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Original Article

Hepatic interferon-gamma-induced protein-10 expression is more strongly associated with liver fibrosis than interleukin-28B single nucleotide polymorphisms in hepatocellular carcinoma resected patients with chronic hepatitis C

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Aim: Single nucleotide polymorphisms (SNP) around *IL-28B* and interferon (IFN)-stimulated gene (ISG) expression are predictors of response to standard therapy involving IFN for chronic hepatitis C virus (HCV) infection. We analyzed the association between these predictors to improve the prediction of the response to IFN therapy after liver resection for hepatocellular carcinoma (HCC).

Methods: Data were collected from 74 patients with HCV-induced HCC. The *IL-28B* genotype and hepatic ISG mRNA levels were analyzed to clarify their association, focusing on the progression of liver fibrosis.

Results: Fifty patients were identified as having major alleles (*rs8099917* TT) and the remaining 24 patients had minor alleles (*rs8099917* TG or GG). Hepatic *ISG15* expression was lower in the *IL-28B* major group than that in the *IL-28B* minor group ($P < 0.005$). *IP-10* expression was similar between the *IL-28B* major and minor groups ($P = 0.44$). *IP-10* expression was elevated with advancing stages of liver fibrosis in HCV infected patients ($P = 0.005$). In patients with mild or no fibrosis, the *IL-28B* major group had lower *IP-10* expression than the *IL-28B* minor group ($P = 0.02$). However, in patients with advanced fibrosis, *IP-10* expression was not different between the *IL-28B* major and minor groups ($P = 0.66$).

Conclusion: Hepatic *ISG15* expression is associated with *IL-28B* polymorphisms, while *IP-10* is strongly affected by liver fibrosis.

Key words: hepatitis C virus, interferon-γ-induced protein-10, interleukin-28B, liver fibrosis, single nucleotide polymorphism

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection affects 170 million people worldwide, and most acute HCV infections become chronic, with some progression to liver cirrhosis or hepatocellular carcinoma (HCC). Current standard therapy against chronic HCV infection includes pegylated interferon (PEG IFN)-α and ribavirin (RBV),[1] which achieve a sustained virological response (SVR) in only 50% of patients chronically infected with the HCV genotype 1.[2–4]

HCV infection induces IFN production via innate immune cascades.[5,6] IFN consists of type I (IFN-α, -β and others), II (only IFN-γ) and III (various IFN-λ; interleukin [IL] or IL-28A/B and IL-29) IFN, and these IFN have antiviral activity by inducing a subset of IFN-stimulated genes (ISG).[7] ISG include some genes which show antiviral activity or are involved in lipid metabolism, apoptosis, protein degradation and inflammation.[8] IFN are effective against HCV infection, and are also essential for innate immunity.

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Recently, a genome-wide association study showed that single nucleotide polymorphisms (SNP), located near *IL-28B*, which encodes for IFN-λ3, are strongly associated with a virological response to IFN therapy and spontaneous clearance of HCV.[9–13] Especially in Japanese patients, *rs8099917* is closely associated with efficacy for IFN therapy; patients with the TT genotype (termed *IL-28B* major) have a stronger response to IFN therapy than those with TG or GG genotypes (termed *IL-28B* minor).[11] Even after liver resection because of HCC[14] or liver transplantation,[15] the efficacy of therapy is associated with *IL-28B* SNP. More recently, *IL-28B* major patients were reported to express hepatic ISG, such as *ISG15* and *OAS*, which were lower than those in *IL-28B* minor patients.[16,17] ISG expression is also known to be strongly associated with the efficacy of IFN therapy.[16,17] However, after liver resection for HCC, the association between hepatic ISG expression and the efficacy of therapy is still unclear.

Similar to *IL-28B* SNP, IFN-γ-induced protein-10 (IP-10) expression is a predictor for IFN therapy, and high IP-10 expression is a poor predictive factor for an IFN response.[18,19] Unlike other ISG, serum IP-10 protein levels have been reported to be associated with differences in *IL-28B* SNP[20,21] and they also have been found not to be associated with differences in *IL-28B* SNP.[22] IP-10 is one of the ISG and it is regulated by inflammatory cytokines, such as tumor necrosis factor (TNF)-α, via nuclear factor (NF)-κB transcription factor.[23] TNF-α is elevated in severe fibrosis patients;[24] therefore, IP-10 expression should be elevated in response to progression of liver fibrosis.

To clarify why IP-10 expression is not correlated with *IL-28B* SNP, we genotyped *IL-28B* SNP and quantitated the hepatic ISG. We then analyzed their association, focusing on the progression of liver fibrosis. We found that IP-10 expression is associated with *IL-28B* SNP only in patients with mild or no fibrosis.

METHODS

Human tissue samples

NON-CANCEROUS TISSUE SAMPLES from 74 patients who were negative for hepatitis B virus (HBV) and HIV, were positive for HCV RNA and had undergone liver resection for HCC at the Department of Surgery and Science at Kyushu University Hospital between February 2004 and September 2010, were analyzed by genotyping, real-time reverse transcription polymerase chain reaction (RT–PCR) and immunohistochemistry. We retrospectively analyzed hepatic gene expression in these patients. For the non-B, non-C (NBNC) hepatitis group, we analyzed non-cancerous tissue samples from 20 patients who were negative for HBV, HCV and HIV, and had undergone liver resection for HCC at our institute. Samples were collected immediately after resection, as previously described,[25] transported in liquid nitrogen and stored at −80°C. The degree of chronic hepatitis in non-cancerous regions was classified as follows. The degree of necroinflammatory activity (grading) was graded from A0 to A3, and the degree of fibrosis (staging) was staged as F0 to F4. Histological diagnosis of the non-cancerous tissues was based on the General Rules for the Clinical and Pathological Study Group of Japan.[26] The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and patient samples were collected after obtaining informed consent, according to an established protocol approved by the Ethics Committee of Kyushu University. The data do not contain any information that could lead to the identification of the patients.

