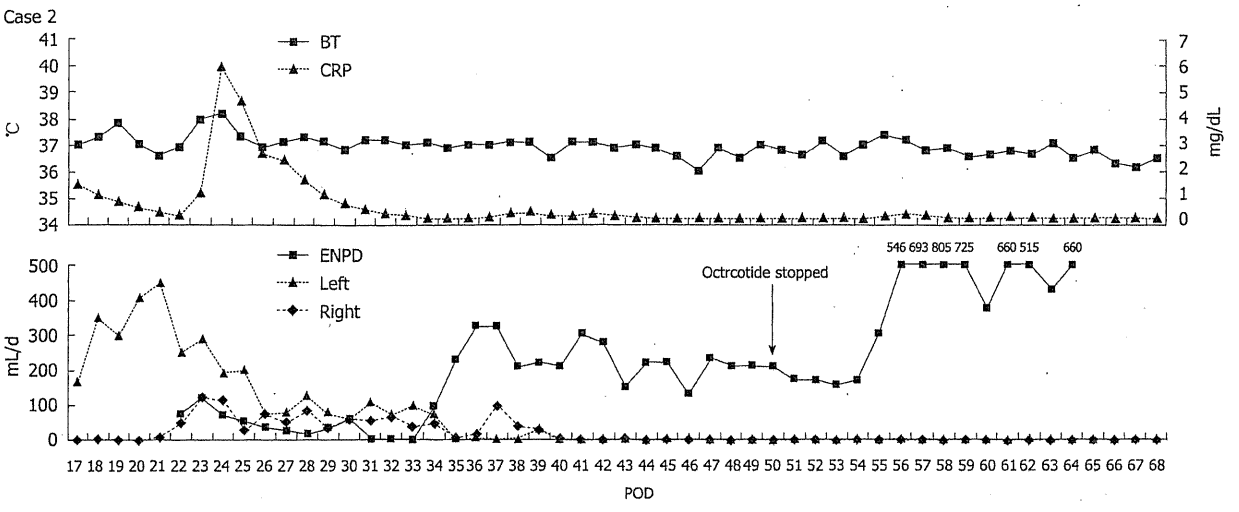

Figure 8 Upper chart shows the body temperature and serum C-reactive protein level. Lower one shows daily output of endoscopic naso-pancreatic drainage (ENPD) tube and the drains at both the edges of the pancreas (Right and Left) in case 2. The pancreatic fistula healed completely on post-ENPD day 38. BT: Body temperature; CRP: C-reactive protein; POD: Postoperative day.

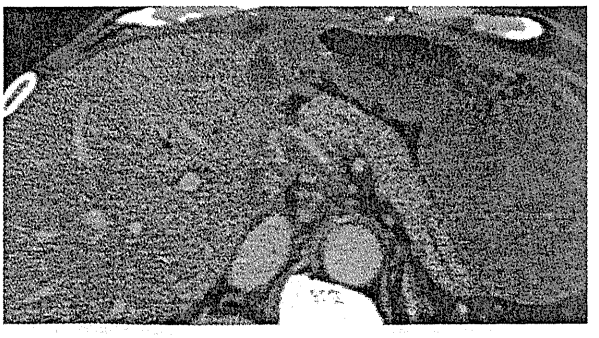

Figure 9 Computed tomography performed 14 d after removal of the drains in case 2 showing no fluid collection around the pancreas.

diagnosed with high-output pancreatic fistula, and ENPD was performed on POD 22 (Figure 7). The drain output decreased very rapidly (Figure 8); therefore, the patient was allowed to consume solid foods on POD 49, and octreotide administration was stopped on POD 50. The ENPD tube was removed on POD 65, and the drains placed at the right and left edges of the pancreas were removed on POD 68 and POD 70, respectively. The patient was discharged on POD 100 without abnormal fluid collection around the pancreas (Figure 9). The patient is well and receiving regular out-patient treatment and is showing no recurrence of pancreatic fistula.

DISCUSSION

Pancreatic fistula is primarily treated by conservative therapy, which includes rapid total infusion or enteral nutrition along with administration of octreotide. The recovery rate after conservative therapy ranges from 44% to 85%[6-8]; thus, a number of cases are not resolved by conservative treatment. Surgical treatment has been performed in such cases. However, surgical treatment is highly invasive and may lead to various complications. Further, surgical treatment is associated with high mortality rates, with the mortality rate being as high as 23%-67% in the cases showing early peritonitis after the operation[9]. Endoscopic drainage of the main pancreatic duct *via* the ampulla of Vater, which was first reported in 1991[10], has drawn considerable attention. Boerma *et al*[11] (2006) reported an excellent recovery rate (87%) after endoscopic treatment of 15 cases of pancreatic fistula. In addition, other studies

have reported recovery rates of about 58%-100% in the cases of pancreatic fistulas that do not respond to conservative therapy and involve endoscopic treatment[12-20]. To date, only 1 death caused by acute pancreatitis has been reported. However, since this death may also have been caused by inadequate drainage, a direct relationship between the death and endoscopic treatment could not be confirmed[13]. Unlike LDLT, endoscopic treatment for pancreatic fistula allows greater accessibility to the ampulla of Vater. Further, endoscopic treatment is less invasive than surgical treatment; therefore, it can easily replace conservative therapy if sufficient drainage is achieved. Thus, patients who undergo endoscopic treatment for pancreatic fistula can be expected to make an early recovery. Irrespective of their merits and demerits, both ENPD and endoscopic pancreatic stenting (EPS) have been referred to in the reports. ENPD causes a sense of discomfort in the pharynx; however, this technique enables easy diagnosis of occlusion and dropout because it allows monitoring of the pancreatic fluid. In contrast, in EPS the diagnosis of occlusion and dropout is difficult; however, this technique causes no sense of discomfort in the pharynx. We selected ENPD to enable safe monitoring of 2 channels of drainage: the endoscopic retrograde pancreatic drain as well as the intraperitoneal drain. In case 1, the drain tube had to be replaced because of the fever caused by occlusion; therefore, the choice of ENPD was considered to be reasonable. The patient in case 1 could have recovered earlier if the endoscopic treatment for pancreatic fistula had been initiated earlier. In each case, the patient recovered within approximately 40 d after ENPD. Further, the treatment had no influence on the patients' general status. Endoscopic treatment is considered to be safe for treating pancreatic fistulas that develop after LDLT. New endoscopic techniques, such as ultrasonography (US)-guided drainage, have also been used to treat refractory cases that do not respond to drainage via the ampulla of Vater; however, only few reports have described these techniques. These new techniques may also be less invasive than surgical treatment[21-22].

In conclusion, we described 2 cases of pancreatic fistula after LDLT that were not responsive to conservative therapy. In each case, the patient recovered within approximately 40 d after ENPD. Thus, endoscopic treatment for pancreatic fistula after LDLT should be adopted because of its high recovery rate and low invasiveness.

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Association of *IL28B* gene polymorphism with development of hepatocellular carcinoma in Japanese patients with chronic hepatitis C virus infection

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ABSTRACT

IL28B single nucleotide polymorphisms (SNPs) are associated with spontaneous and treatment-induced elimination of hepatitis C virus (HCV). To assess whether the *IL28B* rs8099917 SNP also affects the progression of chronic HCV infection, we genotyped 511 Japanese HCV patients, including 69 with hepatocellular carcinoma (HCC). The T/T genotype of rs8099917 was not associated with the development of HCC (p = 0.623), although stepwise logistic regression analysis showed that liver cirrhosis, age greater than 68 years, and serum albumin <4.2 mg/dl were associated with HCC onset. It appears that the *IL28B* SNP does not directly influence hepatocarcinogenesis in chronic HCV infection.

1. Introduction

Nearly 2 million individuals in Japan and 170 million persons worldwide are infected with the hepatitis C virus (HCV). Persistent infection with HCV causes chronic hepatitis and eventually leads to liver cirrhosis and/or hepatocellular carcinoma (HCC) [1]. HCC is one of the major causes of cancer-related death, and patients with HCV-associated HCC account for 70–80% of HCC cases in Japan [2]. It is generally considered that several factors, including viral, host, and environmental elements, are involved in the development of HCV-associated HCC. Recently, single nucleotide polymorphisms (SNPs) around the *IL28B* gene have been identified as strong predictors of spontaneous [3] and treatment-induced HCV clearance (reviewed by Balagopal et al. [4]). Although such studies have confirmed an association of *IL28B* SNPs with the outcome of antiviral therapy, only a few have investigated whether this genetic variant also affects the progression of chronic HCV infection [5–7]. Furthermore, it remains unclear whether *IL28B* SNPs influence the development of HCV-associated HCC. The aim of the present cross-sectional study was to determine the association between the *IL28B* rs8099917 SNP and HCC onset in Japanese patients with chronic HCV infection.

2. Subjects and methods

2.1. Study subjects

A total of 511 consecutively treated Japanese patients with chronic HCV infection who were seen at Shinshu University Hospital (Matsumoto, Japan) between April 2004 and December 2010 were enrolled. The diagnosis of chronic hepatitis C was based on the following criteria: (1) the presence of both anti-HCV antibody and HCV RNA in the serum for at least 6 months; (2) the absence of detectable hepatitis B surface antigen and antibody to the human immunodeficiency virus; and (3) the exclusion of other causes of chronic liver disease, including Wilson's disease and hemochromatosis. Liver cirrhosis was diagnosed by histologic examination and/or characteristic clinical signs of advanced liver disease. HCC was diagnosed by histologic examination and/or imaging studies [8]. Hypertension was defined according to the Seventh Joint National Committee on the Prevention, Detection, Evaluation, and Treatment of Hypertension. Diabetes mellitus was defined according to the American Diabetes Association diagnostic criteria. All participants provided written informed consent for this study, which was approved by our institutional ethics committee.