DNA extraction and IL-28B genotyping

DNA was extracted from the patients' resected liver tissue, and genotyping was performed using Taqman GTXpress Master Mix (Life Technologies, Tokyo, Japan), according to the manufacturer's protocol. The Custom TaqMan SNP Genotyping Assay (Life Technologies) was used for identifying *IL-28B* genetic polymorphism (*rs8099917*).

Measurement of ISG using real-time RT–PCR

Total RNA was extracted from resected liver tissue using reagents for RNA extraction, including ISOGEN and Ethachinmate (Nippon Gene, Tokyo, Japan). The synthesis of first-strand complementary DNA (cDNA) was performed using the SuperScript III First-Strand synthesis system for quantitative RT–PCR (Life Technologies) according to the manufacturer's protocol. Real-time RT–PCR was performed using the QuantiFast SYBR Green PCR kit (Qiagen, Tokyo, Japan). *β-Actin* expression was used as the endogenous reference for each sample. The shown value was normalized using cDNA of the HCV replicon harboring HuH-7 cells, for an internal reference. Primers for each gene were designed as follows: *ISG15*, 5′-AGC GAA CTC ATC TTT GCC AGT ACA-3′ (sense) and 5′-CAG CTC TGA CAC CGA CAT GGA-3′ (antisense); *OAS*, 5′-GGG TGG AGT TCG ATG TGC TG-3′ (sense) and 5′-GGG TTA GGT TTA TAG CCG CCA G-3′ (antisense); *IP-10*, 5′-CTG AAT CCA GAA TCG AAG GCC ATC-3′ (sense) and 5′-TGT AGG GAA

GTG ATG GGA GAG G-3′ (antisense); *TNF*, 5′-CCC AGG GAC CTC TCT CTA ATC A-3′ (sense) and 5′-GCT ACA GGC TTG TCA CTC GG-3′ (antisense); and *β-actin*, 5′-CTG GCA CCA CAC CTT CTA CAA TG-3′ (sense) and 5′-GGC GTA CAG GGA TAG CAC AGG-3′ (antisense).

Immunohistochemistry

Formalin-fixed, paraffin-embedded, 3-μm sections were deparaffinized in xylene, rehydrated through graded ethanol and rinsed in phosphate-buffered saline. Heat-induced epitope retrieval was performed in 10 mM citrate buffer, pH 6.0, in a 650-Watt microwave oven at 80% output for 15 min. Endogenous peroxidase activity was blocked by incubation with 0.3% H_2O_2 for 10 min. Non-specific antibody binding was blocked by incubating the sections with normal rabbit serum (Dako, Glostrup, Denmark) for 10 min. The sections were then incubated with anti-CXCL10 goat polyclonal antibody (1:40, R&D Systems, Minneapolis, MN, USA) overnight at 4°C and labeled with the Envision Detection System (Dako) for 1 h at room temperature. The sections were then developed with 3,3′-diaminobenzidine-tetrachloride (DAB plus; Dako) and counterstained with 10% Mayer's hematoxylin, dehydrated and mounted.

Cell culture and reagents

HuH-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA). The HCV replicon cells were cultured in 0.5 mg/mL G418-containing DMEM supplemented with 10% FBS. The replicon construct was derived from pFK-I377neo/NS3-3′/WT, as previously described.[27] Recombinant human IFN-α2a was a kind gift from F. Hoffmann-La Roche. Recombinant human IFN-λ3 and TNF-α were purchased from R&D Systems. ISG expression in HuH-7 and HCV replicon cells was measured using total RNA that was extracted from cells cultured in the presence of 100 IU/mL IFN-α, 100 ng/mL IFN-λ3 or 1 ng/mL TNF-α for 24 h. Complementary DNA synthesis and real-time RT–PCR reactions were performed as described above.

Statistical analysis

Pearson's χ^2-test was performed for qualitative variables. One-way ANOVA, the *F*-test, and Student's and Welch's *t*-tests were performed for quantitative variables.

RESULTS

Patient characteristics, genotyping for IL-28B (rs8099917) polymorphisms and quantification of ISG expression

TABLE 1 SHOWS the patients' characteristics according to the *IL-28B* genotype. Fifty patients were identified as having major alleles (*rs8099917* TT; *IL-28B* major). The remaining 24 patients had minor alleles (*rs8099917* TG or GG; *IL-28B* minor), and two of 24

Table 1 Patient characteristics and *IL-28B* genotype

Factor	*IL-28B* SNP major ($n = 50$)	*IL-28B* SNP minor ($n = 24$)	*P*-value (univariate)
Sex, male/female	42/8	17/7	0.187
Age (years), mean ± SD	70 ± 8	70 ± 8	0.980
BMI (kg/m^2), mean ± SD	22.5 ± 3.3	22.0 ± 2.5	0.596
ALT (IU/L), mean ± SD	61.0 ± 41.5	61.0 ± 49.1	1.000
γ-GTP (IU/L), mean ± SD	72.2 ± 89.6	77.3 ± 59.5	0.774
Cholesterol (mg/dL), mean ± SD	156.8 ± 25.0	154.3 ± 26.8	0.698
Hemoglobin (g/dL), mean ± SD	13.1 ± 1.6	12.7 ± 1.9	0.311
Platelet count (×10^4/mL), mean ± SD	14.1 ± 4.1	13.6 ± 5.0	0.651
Fibrosis stage, F0/1/2/3/4	3/8/12/17/10	1/6/4/2/11	0.107
Activity grade, A0/1/2/3	1/13/24/12	0/5/14/5	0.900
Differentiation of hepatic cancer (well/moderate/poor)	6/35/9	4/14/6	0.611
Tumor factor, T1/T2/T3/T4	7/26/15/2	6/12/5/1	0.652
HCV genotype, 1/2/unknown	38/8/4	16/4/4	0.516
HCV viral load (log IU/mL), mean ± SD	6.0 ± 1.1	6.1 ± 0.7	0.667

Fibrosis stage and activity grade were classified according to the New Inuyama Classification.
ALT, alanine aminotransferase; BMI, body mass index; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; SD, standard deviation; SNP, single nucleotide polymorphism.