Antibodies to HCV were measured in serum samples via third-generation enzyme immunoassay (EIA; Abbott Laboratories, North Chicago, IL). Serum HCV RNA was determined using Cobas Amplicor assays (sensitivity 50 IL/ml; Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes were determined using INNO-LiPA HCV II assays (Innogenetics, Ghent, Belgium). Alanine aminotransferase,

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albumin, and other relevant blood and biochemical tests were performed using standard methods [9].

Among the 511 patients, 306 (59.9%) underwent interferon (IFN)–based therapy before the study period (Table 1). Of these, 238 were treated with pegylated (PEG) IFN and ribavirin combination therapy, and 75 were treated with either PEG-IFN or IFN monotherapy. A sustained virological response (SVR) was defined as undetectable serum HCV RNA 24 weeks after the discontinuation of IFN-based therapy.

2.2. Genotyping of IL-28 SNP (rs8099917)

Genomic DNA from patients was isolated by phenolic extraction of sodium dodecyl sulfate-lyzed and proteinase K–treated cells and adjusted to 10–15 ng/μl. Genotyping of the rs8099917 SNP was performed using the ABI TaqMan allelic discrimination kit and an ABI 7500 Real Time PCR System (Applied Biosystems) [10].

2.3. Statistical analysis

Statistical analysis of data was performed using StatFlex version 6 software (Artech, Osaka, Japan). Continuous variables were presented as the median (range) and were analyzed using the Mann–Whitney *U* test. Categorical variables were presented as frequency (%) and were analyzed using the χ^2 test with the Yates correction. Fisher's exact probability test was used for groups with fewer than five samples. The model was checked by regression diagnostic plots to verify normality, linearity of the data, and constant variance. Stepwise logistic regression analysis with a forward approach was performed to identify independent factors associated with HCV-induced HCC after continuous variables were separated into two categorical variables by each median. A p value of less than 0.05 was considered statistically significant.

3. Results

The demographic, virological, and clinical characteristics of our cohort are summarized in Table 1. Most patients (76.7%) were infected with HCV genotype 1b, and the majority (76.3%) had chronic hepatitis rather than liver cirrhosis (23.7%). Of the patients, 45 (8.8%) and 126 (24.7%) had diabetes mellitus and hypertension, respectively. A total of 90 patients (17.6%) had a history of regular alcohol intake of more than 20 g/day. The median follow-up period between the first visit and final follow up was 6.9 years. Among the 511 patients studied for the *IL28B* rs8099917 SNP, 365 had the T/T genotype (71.4%), 139 had T/G (27.2%), and 7 had G/G (1.4%). Of the 392 patients infected with HCV genotype 1b, 273 had the T/T genotype (69.6%), 113 had T/G (28.8%), and 6 had G/G (1.6%). Of the 119 patients infected with HCV genotype 2, 92 had the T/T genotype (77.3%), 26 had T/G (21.8%), and 1 had G/G (0.9%). With regard to gender, 176 male patients had the T/T genotype (74.3%), 57 had T/G (24.1%), and 4 had G/G (1.6%). In comparison, 189 female patients had the T/T genotype (69.0%), 82 had T/G (29.9%), and 3 had G/G (1.1%). There were no significant differences related to genotype ($p = 0.359$) or gender ($p = 0.393$). There were also no remarkable differences found between the groups with clinical liver cirrhosis (LC) and non-LC (73%, 87 of 120 vs 71%, 274 of 384; $p = 0.808$) among patients with the T/T or T/G or G/G genotypes.

Table 1
Demographic, virological, and clinical characteristics of 511 patients with chronic HCV infection

Characteristic		Range
Median age (y)	68	(18–96)
Gender (male/female)	237/274	
HCV genotype 1/2	392/119	
Median BMI (kg/m^2)	22.2	(15.8–32.3)
Clinical state, n (%)		
CH without HCC	386 (75.5)	
LC without HCC	56 (11.0)	
HCC	69 (13.5)	
IFN therapy, n (%)	306 (59.9)	
Diabetes mellitus, n (%)	45 (8.8)	
Hypertension, n (%)	126 (24.7)	
Alcohol intake, n (%)	90 (17.6)	
Median serum value		
Albumin (mg/dl)	4.2	(1.5–4.8)
ALT (IU/L)	25	(3–199)
AST (IU/L)	31	(3–237)
GGTP (IU/L)	22	(7–295)
PLT (10^4/μl)	16.0	(1.0–43.1)
Median follow-up (y)	6.9	(0.2–41.6)
IL28B SNPs (rs8099917)		
T/T/T/G/G/G	365/139/7	
Clinical state of LC	87/33/0	

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; LC, liver cirrhosis; PLT, platelet count.

The clinical features of patients with and without HCC are shown in Table 2. Patients with HCC were significantly older and had a higher frequency of liver cirrhosis than those without HCC (both $p < 0.0001$). HCC development in patients with HCV genotype 1b was higher than in those with genotype 2a or 2b ($p = 0.03$). Patients with HCC who had been treated with IFN-based therapy were statistically fewer than those without ($p < 0.0001$), as was the case for SVR rate ($p < 0.0001$). More HCC patients had diabetes mellitus and hypertension than patients without HCC ($p = 0.002$ and $p = 0.003$, respectively). These patients also showed statistically higher alcohol consumption ($p = 0.020$). Regarding laboratory findings, albumin and platelet levels were significantly lower and alanine aminotransferase, aspartate aminotransferase, and γ-glutamyl transpeptidase levels were significantly higher in patients with HCC than in those without. However, there was no significant difference between HCC and non-HCC groups (74%, 51 of 69 vs 71%, 314 of 442; $p = 0.623$) among patients with the T/T genotype and those with the T/G or G/G genotypes. There was also no significant difference between the groups according to allele frequencies (T vs G; $p = 0.495$). We further analyzed the IL-28B SNP among 205 patients who had had never received IFN-based therapy. No significant association was found between HCC and non-HCC groups (74%, 34 of 46 vs 71%, 113 of 159; $p = 0.701$) among patients with the T/T genotype or the T/G or G/G genotypes.

We used a stepwise logistic regression model to determine the relationship between the above-described explanatory factors and complicating HCC in HCV patients. In agreement with the linear model, significant variables were found for liver cirrhosis (odds ratio [OR] = 57.3; $p < 0.0001$), age greater than 68 years (OR = 3.9; $p = 0.0018$), and serum albumin <4.2 mg/dl (OR = 3.6; $p = 0.021$). *IL28B* gene polymorphisms had no effect on the estimated risk of complication with HCC.

4. Discussion

The allelic frequency of the *IL28B* rs8099917 T/T polymorphism was significantly associated with treatment response in patients with chronic hepatitis C [3,4]. However, the influence of *IL28B* polymorphisms on the severity and progression of liver disease remains unclear; it was reported that the rs8099917 T/T genotype was associated with inflammatory activity and fibrosis in patients with chronic hepatitis C in Japan [5], and Fabris et al. reported that this polymorphism was significantly associated with development of HCC in the patients with liver cirrhosis, regardless of etiology [6]. Conversely, Marabita et al., in an Italian prospective study, found that the host genetic background at the *IL28B* locus was not associated with the risk of developing advanced fibrosis [7]. In the current study, the frequency of patients with the rs8099917 T/T genotype did not differ between HCC and non-HCC groups, suggesting that the *IL28B* SNP may play little or no role in HCC development among Japanese HCV patients.

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Table 2
Clinical characteristics of patients with with chronic HCV infection, with and without HCC

Characteristic	With HCC (n = 69)	Without HCC (n = 442)	p
Median age (y)	77	66	<0.0001
Gender (male/female)	36/33	201/241	0.299
Clinical state at LC, n (%)	65 (94.2)	56 (12.7)	<0.0001
HCV genotype 1/2	60/9	332/110	0.030
Median BMI (kg/m^2)	22.4	22.2	0.124
Treatment			
IFN therapy, n (%)	23 (33.3)	283 (64.0)	<0.0001
SVR, n (%)	6 (8.7)	176 (39.8)	<0.0001
Diabetes mellitus, n (%)	13 (18.8)	32 (7.2)	0.002
Hypertension, n (%)	27 (39.1)	99 (22.4)	0.003
Alcohol intake, n (%)	19 (27.5)	71 (16.1)	0.020
Median serum value			
Albumin (mg/dl)	3.4	4.3	<0.0001
ALT (IU/L)	40	27	0.0005
AST (IU/L)	56	29	<0.0001
GGTP (IU/L)	37	24	0.0003
PLT (10^4/μl)	9.6	16.4	<0.0001
IL28B SNPs (rs8099917)			
T/T/T/G + G/G	51/18	314/128	0.623

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; GGTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IFN, interferon; LC, liver cirrhosis; PLT, platelet count; SVR, sustained virological response.