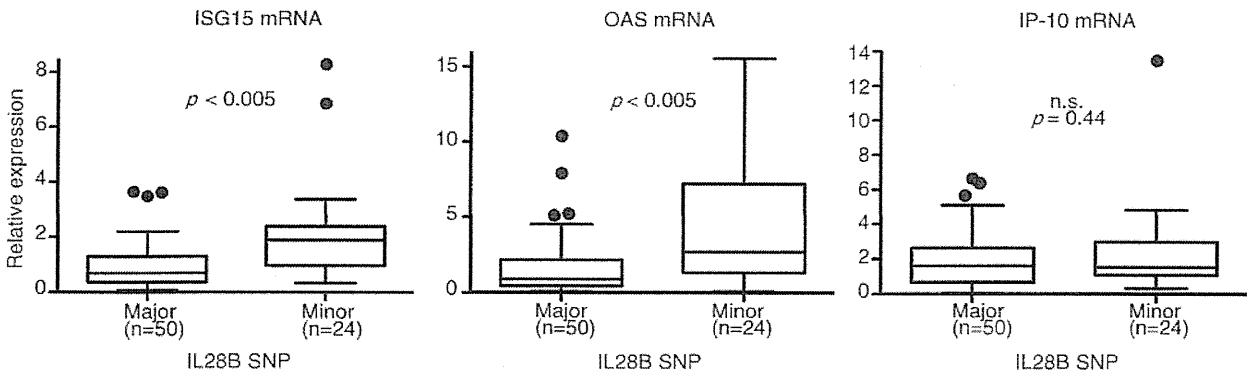

Figure 1 Comparison of hepatic gene expression levels between *IL-28B* major and *IL-28B* minor patients. Expression levels of *IP-10*, *ISG15* and *OAS* are shown. The *P*-values were determined by Welch's *t*-test. SNP, single nucleotide polymorphism.

patients had a minor homozygote (*rs8099917* GG). We measured hepatic *IP-10*, *ISG15* and *OAS* mRNA using non-cancerous liver tissue, and found that *ISG15* and *OAS* mRNA was significantly higher in *IL-28B* minor patients than that in *IL-28B* major patients (Fig. 1; *ISG15*, $P < 0.005$; *OAS*, $P < 0.005$). In contrast, *IP-10* expression in major patients was similar to that in minor patients (Fig. 1; $P = 0.44$). Thirty-one out of 74 patients received IFN therapy prior to liver resection, and could not achieve an SVR. Table S1 shows patient characteristics according to receiving or not receiving IFN therapy.

IP-10 expression and progression of liver fibrosis

To clarify why IP-10 is highly expressed, even in *IL-28B* major patients, we evaluated the association between ISG expression and the stage of fibrosis. We found that *IP-10* expression was significantly higher in F2/3/4 patients than that in F0/1 patients (Fig. 2a; $P = 0.005$). We also separately analyzed the *IL-28B* major and minor groups for the association between ISG expression and the stage of fibrosis. In *IL-28B* major patients, *IP-10* expression was significantly increased as the stage of fibrosis worsened (Fig. S1) (one-way ANOVA, $P = 0.03$). The one-way ANOVA analysis revealed no significant association, but tendency between other ISG and the stage of fibrosis (*ISG15*, $P = 0.10$; *OAS*, $P = 0.09$). There were not enough *IL-28B* minor patients for statistical analysis, but ISG expression was similar between mild and severe fibrosis patients (Fig. S1). To assess whether *IP-10* expression was induced by progression of fibrosis without viral infection, we measured *IP-10* expression in the liver of HCC patients with NBNC hepatitis. Progression of liver fibrosis did not induce *IP-10* expression in the NBNC patients (Fig. 2b; $P = 0.32$). HCV infection induced *IP-10* expression more strongly than NBNC hepatitis (Fig. 2c; $P < 0.001$). Immunohistochemistry using an anti-IP-10 antibody showed that liver sections with mild fibrosis were slightly stained. In contrast, a lot more hepatocytes were stained in liver sections with more severe fibrosis compared with those with mild fibrosis (Fig. 2d). *IP-10* expression in the liver was induced by progression of fibrosis, specifically in HCV infectious patients.

IP-10 expression and IL-28B polymorphisms in mild fibrosis patients

As shown in Figure S1, *IP-10* expression was increased in the F2/3/4 stage liver compared with that in F0/1 stage liver of *IL-28B* major patients. Therefore, to exclude an effect of progression of fibrosis on *IP-10* expression, we compared *IP-10* expression in *IL-28B* SNP major and minor patients, in those with only mild or no fibrosis. In the F0/1 patients, *IL-28B* minor patients had significantly higher IP-10 expression than *IL-28B* major patients (Fig. 3a; $P = 0.02$), similar to *ISG15* expression (Fig. 3a; $P = 0.005$). In the F2/3/4 patients, *ISG15* expression in *IL-28B* minor patients was significantly higher than that in *IL-28B* major patients (Fig. 3b; $P = 0.03$), while *IP-10* expression was not different between these patients (Fig. 3b; $P = 0.66$).

Inflammatory cytokine TNF-α induces IP-10 expression in HCV RNA-replicating HuH-7 cells

We analyzed hepatic *TNF* mRNA expression and association between the expression and stage of fibrosis.