Stepwise logistic regression analysis confirmed that liver cirrhosis, advanced age (>68 years), and low serum albumin (<4.2 mg/dl) were significant predictors of HCC. These risk factors agreed with findings in earlier studies [5,11–13]. No associations of *IL28B* SNPs with HCC development were found.

It may be considered that some HCV patients in our study who were complicated with HCC did not achieve an SVR because they were untreatable with IFN-based therapy. Accordingly, clinicians may opt for early therapeutic intervention in patients with HCV who possess traits that are positively associated with an SVR, such as *IL-28B* polymorphisms. Furthermore, elderly patients should be carefully monitored for HCC once liver cirrhosis is detected, regardless of *IL-28B* genotype.

Recently, two genome-wide association studies from different Japanese groups showed that an SNP in the 5′ flanking region of the MHC class I polypeptide-related sequence A on chromosome 6 [14] and an SNP within isoform 1 in the DEP domain-containing protein 5 on chromosome 22 [15] were associated with HCC risk in HCV patients. Further association studies of such gene polymorphisms are needed both in Japan and abroad.

In summary, our findings showed that the *IL28B* rs8099917 genotype did not affect the development of HCC in Japanese patients with chronic HCV infection, whereas liver cirrhosis, advanced age, and low serum albumin were confirmed to influence disease progression.

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Original Article

Serum chemokine levels are associated with the outcome of pegylated interferon and ribavirin therapy in patients with chronic hepatitis C

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Aim: Serum chemokine levels and amino acid substitutions in the interferon-sensitivity determining region (ISDR) and core region have been associated with treatment outcome of pegylated interferon and ribavirin therapy in genotype 1 hepatitis C virus (HCV)-infected patients. The present study was conducted to clarify the association between serum chemokines and treatment outcome in patients with chronic HCV-1 infection in a Japanese cohort.

Methods: A total of six serum chemokines were quantified before, during and after pegylated interferon and ribavirin treatment in 79 genotype 1 chronic HCV patients using a multiple bead array system. Viral ISDR and core region variants were determined by direct sequencing.

Results: The baseline serum levels of eotaxin, IP-10 and RANTES were significantly higher in chronic HCV patients than in controls. High levels of eotaxin and macrophage inflammatory protein (MIP)-1β before therapy and more than two mutations in the ISDR were associated with a sustained virological response, and patients with more than two mutations in the ISDR also had significantly higher MIP-1β levels. Receiver–operator curve analysis showed a 77% sensitivity and 73% specificity for predicting an SVR using MIP-1β values.

Conclusion: Serum MIP-1β levels may predict the response to HCV treatment with pegylated interferon and ribavirin and are associated with amino acid substitutions in the ISDR.

Key words: chemokines, core, interferon sensitivity determining region, MIP-1β, pegylated interferon, ribavirin

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is a major cause of chronic liver disease that leads to liver cirrhosis and/or hepatocellular carcinoma (HCC).[1] HCC is ranked fourth in men and fifth in women as a cause of death from malignant neoplasms in Japan.[2,3] Interferon (IFN)-based therapy can achieve HCV eradication and decrease the risk of HCC to improve prognosis; with pegylated (PEG) IFN and ribavirin therapy, approximately 50% of patients with genotype 1 HCV infection achieve a sustained virological response (SVR).[4,5]

Chemokines and their receptors play an important role in the pathogenesis of HCV infection.[6,7] Despite the growing amount of published research supporting the complex interactions of these inflammatory biomarkers in the outcome of antiviral therapy, the majority of recent studies have nearly exclusively concentrated on only one or a few markers. Thus, it is possible that a test evaluating several biomarkers may prove to be of greater value in predicting responses to therapy.

In the present study, we sought to determine the levels of six chemokines in patients with chronic HCV-1b

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infection who underwent treatment with PEG IFN and ribavirin using a broad-spectrum bead-based multiplex immunoassay.

METHODS

Subjects

A TOTAL OF 79 treatment-naïve patients with chronic hepatitis C (40 men and 39 women; median age 60 years [range: 17–74]) were seen at Shinshu University Hospital or its affiliated hospitals in the Nagano Interferon Treatment Research Group. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyl transpeptidase (γ-GTP) were tested using standard methods.[8] All patients, who were infected with genotype 1b HCV, received PEG IFN-α-2b (PegIntron; Schering-Plough, Tokyo, Japan; 1.5 μg/kg of bodyweight) and ribavirin (Rebetol; Schering-Plough; 600–1000 mg/day) adjusted to bodyweight for 48 weeks as described previously.[9] The pretreatment median value for ALT was 54 IU/L (range: 22–389), AST was 44 IU/L (range: 20–288) and HCV RNA was 1700 KIU/mL (range: 11–5100), as measured by COBAS AMPLICOR assays (Roche Diagnostic Systems, Tokyo, Japan). A group of 26 healthy individuals (13 men and 13 women; mean age 54 years [range: 28–60]) with hepatitis B and C negative serologies and normal transaminases were used as the control. All patients and controls were negative for the antibody to HIV. The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine and all patients provided written informed consent. All serum samples were immediately stored at −70°C and remained in storage until testing.

Definition of treatment outcome

An SVR was concluded in those whose serum HCV RNA was undetectable 24 weeks after completing therapy. Post-treatment relapse was defined as the reappearance of HCV RNA in the serum after treatment in patients whose HCV RNA was undetectable during or at the completion of therapy. A non-response was defined as a decrease in HCV RNA to less than 2 log copies/mL at week 12 and detectable HCV RNA during the treatment course.

Detection of amino acid substitutions in core and interferon-sensitivity determining regions (ISDR)

The sequences of 1–191 amino acids (a.a.) in the core protein and 2209–2248 a.a. in the NS5A region of genotype 1b HCV were determined by direct sequencing using stored serum samples obtained before therapy, as reported previously. Nucleotide and a.a. sequences were compared with the nucleotide sequences of genotype 1b HCV-J.[10] Substitutions of a.a.70 arginine (Arg70) and glutamine (Gln70) or a.a.91 leucine (Leu91) and methionine (Met91)[11] and the number of a.a. substitutions in the ISDR were defined as wild-type (0), intermediate-type (1) and mutant-type (≥2).[12] Of the 79 patients, 75 were determined to have substitutions at a.a.70 and a.a.91, and 76 could be sequenced for their ISDR.

Detection of chemokines

Six chemokines (macrophage inflammatory protein [MIP]-1α, MIP-1β, eotaxin, IP-10, RANTES and interleukin [IL]-8) were quantified using Luminex Multiplex Cytokine Kits (Procarta Cytokine assay kit; Panomics, Fremont, CA, USA) from serum samples obtained before the start of treatment, 4 weeks after the start of treatment and 24 weeks after the completion of treatment, according to the manufacturer's instructions.[13]

Statistical analysis

The Mann–Whitney *U*-test and Kruskal–Wallis test were used to analyze continuous variables as appropriate. The Wilcoxon rank sum test and the Friedman test were used to evaluate changes in serum chemokine levels over time. Spearman's rank correlation coefficients were used to evaluate the relationship between each pair of markers. The χ^2-test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was less than 5, Fisher's exact test was used. $P \leq 0.05$ was considered significant. To predict treatment outcome, each cut-off point for continuous variables was determined by receiver–operator curve (ROC) analysis. Statistical analyses were performed using SPSS ver. 18.0J.

RESULTS

OF THE 79 patients receiving PEG IFN and ribavirin therapy, 31 (39%) achieved an SVR, 23 (29%) relapsed, and 25 (32%) did not respond to treatment and were termed null viral responders (NVR). When stratified into three groups based on treatment outcome, patients with an NVR had a higher female ratio ($P = 0.030$) (Table 1). Before treatment, the median AST and γ-GTP levels in the SVR group were significantly lower than those in the relapsed and NVR groups. Substitutions of a.a.70 in the core region

Table 1 Clinical characteristics of patients with chronic hepatitis C

Characteristic	SVR ($n = 31$)	TR ($n = 23$)	NVR ($n = 25$)	P
Mean age, years (range)	55 (28–72)	57 (17–71)	59 (22–74)	0.20
Sex, male : female	23:8	9:14	8:17	0.030
Mean values (range)				
ALT (IU/L)	58 (24–172)	76 (24–389)	90 (22–357)	0.43
AST (IU/L)	41 (21–133)	57 (20–218)	78 (25–288)	0.042
γ-GTP (IU/L)	40 (13–147)	47 (12–167)	81 (17–439)	0.027
HCV RNA (10^3 IU/mL)	1962 (110–>5100)	2379 (360–>5100)	1934 (220–>5100)	0.23
Substitutions				
Core a.a. 70 (Arg70/Gln70)	22/6	14/8	11/14	0.034
Core a.a. 91 (Leu91/Met91)	20/8	17/5	17/8	0.78
ISDR of NS5A (0–1/≥ 2)	20/9	20/2	23/2	0.040

a.a., amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; ISDR, interferon-sensitivity determining region; NVR, null virological response; SVR, sustained virological response; TR, transient response; γ-GTP, γ-glutamyl transpeptidase.

($P = 0.034$) and in the ISDR ($P = 0.040$) were both significantly associated with treatment outcome. Six serum chemokines were assessed before therapy in all patients and in 26 healthy controls, revealing that the median serum levels of eotaxin, IP-10 and RANTES were significantly higher in HCV-afflicted patients. The median serum IL-8 level in cases with chronic HCV infection was significantly lower compared with the control group (Table 2).