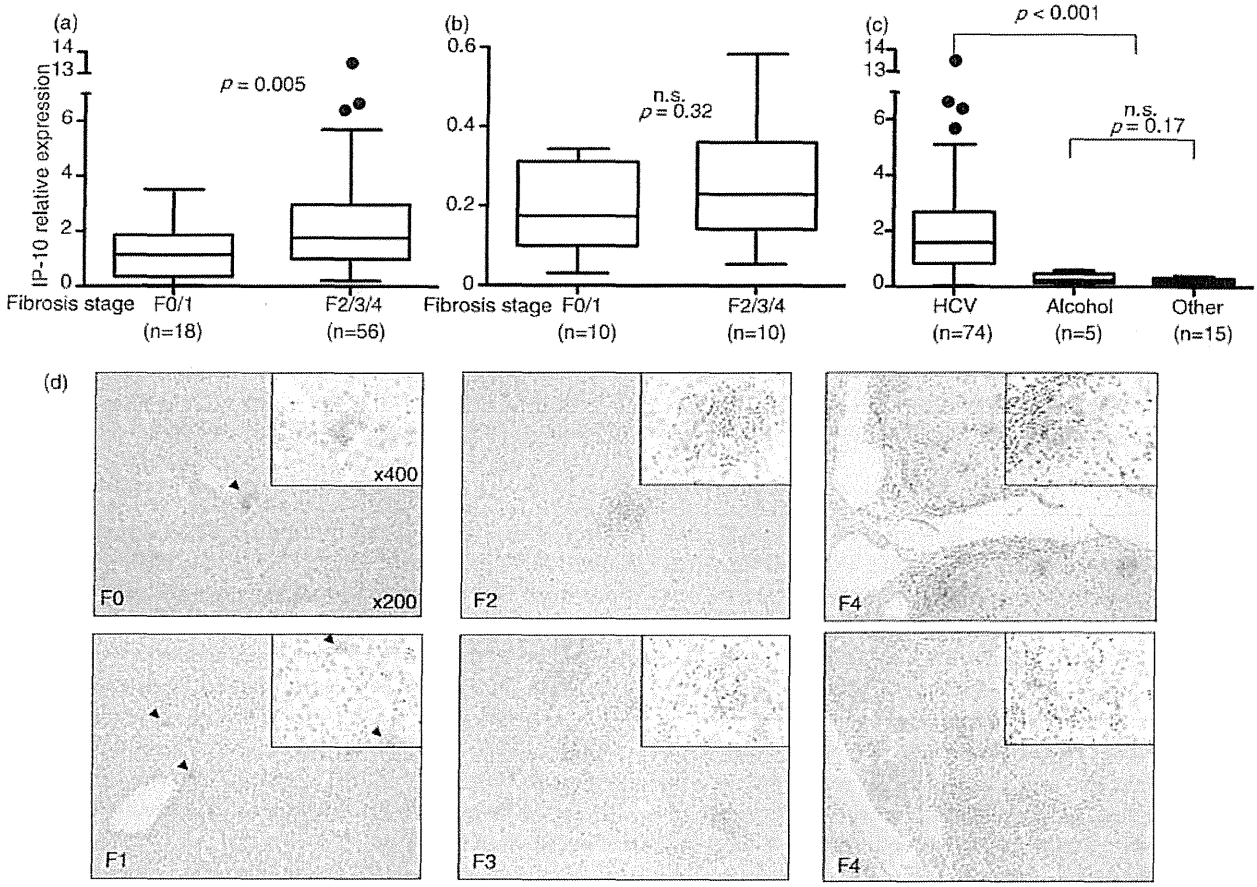

Figure 2 Comparison of hepatic *IP-10* expression levels between F0/1 and F2/3/4 patients. (a) Expression level of *IP-10* in chronic hepatitis C patients. (b) Expression level of *IP-10* in non-B, non-C hepatitis patients. (c) Comparison of *IP-10* expression levels in patients with different causes of hepatitis: hepatitis C virus (HCV), alcohol and others. Bars indicate the median of these values. (d) Hepatic interferon-γ-induced protein-10 (IP-10) protein expression determined by immunohistochemistry is shown. Stained tissue sections from patients with differential fibrosis are shown. Arrows indicate IP-10 positive cells.

TNF expression was significantly higher in F2/3/4 patients than that in F0/1 patients (Fig. S2, $P = 0.02$), like *IP-10* expression. *IP-10* expression in the liver was mainly induced in parenchymal cells (Fig. 2d). Therefore, hepatoma cell line, HuH-7 cells and the HCV subgenomic replicon harboring HuH-7 cells were incubated with IFN or TNF-α, to confirm the capability of *IP-10* induction by these stimulators. We observed that TNF-α induced *IP-10* expression much higher than type I and III IFN (Fig. 4a). *ISG15* expression was also measured in the IFN- or TNF-α-treated cells. We found that *ISG15* was induced by IFN, but not by TNF-α (Fig. 4b). These results indicate that *IP-10* and *ISG15* expressions are separately regulated in the liver with severe fibrosis. Therefore, *IP-10* expression could be used as the representative of hepatic ISG in patients with mild or no fibrosis. However, *IP-10* expression cannot be used in patients with severe fibrosis.

DISCUSSION

IN THE PRESENT study, we showed that after liver resection, ISG expression was associated with *IL-28B* polymorphisms. *IP-10* expression was significantly associated with *IL-28B* polymorphisms in patients with mild or no fibrosis, and it was affected by the progression of fibrosis more strongly than the polymorphisms.

Hepatitis C virus-induced HCC occurs among patients with cirrhosis or bridging fibrosis. However, all the patients in our study were only HCC resectable patients,

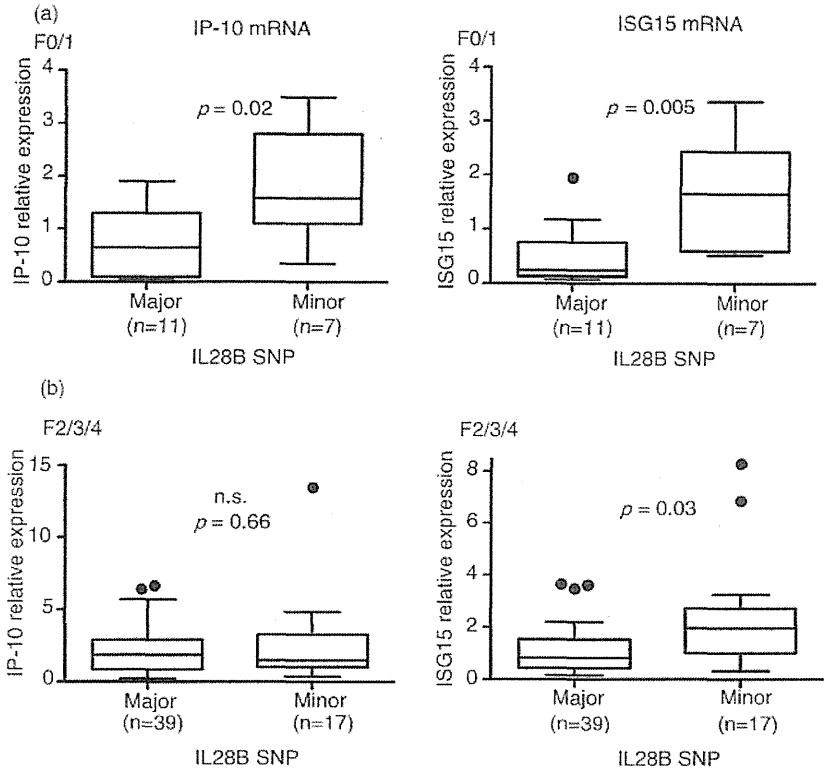

Figure 3 Comparison of hepatic *IP-10* and *ISG15* expression between *IL-28B* major and *IL-28B* minor patients in subgroups of mild or no fibrosis (a) and more progressed fibrosis (b). The *P*-values were determined by Welch's *t*-test. IL, interleukin; IP-10, interferon-γ-induced protein-10; ISG, interferon-stimulated genes; SNP, single nucleotide polymorphism.

and therefore, patients with mild fibrosis would be selected, and the stage of fibrosis was widely distributed (Table 1). Hepatic *ISG15* and *OAS* expressions were higher in *IL-28B* SNP minor patients than those in *IL-28B* SNP major patients, as previously reported.[16,17] However, *IP-10* expression was not associated with *IL-28B* SNP (Fig. 1). Suppression of *IP-10* expression during IFN therapy was reported,[19] and some patients received IFN therapy prior to liver resection. The patients who had received IFN therapy prior to the resection had HCC resected at least 4 weeks after interrupting the therapy, and therefore, *IP-10* expression was similar between patients who experienced IFN therapy and naïve patients (Table S1). We found that *IP-10* expression was significantly higher in patients with progressed fibrosis than in those with mild or no fibrosis (Fig. 2a,d). *IP-10* induction by progression of fibrosis complicates the association between *IP-10* expression

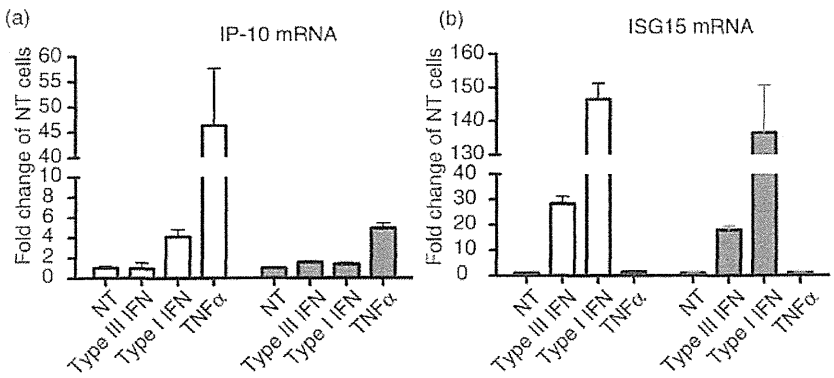

Figure 4 *IP-10* (a) and *ISG15* (b) expression levels were measured using real-time reverse transcription polymerase chain reaction. The values shown are the mean fold change induction compared with the mRNA level of human *β-actin* and the fold change induction compared with untreated cells. ▭, HCV replicon; ▬, Huh-7. IFN, interferon; NT, non-treated; TNF, tumor necrosis factor.

and *IL-28B* SNP. In fact, in mild fibrosis patients, *IP-10* expression was significantly higher in *IL-28B* SNP minor patients than that in *IL-28B* SNP major patients. However, in progressed fibrosis patients, *IP-10* expression was similar among the patient groups who possessed different SNP (Fig. 3a,b).

In chronic hepatitis C patients, TNF-α expression is elevated with progression of liver fibrosis.[24] Immunohistochemistry showed that IP-10 expression was mainly induced in hepatocytes (Fig. 2d), and only a few inflammatory cells were stained (data not shown). Therefore, we evaluated the effect of TNF-α and IFN on *ISG15* and *IP-10* expression in the human hepatoma cell line HuH-7 and HuH-7-derived HCV replicon cells (Fig. 4). Transforming growth factor (TGF)-β is also a fibrosis-related cytokine, however, TGF-β did not induce *IP-10* expression in the HuH-7 cells (data not shown). We found that TNF-α induced *IP-10* expression more strongly than IFN signaling in these cells. These results suggest that even in the liver of *IL-28B* SNP major and progressed fibrosis patients, TNF-α or other fibrosis-related stimulator induce *IP-10* expression. In the liver of mild fibrosis patients with *IL-28B* minor genotype, some *IL-28B* SNP-related stimulator would induce *IP-10* expression. Thus, *IP-10* expression was associated with *IL-28B* SNP only in patients with mild or no fibrosis. In contrast, *ISG15* was not induced by TNF-α, suggesting that *ISG15* is strongly regulated by IFN (Fig. 4b). Therefore, irrespective of progression of fibrosis, *ISG15* expression is higher in *IL-28B* SNP minor patients than that in *IL-28B* SNP major patients (Fig. 3).