The median serum chemokines of our cohort are shown in Table 3. Before treatment, the median serum levels of three chemokines (eotaxin, MIP-1β and RANTES) were significantly higher in patients who achieved an SVR than in those who did not. Patients with a virological response had significantly higher MIP-1α (39.0 vs 25.9 pg/mL; $P = 0.001$) and MIP-1β (192.7 vs 110.0 pg/mL; $P < 0.001$) compared with non-responders.

Table 2 Serum chemokines in patients with chronic hepatitis C and healthy controls

Chemokine	Chronic hepatitis C ($n = 79$)	Control ($n = 26$)	P
MIP-1α	36.4 (2.4–5021.3)	34.6 (10.6–92.8)	0.46
MIP-1β	160.2 (14.4–3341.6)	122.3 (21.1–1677.6)	0.18
Eotaxin	100.0 (2.4–1296.0)	19.8 (18.3–25.0)	<0.001
IP-10	1642.8 (57.7–11 487.0)	31.1 (21.3–80.6)	<0.001
RANTES	31 755.3 (17.9–83 248.0)	3460.0 (191.5–40 001.0)	<0.001
IL-8	12.9 (2.4–6324.3)	41.8 (2.4–327.6)	<0.001

Data are expressed as median (interquartile range) values (pg/mL).
IL, interleukin; MIP-1, macrophage inflammatory protein-1.

Table 3 Serum chemokines in treatment outcome to antiviral therapy

Chemokine	SVR ($n = 31$)	TR ($n = 23$)	NVR ($n = 25$)
MIP-1α	36.4 (32.3–99.3)	39.0 (33.7–52.9)	25.9 (17.7–40.8)
MIP-1β	264.4 (176.3–371.6)	155.0 (112.1–300.0)	110.2 (81.0–150.2)
Eotaxin	107.0 (66.9–180.4)	44.8 (28.1–87.6)	120.1 (50.7–234.5)
IP-10	1964.4 (956.4–5485.4)	1088.2 (818.6–2006.4)	1879.8 (653.4–2969.0)
RANTES	83 248.0 (31 755.3–83 248.0)	8633.4 (3469.1–22 498.6)	30 970.8 (3638.7–83 248.0)
IL-8	12.5 (8.7–24.2)	10.6 (2.4–17.8)	13.6 (12.1–15.2)

Data are expressed as median (interquartile range) values (pg/mL).
NVR, null virological response; SVR, sustained virological response; TR, transient response.

Table 4 Serum chemokine level changes before, during and after treatment in patients with chronic hepatitis C

Chemokine	Treatment outcome	Baseline	Week 4	Week 72	*P*
MIP-1α	SVR	36.4 (32.3–99.3)	34.4 (20.3–60.5)	17.4 (5.6–27.9)	<0.001
	Non-SVR	36.1 (25.2–49.2)	28.8 (22.2–45.0)	29.3 (23.2–46.1)	0.331
MIP-1β	SVR	264.4 (176.3–371.6)	161.7 (112.0–223.3)	158.7 (78.8–249.6)	<0.001
	Non-SVR	131.2 (97.0–187.8)	83.6 (59.2–108.9)	105.8 (79.9–148.0)	<0.001
Eotaxin	SVR	107.0 (66.9–180.4)	190.3 (115.4–274.7)	161.8 (101.5–221.2)	0.044
	Non-SVR	78.7 (30.4–141.2)	142.7 (76.3–226.4)	103.6 (30.6–228.5)	0.030
IP-10	SVR	1964.4 (956.4–5485.4)	2322.6 (1222.1–3411.2)	1085.2 (718.5–2314.4)	<0.001
	Non-SVR	1422.7 (766.8–2645.8)	1168.9 (654.3–1713.5)	1458.5 (525.0–3045.6)	0.047
RANTES	SVR	83 248.0 (57 501.7–83 248.0)	83 248.0 (31 037.0–83 248.0)	83 248.0 (17 542.9–83 248.0)	0.091
	Non-SVR	14 670.7 (3730.4–55 199.4)	25 377.2 (11 272.6–83 248.0)	21 707.6 (8746.5–83 248.0)	0.057
IL-8	SVR	12.5 (9.3–22.2)	11.4 (8.9–16.1)	8.2 (6.6–12.0)	<0.001
	Non-SVR	13.1 (10.0–16.3)	12.7 (10.3–14.2)	12.5 (9.3–14.7)	0.418

Data are expressed as median (interquartile range) values (pg/mL).
IL, interleukin; MIP-1, macrophage inflammatory protein-1; SVR, sustained virological response.

We also measured chemokine levels 4 weeks after the initiation of therapy and 6 months after its completion (Table 4). The serum levels of MIP-1α ($P < 0.001$, Friedman test), MIP-1β ($P < 0.001$), eotaxin ($P = 0.044$), IL-8 ($P < 0.001$) and IP-10 ($P < 0.001$) were significantly decreased in samples collected from patients who achieved an SVR from baseline to 6 months after completion. The levels of MIP-1β ($P < 0.001$), eotaxin ($P = 0.03$) and IP-10 ($P = 0.047$) were lower in patients with a non-SVR as well. In addition, MIP-1α ($P = 0.004$, Wilcoxon rank sum test), MIP-1β ($P < 0.001$) and IL-8 ($P = 0.045$) levels were significantly decreased in samples collected from patients who achieved an SVR from pretreatment to 4 weeks after the start of therapy. MIP-1β ($P < 0.001$) was similarly decreased in patients with a non-SVR.

Several demographic (age and sex) and clinical (ALT, AST, viral load and histology) findings were examined for their correlation with serum chemokines in patients with HCV infection. Serum IP-10 levels significantly correlated with ALT ($P = 0.038$, $r = 0.234$), AST ($P = 0.015$, $r = 0.284$) and fibrosis ($P = 0.045$, $r = 0.257$). Serum MIP-1β was significantly correlated with MIP-1α ($P < 0.001$, $r = 0.451$) and RANTES ($P < 0.001$, $r = 0.443$).

The frequency of Gln70 in the core region was significantly higher in patients with a non-SVR than in those with an SVR (22/47 vs 6/28; $P = 0.028$). Mutant ISDR was significantly prevalent in patients with an SVR (9/29 vs 4/47; $P = 0.026$). We next analyzed whether substitutions in the ISDR and core region were associated with serum chemokine levels because substitutions in these regions have been linked with treatment outcome in patients with chronic hepatitis C. The median baseline serum level of MIP-1β was significantly higher in patients with a mutant-type than in those with intermediate- or wild-type (249.2 vs 155.0 pg/mL; $P = 0.039$) (Table 5). Other chemokines

Table 5 Serum chemokine levels according to substitutions in the ISDR

Chemokine	Mutant-type ($n = 63$)	Intermediate- and wild-type ($n = 13$)	*P*
MIP-1α	67.3 (29.2–247.2)	36.4 (25.9–47.4)	0.57
MIP-1β	249.2 (185.1–371.0)	155.0 (106.9–275.5)	0.039
Eotaxin	100.0 (70.0–188.8)	101.1 (41.9–157.7)	0.18
IP-10	1809.4 (1166.7–6437.8)	1576.2 (818.6–3138.4)	0.12
RANTES	83 248.0 (6309.0–83 248.0)	29 705.6 (6713.2–83 248.0)	0.07
IL-8	20.3 (10.4–46.3)	12.9 (8.7–15.7)	0.38

Data are expressed as median (interquartile range) values (pg/mL).
IL, interleukin; MIP-1, macrophage inflammatory protein-1.

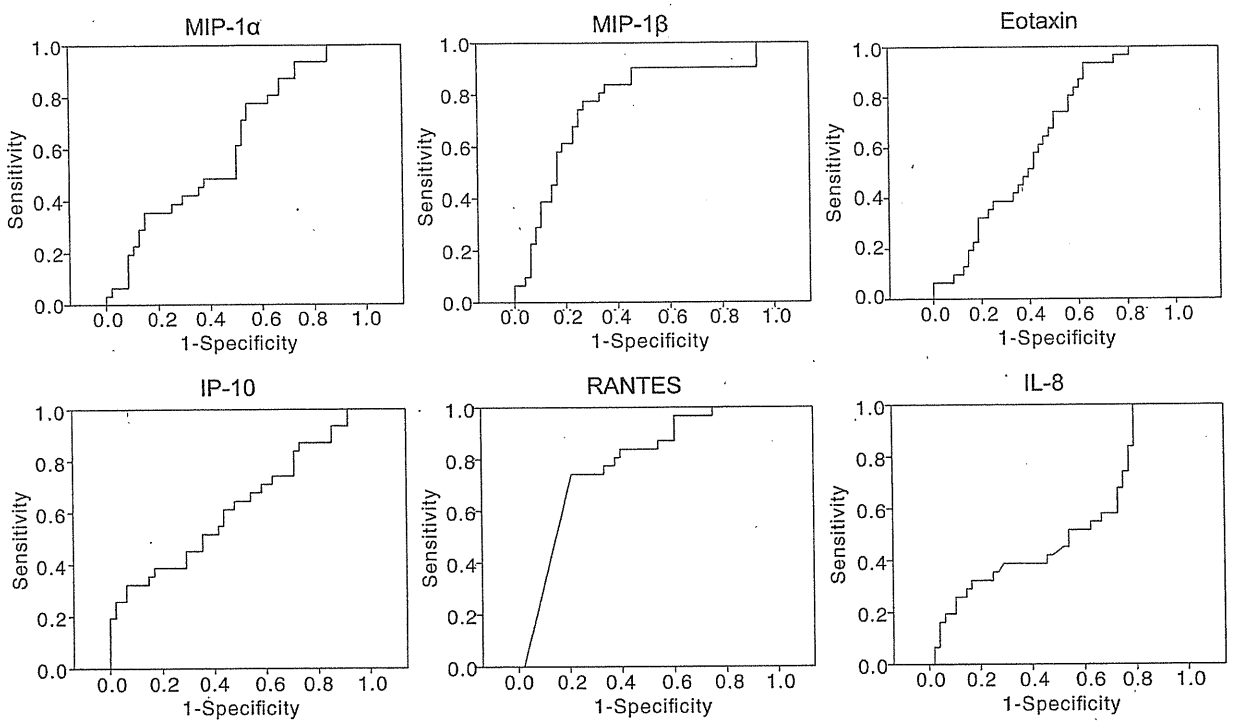

Figure 1 Receiver–operator curves for serum chemokine levels on treatment outcome. The areas under the curve for macrophage inflammatory protein (MIP)-1α, MIP-1β, eotaxin, IP-10, RANTES and interleukin (IL)-8 were 0.612, 0.756, 0.629, 0.623, 0.739 and 0.530, respectively.

were not significantly correlated with substitutions in the ISDR or core region.

Lastly, ROC curve analyses were performed to determine whether serum chemokines could predict an SVR (Fig. 1). MIP-1β only had a significant area under the curve, with values of sensitivity and specificity being 77.4% and 72.9%, respectively. The positive and negative predictive values for MIP-1β were 64.9% and 83.3%, respectively. The area under the curve (AUC) value was high at 0.76 (95% confidence interval = 0.64–0.87), indicating a strong predictive association.

DISCUSSION

IN THIS STUDY, we measured the levels of six chemokines in patients with genotype 1 chronic hepatitis C and analyzed their association with the outcome of PEG IFN and ribavirin therapy. Our data showed that baseline serum levels of eotaxin, IP-10 and RANTES were higher in HCV patients compared to healthy controls. Furthermore, elevated levels of eotaxin and MIP-1β before therapy were associated with an SVR. Serum cytokines have also been associated with pathogenesis in HCV infection. Because an association between serum cytokines and treatment outcome in HCV patients has already been reported in a prior study, only chemokines were assessed in this report.

As CC chemokines, MIP-1α, MIP-1β and RANTES are important in hepatic immunity because they are expressed on the portal vessel endothelium to provide a mechanism for the recruitment of CCR5 memory T cells in portal areas during immune surveillance and against inflammatory liver diseases.[14] Therefore, in the present study, lower MIP-1α and MIP-1β serum levels following treatment suggests that a decrease in the trans-endothelial migration of leukocytes occurs in responsive patients, which may preclude the retention and survival of lymphocytes in the liver and, thereby, ameliorate tissue damage and fibrosis. In particular, patients with an SVR had significantly higher MIP-1β compared to those without, in agreement with a previous study.[15]

The association between substitutions in the NS5A region of the ISDR and elevated MIP-1β levels that

was seen in our study is intriguing. Ahlenstiel *et al.*[16] reported that only HCV proteins, such as HCV core and NS5A, can modify RANTES secretion by altering RANTES promoter activity. To explain the observed association between MIP-1β and substitutions in the NS5A region of ISDR, one could hypothesize that IFN induces high levels of chemokines or other antiviral mediators that preferentially kill HCV; however, such a notion is highly speculative and would require additional studies to establish its validity. MIP-1β-mediated T-cell infiltration is essential for the delivery of IFN-γ to mediate protective downstream responses against HCV infection in the liver. It has been shown from the intrahepatic gene expression profiles of chimpanzees that MIP-1β was upregulated during acute infection at the time of viral clearance, but not in those who failed to eradicate the virus,[17] and previous studies have shown that HCV-infected individuals have a diminished response to MIP-1β in the liver.[18] As ROC analysis showed that MIP-1β could predict an SVR in our cohort, our data support that elevated serum levels of MIP-1β at baseline might be a favorable indicator of treatment outcome in patients with chronic hepatitis C.

Eotaxin is a chemokine that is thought to selectively attract eosinophils by activating CCR3 receptors. Several studies have shown that eotaxin is involved in the pathogenesis of inflammatory processes during liver diseases as well.[19,20] Vargas *et al.* recently analyzed the association between chemokines and virological response to IFN and ribavirin in HIV and HCV co-infected patients;[21] in patients achieving an SVR, plasma eotaxin levels before therapy were statistically higher than in non-responders. Thus, both our and their studies suggest that eotaxin may also be a useful marker in predicting an SVR to HCV treatment with PEG IFN and ribavirin.

There have been reports of increased serum and intrahepatic levels of IP-10 in HCV genotype 1-infected individuals.[22,23] Related studies have found elevated IP-10 to be associated with increased liver damage, and it has also been shown that serum IP-10 concentrations are higher in non-responders to HCV therapy than in those who achieve an SVR.[24–29] The serum level of IP-10 was not significantly associated with treatment outcome in our study, but the degree of fibrosis was well correlated with IP-10, as in a previous study.[30] These conflicting findings may reflect patient selection, sample size or racial differences.

Overall, the serum levels of eotaxin, IL-8, IP-10, MIP-1α and MIP-1β decreased during treatment and remained low in patients with an SVR. Because no direct correlation between chemokine levels and HCV RNA viral load was noticed, it is possible that chemokines may in fact compromise host immune responses to the virus.

One limitation of this study is a small sample size. Because we could not perform multivariate statistical analysis, it was difficult to draw a definitive conclusion on the most relevant chemokine. Hence, ROC analysis only was performed in our study. Larger studies are needed in the future. Another limitation of our findings is that we could not confirm if the stored serum chemokine levels were consistent with the original fresh serum samples. However, we can presume that this effect was minimal because all samples were stored immediately at −70 °C until use. Furthermore, our prior study with the same samples showed data consistent with those of other published work for the Luminex bead assay.

In conclusion, our data show that chemokines, especially MIP-1β, eotaxin and IP-10, have the potential to be effective and non-invasive markers of an SVR and potential prognostic surrogates for therapeutic outcome. Assessing chemokines may help elucidate the pathogenic processes of this disease on an individual basis, thereby assisting with prognostication and treatment decisions.

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Original article

Serum interleukin (IL)-10 and IL-12 levels and *IL28B* gene polymorphisms: pretreatment prediction of treatment failure in chronic hepatitis C

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Background: Both *IL28B* gene polymorphisms and serum levels of interleukin (IL)-10, IL-12p40 and IL-18 have been reported to affect the outcome of natural and pegylated interferon and ribavirin-treated HCV infection.
Methods: To clarify their association and predictive value in treatment outcome of genotype 1 HCV-infected patients, we measured pretreatment serum IL-10, IL-12p40 and IL-18 levels using multiplex assays and determined *IL28B* gene polymorphisms (rs 8099917) in 52 cases with chronic hepatitis C.
Results: High baseline levels of IL-10 ($P<0.001$) and low levels of IL-12p40 ($P<0.001$) were significantly associated with a non-virological response (NVR) in our cohort. The *IL28B* polymorphism was tested and TT, TG or GG genotypes were found in 60%, 38% and 2% of patients, respectively, with corresponding NVR rates of 10%, 60% and 100% ($P<0.001$). Serum cytokine levels were significantly correlated with *IL28B* gene polymorphisms. When serum IL-10 levels were stratified at 5.0 pg/ml, NVR rates were 50% versus 0% ($P=0.004$) for the TT genotype and 87% versus 0% ($P=0.001$) for the TG or GG genotypes. Similarly, low IL-12p40 levels were associated with an NVR in patients with TG or GG genotypes ($P=0.006$). In multivariate analysis, high IL-10, low IL-12p40 and *IL28B* TG or GG genotypes were independently associated with an NVR.
Conclusions: Serum IL-10 and IL-12p40 levels in combination with *IL28B* genotype, especially G-allele carriage, are strong predictive markers of an NVR to HCV treatment with pegylated interferon and ribavirin.

Introduction

Chronic HCV infection often develops into chronic hepatitis leading to liver cirrhosis and/or hepatocellular carcinoma [1–3]. The successful eradication of HCV, defined as a sustained virological response (SVR), is therefore considered important. Despite recent advances, however, approximately 50% of patients with genotype 1 HCV infection do not achieve an SVR by conventional pegylated interferon (PEG-IFN) and ribavirin therapy [4,5].

It is considered beneficial to predict the response of patients with genotype 1 HCV and high viral load to PEG-IFN and ribavirin therapy before commencement of treatment because therapy can be long, costly and have many side effects. To date, many predictive factors have been reported for treatment response. Regarding viral factors, substitutions at core amino acids 70 and 91 [6] or the IFN sensitivity determining region (ISDR) have been reported [7]. Concerning host factors, Ge *et al.* [8] recently identified single nucleotide polymorphisms (SNPs) located 5′ to the *IL28B* gene that affect response to combination therapy using a genome-wide association study. Similarly, three other groups independently reported that these SNPs are associated with the effectiveness of combination treatment [9–11]. Thomas *et al.* [12] also reported that the same SNPs are associated with spontaneous clearance of HCV.