In the NBNC patients, *IP-10* expression was rarely induced by progression of fibrosis and was much lower than that in HCV infectious patients (Fig. 2c). Some reported that the HCV infected patients, rather than the HBV infections, expressed *IP-10* higher.[24,28] At least clinically, *IP-10* induction would occur dramatically by HCV infection, and may be quiescent without any viral infection. Thus, *IP-10* expression in the NBNC patients could be scarcely induced or be near the detection limit. With TNF-α stimulation, HCV replicon cells expressed *IP-10* mRNA more strongly than HCV RNA negative HuH-7 cells. These data suggest that *IP-10* induction by fibrosis-related stimulation could be enhanced by HCV infection or replication. Recently, Thomas *et al.* showed that HCV infection induces IFN-λ expression in the liver of chimpanzees.[29] IFN-λ and fibrosis-related stimulations could induce *IP-10* expression coordinately.

We then evaluated whether *IP-10* expression predicted therapeutic outcomes. *IL-28B* SNP is associated with IFN therapy outcomes, especially among HCV genotype 1 infected patients, and 54 patients were infected with HCV genotype 1. Thirty-six patients did not receive IFN therapy: 19 patients due to low platelet counts, 11 patients, due to tumor recurrence and six patients rejected the therapy. Eighteen patients with genotype 1 infection were administrated PEG IFN-α-2b and RBV combination therapy for 48 weeks after liver resection. *IL-28B* SNP was significantly associated with IFN therapeutic outcome, and the hepatic mRNA expressions of *ISG15* and *OAS* at resection were significantly lower in the SVR group than those in the non-SVR group (data not shown). However, *IP-10* expression was not associated with outcome. Lagging *et al.*[20] and Fattovich *et al.*[21] reported that serum IP-10 protein levels are higher in IL-28B SNP minor patients than those in IL-28B SNP major patients. Liver fibrosis of the patients in their study was less severe than that in the patients in our study. A correlation between hepatic *IP-10* expression and serum IP-10 protein levels was reported by Askarieh *et al.*,[30] and hepatic and serum *IP-10* expression has been reported as a predictor of IFN therapy outcome.[18,19] However, IP-10 expression as a predictor of outcome would not be beneficial in patients with severe fibrosis. Therefore, to predict efficacy using *IP-10* expression, progression of fibrosis should be considered.

The mechanism of how *IL-28B* SNP minor patients express excessive ISG is unclear. Excessive and probably maximal ISG expression before treatment would prevent the activity of IFN. Sugiyama *et al.* showed that long TA repeats in the promoter region of *IL-28B* possess strong *IL-28B* induction capability.[31] However, Smith *et al.* reported that *IL-28B* polymorphism affects methylation in the transcription factor binding sites and results in high *IL-28B* expression in *IL-28B* SNP minor patients.[32] *IL-28B* and IFN-λ3 have antiviral activity, and PEG IFN-λ has been previously used in a clinical trial and antiviral activity was reported.[33] However, an association between PEG IFN-λ efficacy and *IL-28B* SNP has not been reported. Investigation of PEG IFN-λ efficacy in *IL-28B* SNP minor patients may help clarify the function of IFN-λ in HCV infection. Further studies are required to determine this issue, and this could improve the health of chronic HCV infected patients.

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SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's web-site:

Figure S1 Comparison of hepatic interferon-stimulated genes (ISG) expression in the indicated fibrosis patients with *IL-28B* major polymorphism (upper graphs) and *IL-28B* minor polymorphism (lower graphs).
Figure S2 Comparison of hepatic *TNF* expression levels between F0/1 and F2/3/4 patients.
Table S1 Patient characteristics and interferon (IFN) therapy prior to liver resection.

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Original Article

Early extensive viremia, but not rs8099917 genotype, is the only predictor for cholestatic hepatitis C after living-donor liver transplantation

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Aim: Cholestatic hepatitis C is one of the most serious but still unaddressed disorders after liver transplantation.

Methods: In this study, we analyzed 49 patients who underwent living-donor liver transplantation (LDLT) to treat hepatitis C virus (HCV) infection.

Results: Five patients developed cholestatic hepatitis C, with total bilirubin of 15.2 ± 3.1 mg/dL at diagnosis 6.2 ± 1.0 weeks after LDLT. Univariate analysis showed that larger graft to standard liver volume ratio, higher HCV RNA titer at 2 weeks, earlier peak HCV RNA titer and cytomegalovirus infection were the significant risk factors. The development of cholestatic hepatitis C was not significantly associated with interleukin-28B genotype (rs8099917); four out of five affected patients had the T/T genotype. Multivariate analysis showed that higher HCV RNA titer at 2 weeks was the only significant factor ($P = 0.026$) for the development of cholestatic hepatitis C. Receiver–operator curve analysis showed that that HCV RNA titer of more than 7.2 $\log_{10}$IU/mL was the optimal cut-off for characterizing cholestatic hepatitis C. All of the patients were serum HCV RNA negative after treatment with pegylated interferon and ribavirin and all the patients are alive.

Conclusion: Early extensive viremia, but not the rs8099917 genotype, was the only predictor for cholestatic hepatitis C after LDLT.

Key words: cholestatic hepatitis, hepatitis C, interleukin 28B, liver transplantation, living donor, splenectomy

INTRODUCTION

ALTHOUGH END-STAGE LIVER disease secondary to hepatitis C virus (HCV) is the leading indication for liver transplantation (LT), re-infection of HCV is a widespread, unaddressed and serious event.[1] It has been reported that approximately one-quarter of patients develops cirrhosis within 10 years after LT for HCV; therefore, graft outcomes after LT for HCV are inferior to those for other indications.[2]

Nevertheless, recurrent hepatitis C after LT is represented by a spectrum of disorders, including mild to severe inflammation with various degrees of fibrosis progression over several years.[1,2] Of note, HCV re-infection can result in very aggressive hepatitis in a small number of patients, and is usually characterized by rapid progression of cholestasis with fibrosis resulting in graft failure and death.[3,4] This outcome has been termed post-transplant cholestatic hepatitis C and its risk factors include higher donor age, HCV genotype 1, extremely high viral titers and bolus steroid administration for acute rejection.[3,4] More recently, two reports

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have shown that single nuclear polymorphism (SNP) in the interleukin (IL)-28B gene was a significant risk factor for the disease process.[5,6] To date, however, the pathogenesis of recurrent cholestatic hepatitis C after LT has not been elucidated.