Interleukin (IL)-28A, IL-28B and IL-29 gene products belong to the IFN-λ family. These cytokines are functionally considered to be IFNs, but have been reported to be structurally related to the IL-10 family, which include IL-10, IL-22 and IL-26, and the

Table 1. Demographic and clinical characteristics of patients with chronic hepatitis C

Characteristic	All (*n*=52)	VR (*n*=36)	NVR (*n*=16)	*P*-value
Age, years	58 (17–74)	57 (17–72)	60 (45–74)	0.781
Male, *n* (%)	24 (46)	18 (50)	6 (38)	0.404
Body mass index, kg/m^2	23 (18–30)	24 (18–30)	22 (19–29)	0.115
White blood cell count, cells/μl	4,470 (1,980–7,890)	4,810 (1,980–7,890)	3,700 (2,270–5,180)	0.007
Haemoglobin, g/dl	14.7 (12–18)	15.0 (13–18)	14.1 (12–16)	0.094
Platelet count, 10^4/μl	17.5 (8–30)	17.9 (8–30)	16.7 (9–27)	0.420
ALT, IU/l	75 (22–389)	68 (24–389)	91 (22–357)	0.663
AST, IU/l	58 (20–288)	49 (20–218)	78 (25–288)	0.092
HCV RNA, 10^5 IU/ml	21 (1.1–>50)	20 (1.1–>50)	18 (2.9–>50)	0.469
Core aa 70 (Arg70/Gln70/ND), *n*	30/21/1	23/12/1	7/9/0	0.139
Core aa 91 (Leu91/Met91/ND), *n*	37/14/1	26/9/1	11/5/0	0.463
ISDR of NS5A (wild/mutant), *n*	44/8	29/7	15/1	0.218
rs8099917 allele (TT/TG/GG), *n*	31/20/1	28/8/0	3/12/1	<0.001

Data are mean (range) unless indicated otherwise. aa, amino acid; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ISDR, interferon-sensitivity determining region; NVR, non-virological response; VR, virological response.

IFN-λ family. The ligand-binding chains for IL-22, IL-26 and IFN-λ are distinct from that used by IL-10. However, all of these cytokines use a common second chain, IL-10 receptor-2, to assemble their active receptor complexes. Thus, IL-10 receptor-2 is a shared component in at least four distinct class II cytokine-receptor complexes [13]. Although IL-10 was originally described as a cytokine synthesis inhibitory factor [14,15], recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathological effects of Th17 [16,17]. Furthermore, increased IL-10 levels are associated with a high risk of inefficient HCV clearance and resistance to IFN treatment [18–21]. Our recent study showed that low serum IL-10 levels as well as high IL-12p40 and IL-18 levels at baseline were independent predictive factors for a SVR to combination therapy [22]. Therefore, in the present study, we investigated the association between treatment outcome and the influence of *IL28B* genotype and serum cytokine levels in combination therapy.

Methods

Subjects

A total of 52 consecutive treatment-naive patients with genotype 1 chronic hepatitis C were included in this study. Diagnosis of chronic hepatitis C was based on the following criteria as reported previously [23]: presence of serum HCV antibodies and detectable viral RNA, absence of detectable hepatitis B surface antigen and antibody to HIV and exclusion of other causes of chronic liver disease. No patients had a history of or developed decompensated cirrhosis or hepatocellular carcinoma. The baseline characteristics of the patients are shown in Table 1.

Laboratory testing

Antibodies to HCV were measured in serum samples via third-generation enzyme-linked immunosorbent assays (EIA-3; Abbott Laboratories, North Chicago, IL, USA). Serum levels of HCV RNA were determined using the Cobas Amplicor assays (sensitivity 50 IU/ml; Roche Diagnostic Systems, Tokyo, Japan). HCV genotypes were determined using INNO-LiPA HCV II (Innogenetics, Gent, Belgium). All patients in our test cohort were infected with genotype 1b. Alanine aminotransferase, aspartate aminotransferase and other relevant biochemical tests were performed using standard methods [24].

Antiviral therapy and definition of treatment outcome

All patients received bodyweight-adjusted PEG-IFN-α2b (PegIntron; Schering–Plough KK, Tokyo, Japan; ≤45 kg, 60 μg/dose; 46–60 kg, 80 μg/dose; 61–75 kg, 100 μg/dose; 76–90 kg, 120 μg/dose; ≥91 kg, 150 μg/dose), and ribavirin (Rebetol; Schering–Plough KK; ≤60 kg, 600 mg/day; 61–80 kg, 800 mg/day; ≥81 kg, 1,000 mg/day) for 48 weeks.

The response to therapy categories were defined as follows: an SVR was defined as undetectable serum HCV RNA 24 weeks after completing therapy. Relapse was defined as a reappearance of serum HCV RNA after treatment in patients whose HCV RNA level was undetectable during or at the completion of therapy. A non-virological response (NVR) was defined as a decrease in HCV RNA of <2 log copies/ml at week 12 and detectable HCV RNA during the treatment course.

Detection of amino acid substitutions in the core and NS5A regions

Core region and ISDR were determined by direct sequencing after amplification by reverse transcription and PCR as reported previously [22]. Amino acids at

positions 70 and 91 of the core region identical to the reference sequence HCV-J D90208 [25] were considered wild type [6]. The number of amino acid substitutions in the ISDR was defined as in Enomoto *et al.* [7].

Detection of serum IL-10, IL-12p40 and IL-18

Serum IL-10, IL-12p40 and IL-18 were quantified using Luminex® Multiplex Cytokine Kits (Procarta Cytokine assay kit; Panomics Inc., Fremont, CA, USA) for serum samples obtained before the start of treatment as reported previously [22]. All collected samples were immediately stored at -70°C prior to testing.

Genotyping of *IL28B*

Genomic DNA was isolated from the whole blood of patients using QuickGene-800 (Fujifilm, Tokyo, Japan). The concentration of genomic DNA was adjusted to 10–15 ng/μl for the TaqMan SNP genotyping assay. Genotyping of *IL28B* SNP (rs 8099917) was performed with a TaqMan 5′ exonuclease assay using primers supplied by Applied Biosystems (Carlsbad, CA, USA). Probe fluorescence signals were detected with a TaqMan assay for real-time PCR (7500 Real Time PCR System; Applied Biosystems) according to the manufacturer's instructions.

The protocol of this study was approved by the ethics committee of Shinshu University School of Medicine and all patients provided written informed consent.

Statistical analyses

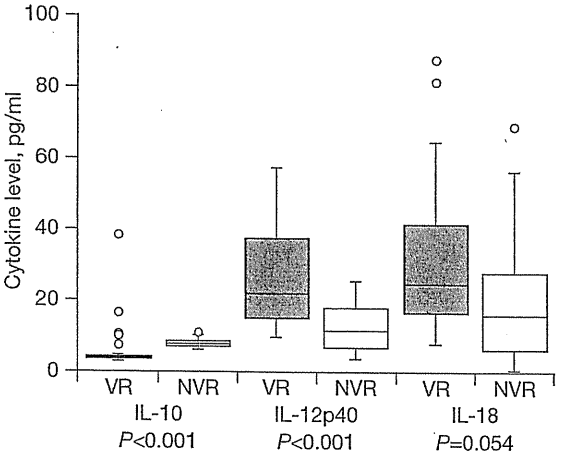
The Mann–Whitney U test was used to analyse continuous variables. The χ^2 test with Yate's correction was used for the analysis of categorical data. In cases where the number of subjects was <5, Fisher's exact test was used. A *P*-value of ≤0.05 was considered statistically significant. To predict treatment outcome, we analysed receiver operating characteristic (ROC) curves for serum levels of IL-10, IL-12p40 and IL-18. Optimal cutoff values were chosen as serum cytokine levels with the highest diagnostic accuracy, that is, when the sum of the false-negative and false-positive rates was minimized. The respective overall diagnostic values were expressed using the area under the curve (AUC). Multivariate analysis was performed using a logistic regression model with stepwise method. Statistical analyses were performed using PASW Statistics 18.0J (IBM, Tokyo, Japan).

Results

Treatment outcome in patients with chronic hepatitis C

Of the 52 patients receiving PEG-IFN and ribavirin therapy, 22 (42%) achieved an SVR. Among the 30 remaining patients, 14 had a relapse and 16 had an NVR. Before treatment, the median white blood cell count in the virological response group was significantly higher than that in the NVR group (Table 1). Haemoglobin value (15.4 versus 14.1 g/dl; *P*=0.021) was significantly higher in the SVR group compared to the NVR group as well. Substitutions in the ISDR and of aa70 and aa91 in the core region were not associated with treatment outcome.

Figure 1. Detection of serum cytokines related to treatment outcome



Boxes represent the IQR of the data, lines across the boxes indicate the median values and the hash marks above and below the boxes indicate the 90th and 10th percentiles, respectively for each group. Open circles indicate outliers. Serum interleukin (IL)-10, IL-12p40 and IL-18 levels were detected in 36 patients with a virological response (VR) and 16 patients without. NVR, non-virological response.

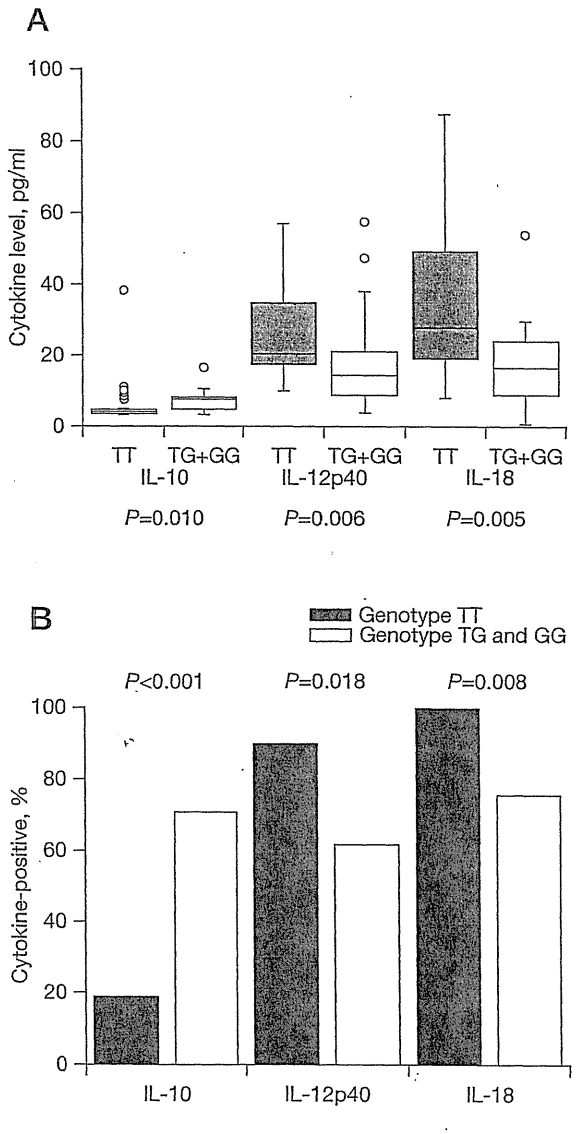
Effects of antiviral therapy on serum cytokine levels

Serum samples obtained prior to antiviral therapy were examined for the presence of IL-10, IL-12p40 and IL-18 by multiplex assays. NVR patients showed significantly higher baseline IL-10 concentrations (8.1 pg/ml) than virological responders (4.1 pg/ml; *P*<0.001; Figure 1). The median baseline serum levels of IL-12p40 (22.1 versus 11.7 pg/ml; *P*<0.001) and IL-18 (24.8 versus 16.0 pg/ml; *P*=0.054) were higher in patients who achieved a virological response than in those with an NVR (Figure 1). Furthermore, serum IL-10 level (4.0 versus 8.1 pg/ml; *P*<0.001) was significantly lower and serum IL-12p40 (25.0 versus 11.7 pg/ml; *P*<0.001) and IL-18 (31.6 versus 16.0 pg/ml; *P*=0.010) levels were significantly higher in the SVR group compared with the NVR group. We also analysed whether pretreatment serum cytokines were correlated with time to clearance of HCV RNA. Serum baseline IL-10 level was significantly lower in patients who eradicated HCV RNA 12 weeks after the start of treatment (*P*=0.002).

IL28B genotype and treatment outcome

Among the 52 patients studied for rs8099917, 31 had the TT genotype (60%), 20 had the TG genotype (38%) and 1 had the GG genotype (2%). Responses to combination therapy for rs8099917 are shown in Table 1. Overall SVR rates in patients with the TT genotype (16/31, 53%) and with the TG or GG genotypes (6/21, 29%) were not significantly different (P=0.09).

Figure 2. Serum cytokines related to *IL28B* gene polymorphisms



(A) Boxes represent the IQR of the data, lines across the boxes indicate the median values and the hash marks above and below the boxes indicate the 90th and 10th percentiles, respectively, for each group. Open circles indicate outliers. Serum interleukin (IL)-10, IL-12p40 and IL-18 were detected in 31 patients with the TT *IL28B* genotype and 16 patients with the TG or GG genotypes. (B) The prevalence of high serum IL-10, IL-12p40 and IL-18 levels in 31 patients with the TT *IL28B* genotype and in 16 patients with the TG or GG genotypes.

However, NVR rates in patients with either TG or GG (13/21, 62%) were significantly higher than in those with TT only (3/31, 10%; P<0.001).

Association of *IL28B* genotype and serum cytokine levels

Median serum IL-10 levels were significantly higher in patients with the TG or GG genotypes (7.7 pg/ml) compared to those with TT (4.1 pg/ml; P=0.010; Figure 2A). Conversely, patients with TT had significantly higher median IL-12p40 (20.6 versus 14.5 pg/ml; P=0.006) and IL-18 (27.9 versus 16.6 pg/ml; P=0.005) levels than patients with TG or GG (Figure 2A).

ROC curve analyses were performed to determine the optimal threshold values of serum cytokines for predicting treatment outcome among the 16 NVR patients and 36 cases with a virological response in our cohort (Figure 3). The optimal threshold value of IL-10 was identical to the 5.0 pg/ml that we had reported in a prior study [22]. The cutoff values for IL-12p40 and IL-18 were 12.1 pg/ml and 6.4 pg/ml, respectively. The calculated AUC for IL-10, IL-12p40 and IL-18 was 0.89 (95% CI 0.77–0.96), 0.81 (95% CI 0.67–0.90) and 0.67 (95% CI 0.52–0.79), respectively, as shown in Figure 3.

The presence of high IL-10 levels (≥5.0 pg/ml) was significantly greater among patients with TG or GG genotypes (71%, 15 of 21) than among those with TT (19%, 6 of 31; P<0.001; Figure 2B). High IL-12p40 levels (≥12.1 pg/ml) were significantly less prevalent (P=0.018) among patients with TG or GG (62%, 13 of 21) than among those with TT (90%, 28 of 31). High IL-18 levels (≥6.5 pg/ml) were found in 100% (31 of 31) of patients with TT but only 76% (16 of 21) patients with TG or GG (P=0.008).

Predicting treatment outcome by serum cytokine levels in combination with *IL28B* genotype

The NVR prediction rate by serum IL-10 in combination with rs8099917 genotype is shown in Figure 4. In patients with TT, a significantly higher proportion of patients with high serum IL-10 levels (50%, 3 of 6) showed an NVR than patients with low IL-10 (0%, 0 of 25; P=0.004). Similarly, an NVR was significantly more likely in high versus low IL-10 levels (87%, 13 of 15 versus 0%, 0 of 6; P=0.001) in patients with TG or GG (Figure 4A).

NVR rates by serum IL-12p40 levels and IL-18 levels in combination with rs8099917 genotype are shown in Figure 4B and 4C. Among patients with the TT genotype, the NVR rate did not differ between low and high IL-12p40 levels (0% versus 11%; P=0.729) or IL-18 levels (0% versus 10%). In cases with TG or GG genotypes, the NVR rate was significantly higher for low IL-12p40 levels compared with high IL-12p40 levels (100% versus 38%; P=0.006). Patients with low serum

IL-18 had a higher NVR rate, but this difference was not statistically significant (100% versus 50%; P=0.063).

Factors associated with an NVR to PEG-IFN and ribavirin therapy

All factors found to be associated with an NVR were evaluated for independence in multivariate analysis. Genotype TG or GG (OR 10.43, 95% CI 1.73–62.96; P=0.011), serum IL-10 levels ≥5.0 pg/ml (OR 1.21, 95% CI 1.03–1.41; P=0.018) and IL-12p40 levels ≥17.4 mg/dl (OR 0.84, 95% CI 0.72–0.97; P=0.020) were all independent predictive factors of an NVR.