Therefore, in the current study, we examined the clinical characteristics of patients who developed this rare type of recurrent cholestatic hepatitis C after living-donor liver transplantation (LDLT). We investigated whether its pathogenesis could be attributed to viral factors, host factors, including IL-28B genotypes or graft-related factors.

METHODS

Patients

LIVING-DONOR LIVER TRANSPLANTATION was performed in 54 patients positive for the HCV antibody at Kyushu University Hospital between February 2007 and July 2012. All procedures were approved by the Ethics and Indications Committee of Kyushu University. Forty-nine patients who were HCV RNA positive before LDLT were included in the current study. The mean follow-up time was 2.8 ± 1.1 years.

Transplantation and postoperative care

The surgical procedures for both the donors and the recipients are described in more detail elsewhere.[7,8] The graft type, either left or right lobe, was determined based on the need for a graft volume (GV) of more than 35% of the recipient's standard liver volume (SLV).[7] Splenectomy was performed for 47 (95.9%) recipients to prevent pancytopenia caused by interferon (IFN) therapy.[9] A biliary stent over the biliary anastomosis was placed during the surgery and was kept in place for 3–4 months after LDLT to prevent early stricture.[10]

The immunosuppression regimen consisted of tacrolimus or cyclosporin with mycophenolate mofetil and steroids as previously reported.[8] The immunosuppression level was maintained at a standard level to prevent acute rejection; unfortunately, this hinders the diagnosis and treatment of hepatitis C after LDLT. The tacrolimus level was maintained at 10–14 ng/mL for 1 month after LDLT and was then decreased to 7–10 ng/mL over the next few months. The cyclosporin level was maintained at 150–250 ng/mL for 1 month after LDLT and then decreased to 100–150 ng/mL over the next few months. Mycophenolate mofetil at the dose of 2 g/day, was then tapered down to 1 g daily over 1–3 months and tapered off at 6 months. All the patients received steroids during the study period. Methylprednisolone (1 g) was given after reperfusion, and titrated from 200 mg/day to 20 mg/day in a week, then switched to oral prednisolone, and tapered off by 6 months. The immunosuppression protocol for blood type-incompatible LDLT consisted of pretransplant rituximab and plasma exchanges with tacrolimus or cyclosporin and mycophenolate mofetil and steroids, as previously described.[11]

Antiviral treatment

Interferon was indicated for recurrent hepatitis C associated with serum HCV RNA positivity, abnormal liver function tests and histological evidence of recurrent hepatitis C. Preemptive antiviral treatment was not performed.

Antiviral treatment consisted of pegylated (PEG) IFN-α-2b with ribavirin (Pegintron with Rebetol; Merck, Whitehouse Station, NJ, USA) or PEG IFN-α-2a with ribavirin (Pegasys with Copegus; Chugai Pharmaceutical, Tokyo, Japan) was used for antiviral treatment. Although PEG IFN-α-2b was primarily used for post-transplant induction of antiviral treatment, PEG IFN-α-2a could also be used for refractory or severe cases. The type of PEG IFN drug, regarding conversion between the products, was determined for individual cases. PEG IFN-α-2b and ribavirin were started at doses of 0.5–1.0 mcg/kg per week and 200–400 mg/day, respectively. The doses were escalated in a stepwise manner, in accordance with the individual's tolerability, to 1.5 mcg/kg per week and 800 mg/day, respectively. PEG IFN-α-2a and ribavirin were started at doses of 90–120 mcg/week and 200–400 mg/day, respectively, to 180 mcg/week and 800 mg/day respectively. The recommended duration of treatment was 48 weeks after achieving viral response (VR), defined as undetectable serum HCV RNA.

Measurement of the serum HCV RNA titer

The serum HCV RNA titer was determined by a real-time HCV assay (AccuGene HCV; Abbott Molecular, Des Plaines, IL, USA). The lower and higher limits of quantification for this assay are 1.08 log IU/mL and 8.0 log IU/mL, respectively. The serum HCV RNA titer was measured before LDLT, 2 weeks after LDLT and monthly thereafter.

IL-28B genotyping assay

DNA from the donors and the recipients was extracted from a biopsy or explanted liver tissue obtained during LDLT, and genotyping was performed using TaqMan

GTX press Master Mix (Life Technologies, Tokyo, Japan), in accordance with the manufacturer's instructions. The Custom TaqMan SNP Genotyping Assay (Life Technologies) was used to identify IL-28B genetic polymorphisms. We used rs8099917 as the representative SNP for IL-28B because of its higher sensitivity and specificity for IFN sensitivity in Asian individuals.[12] The T/T genotype of rs8099917 was defined as the major allele, while the T/G and G/G genotypes were regarded as the minor alleles.

Diagnosis of cholestatic hepatitis

Cholestatic hepatitis C was defined according to the factors as proposed by Wiesner *et al.*[13] with minor modifications: (i) total bilirubin of more than 6 mg/dl; (ii) elevated biliary enzymes with alkaline phosphatase (ALP) and/or γ-glutamyltransferase (GGT) of more than 5 times the upper limit of normal; (iii) very high serum HCV RNA titer of more than 6 log IU/mL; (iv) histological findings that include predominant ballooning of hepatocytes in the perivenular zone and limited inflammation; (v) occurring between 1 and 6 months after LT; and (vi) absence of surgical complications at the time of diagnosing cholestatic hepatitis C.

Percutaneous liver biopsy was obtained and evaluated for patients with abnormal liver function tests suggestive of recurrent hepatitis C or acute rejection. Biopsies were also obtained every year in accordance with the established protocol.