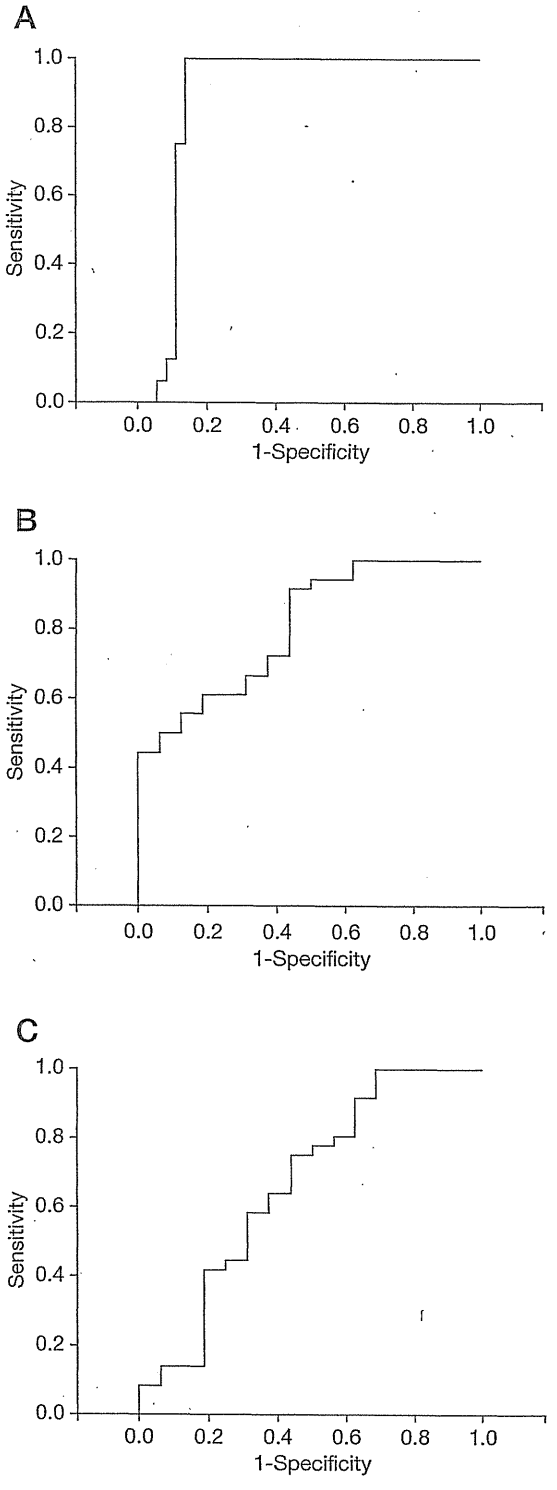
Discussion

This study examined the *IL28B* (rs8099917) genotype and serum levels of IL-10, IL-12p40 and IL-18 in patients with chronic hepatitis C to assess their predictive value in treatment outcome with PEG-IFN and ribavirin. The key findings were as follows: *IL28B* G-allele carriers were associated with an NVR to PEG-IFN and ribavirin therapy in patients infected with HCV genotype 1, consistent with recent findings; *IL28B* genotype was associated with baseline serum IL-10, IL-12p40 and IL-18 levels; in carriers of an *IL28B* G-allele, NVR rates were high (80–100%) and associated with increased IL-10 and decreased IL-12p40 and IL-18 levels, thus providing new predictive markers of an NVR in PEG-IFN and ribavirin therapy; and *IL28B* genotype, high serum IL-10 levels, and low serum IL-12p40 levels were all independent factors related to an NVR in multivariate analyses.

IL28B gene polymorphisms have recently been linked to the outcome of HCV infection during spontaneous and treatment-induced elimination of HCV [8–10,12]. In particular, carriage of a G-allele at the *IL28B* gene SNP (rs8099917) is associated with an NVR to PEG-IFN and ribavirin therapy in Japanese patients infected with HCV genotype 1 [10]. This finding was confirmed in our cohort with NVR rates of 62% with GT or GG genotypes versus 10% with TT genotypes (P<0.001). Therefore, detection of the *IL28B* genotype is a useful marker to predict the outcome of PEG-IFN and ribavirin therapy in patients with chronic hepatitis C. Data for *IL28B* SNP in healthy subjects were not available for this study.

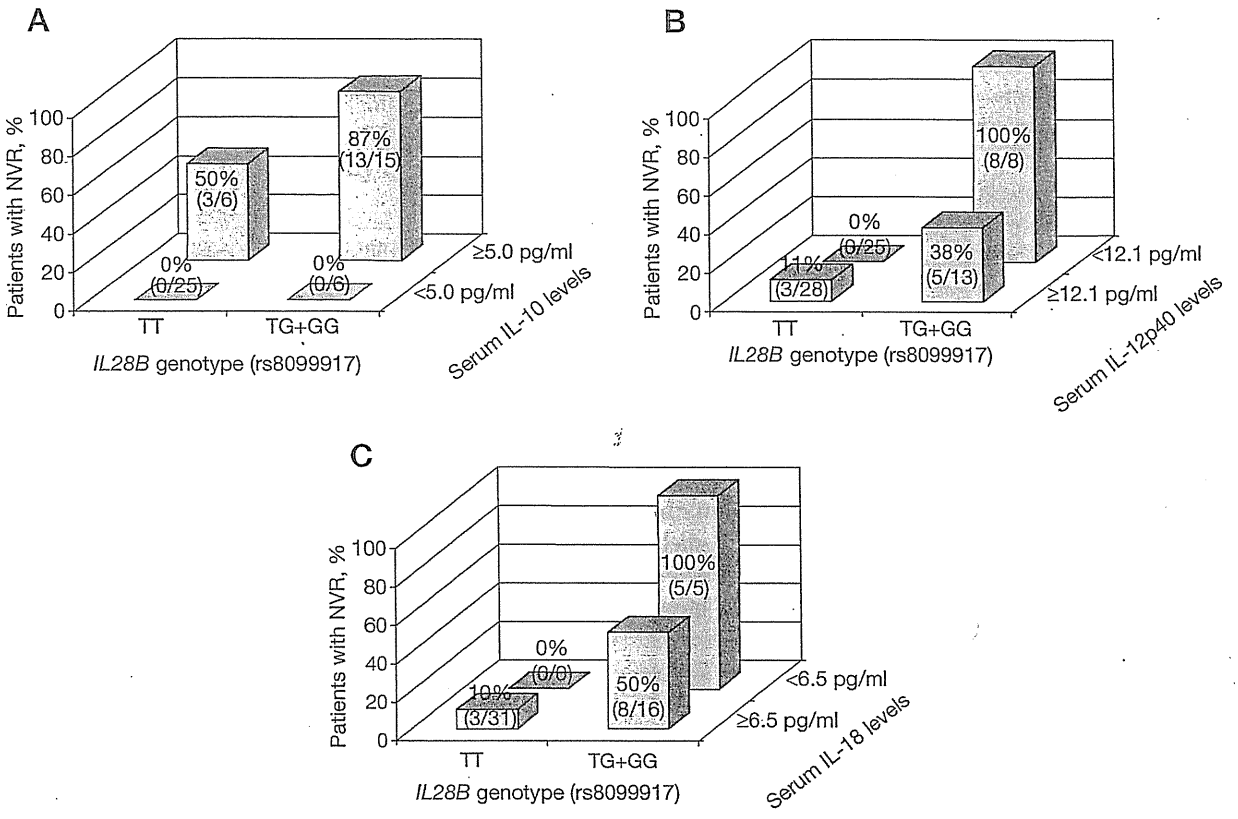
IFN-λ produces an antiviral state by triggering a cascade through the JAK-STAT pathway that up-regulates IFN-stimulated genes. *IL28B* binds to a distinct receptor that may up-regulate a different set of IFN-stimulated genes [26,27]; the precise role of IFN-λ in controlling multiple viral infections, including HCV, is currently under way. Further studies are also needed on how SNPs affect the function of *IL28B* and other cytokines.

Figure 3. Receiver-operating characteristic curves for serum cytokine levels on treatment outcome



The areas under the curve for (A) interleukin (IL)-10, (B) IL-12p40 and (C) IL-18 were 0.89, 0.81 and 0.67, respectively. All areas under the curve values were significantly higher than a 0.50 non-predictive value (P<0.01 for all comparisons). IL-10 was predictive of a non-response. IL-12p40 and IL-18 were predictive of a virological response.

Figure 4. Non-virological response rate determined by serum cytokine levels and *IL28B* gene genotype



The prevalence of a non-virological response (NVR) in patients with high or low serum (A) interleukin (IL)-10, (B) IL-12p40 and (C) IL-18 levels according to *IL28B* genotype.

A strong association between high IL-10, low IL-12p40 and low IL-18 levels and an NVR to PEG-IFN and ribavirin therapy was found in this study, which is consistent with previous studies [22,28–30]. In ROC curve analyses, AUCs were high, especially for IL-10 (AUC=0.89) and IL-12p40 (AUC=0.81), confirming that these cytokines are strong predictive markers for an NVR. This study showed a strong correlation between the *IL28B* genotype and serum IL-10, IL-12p40 and IL-18 levels at baseline. Most strikingly, all patients who had low pretreatment IL-10 levels achieved a virological response regardless of *IL28B* genotype. By contrast, among patients with high IL-10 levels (≥5.0 pg/ml), NVR rates were 87% in *IL28B* G-allele carriers and 50% for the *IL28B* TT genotype. Additionally, all *IL28B* G-allele carriers showed an NVR when pretreatment serum IL-12p40 and IL-18 levels were <12.1 pg/ml and <6.5 pg/ml, respectively. It is unclear how serum IL-10, IL-12p40 and IL-18 are associated with an NVR to antiviral therapy in patients with chronic hepatitis C. Although IL-10 was originally described as a cytokine synthesis inhibitory factor, recent studies have demonstrated that IL-10 produced by Th17 cells restrains the pathological effects of Th17 [31]. Production of IL-12p40 is directed towards the elimination of intracellular pathogens and viruses because IL-12p40 is a proinflammatory cytokine that promotes the differentiation of Th1 cells, suppresses Th2 function and amplifies the cytotoxicity of cytotoxic T-lymphocytes and natural killer cells [32]. Megjugorac *et al.* [33] reported that IL-29-treated plasmacytoid dendritic cells inhibiting production of IL-13, IFN-γ and IL-10 by allogeneic T-cells were consistent with a role for this cytokine in plasmacytoid dendritic cell maturation and activation. Very recently, another report has been published demonstrating that IL-29 enhances IL-12p40 by macrophages and that IL-29 pretreatment primes the activation of macrophages induced by IFN-γ [34]. However, the association between IL-28B and such cytokines has not been studied. To explain this relationship, further studies are needed to clarify whether a direct or indirect interaction exists between pretreatment levels of these cytokines and *IL28B* genotype.