Statistical analysis

Values are expressed as the mean ± standard deviation. Variables were analyzed using the χ^2-test for categorical values or the Mann–Whitney *U*-test for continuous variables. Multivariate analyses were performed using the logistic regression model and odds ratios were calculated. $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of patients with cholestatic hepatitis C

FIVE PATIENTS DEVELOPED cholestatic hepatitis C after LDLT (Table 1). The mean ages of the donors and the recipients were 58.2 ± 7.7 years and 29.2 ± 10.0 years, respectively. The mean GV/SLV ratio was 45.0 ± 7.3%. Donor age was less than 40 years old in all of the cases except for case 5. GV/SLV was more than 35% in all of the cases, except in case 3. Splenectomy was performed in all five cases.

Hepatitis C virus genotype was type 1b, except in case 4 (2a) and the mean HCV RNA titer before LDLT was 5.2 ± 0.7 $\log_{10}$IU/mL. The HCV RNA titer was more than 5 $\log_{10}$IU/mL in all the cases except case 5. The IL-28B (rs8099917) genotype was T/T in both the donors and recipients except in case 2, where the donor and recipient both had the T/G genotype.

The mean values of liver function parameters were 15.2 ± 3.1 mg/dL for total bilirubin, 357 ± 79 IU/L for aspartate aminotransferase (AST) and 859 ± 497 IU/L for GGT. The peak HCV RNA titer was 7.9 ± 0.1 $\log_{10}$IU/mL and more than 7.7 $\log_{10}$IU/mL in all five patients at diagnosis of cholestatic hepatitis C, 6.2 ± 1.0 weeks after LDLT. Although cases 1 and 5 had biliary anastomotic stenosis after LDLT, this complication occurred after treatment for cholestatic hepatitis C.

All of the five patients were treated with PEG IFN with ribavirin after histological confirmation of cholestatic hepatitis C. PEG IFN-α-2b was used in two patients and PEG IFN-α-2b was used in three patients. VR was observed in all of the patients. Among the patients who received IFN ($n = 41$) after LDLT, the total dosage of IFN was larger in patients with ($n = 5$) cholestatic hepatitis C (10.5 ± 3.0 vs 6.0 ± 4.6 mg, $P = 0.040$), compared with those without ($n = 36$). However, the total dosage of ribavirin (24.6 ± 26.1 vs 24.4 ± 20.7 g, $P = 0.981$) and the treatment period (90.0 ± 44.7 vs 62.2 ± 38.8 g, $P = 0.147$) was not different between the groups. Discontinued antiviral treatment was observed in no case in the patients with cholestatic hepatitis ($n = 5$) and 10 cases (27.8%) in the patients without ($n = 36$) due to intolerance and adverse reactions. Dose modification of IFN during the treatment course was observed in three patients (60%) and 18 patients (50.0%), respectively.

Risk factors for cholestatic hepatitis C

We next determined possible risk factors for cholestatic hepatitis C after LDLT. In univariate analyses, larger GV/SLV (45.0 ± 7.3% vs 39.2 ± 5.9%, $P = 0.049$), higher HCV RNA titer at 2 weeks after LDLT (7.7 ± 0.4 vs 5.8 ± 1.3 $\log_{10}$IU/mL, $P = 0.002$), earlier period for having peak HCV RNA titer (3.7 ± 2.3 vs 9.4 ± 5.6 weeks, $P = 0.031$) and cytomegalovirus infection (80.0% vs 27.2%, $P = 0.017$) were significantly associated with cholestatic hepatitis C after LDLT. By contrast, donor and recipient age, cold and warm ischemic time, HCV genotype, and donor and recipient IL-28B genotype were not associated with the occurrence of cholestatic hepatitis C (Table 2).

Table 1 Clinical characteristics of the five cases of cholestatic hepatitis C

Case	1	2	3	4	5
Recipient age, sex	54, F	62, F	52, M	53, F	70, F
MELD score	16	18	8	18	12
Hepatocellular carcinoma	Yes	Yes	Yes	No	Yes
Splenectomy	Yes	Yes	Yes	Yes	Yes
Donor age, sex	21, F	36, M	20, M	23, M	43, F
Immunosuppression regimen	FK-based	CyA-based	CyA-based	CyA-based	CyA-based
ABO incompatible	No	Yes	Yes	No	No
Graft type	Left	Left	Left	Right	Right
GV (g)	460	440	510	598	502
GV/SLV (%)	39.9	44.0	37.0	55.4	48.9
HCV genotype	1b	1b	1b	2a	1b
HCV RNA titer (log_{10}IU/mL)	5.7	5.7	5.3	5.5	3.9
Recipient IL-28B genotype	T/T	T/G	T/T	T/T	T/T
Donor IL-28B genotype	T/T	T/G	T/T	T/T	T/T
Peak liver function tests					
Total bilirubin (mg)	17.4	13.6	19.1	16.7	9.0
AST (IU/L)	354	382	486	163	399
GGT (IU/L)	519	1939	415	1023	401
HCV RNA (log_{10}IU/mL)	7.7	7.7	8.0	8.0	7.7
Weeks after LDLT	4	8	6	6	7
Histological findings					
Hepatocyte ballooning	++	++	++	+++	++
Cholestasis	+	–	–	–	–
Perivenulitis	+++	+	++	+	–
Portal infiltration	+	+	–	–	+
Ductular reaction	+	+	+	–	+
Interferon treatment					
Type and dose (μg/week)	α-2b (50)	α-2a (180)	α-2b (90)	α-2a (180)	α-2a (180)
Ribavirin dose (mg/day)	400	0	400	200	200
Response (weeks)	VR (130)	VR (17)	VR (15)	VR (49)	VR (23)
On treatment (weeks)	Yes (170)	Yes (74)	Yes (70)	Yes (69)	Yes (68)
Graft outcomes (years)	Alive (3.4)	Alive (1.6)	Alive (1.5)	Alive (1.5)	Alive (1.5)

AST, aspartate aminotransferase; CyA, cyclosporin; FK, tacrolimus; GGT, γ-glutamyltransferase; GV, graft volume; HCV, hepatitis C virus; IL, interleukin; LDLT, living-donor liver transplantation; MELD, Model for End-Stage Liver Disease; SLV, standard liver volume; VR, viral response.

In multivariate logistic regression analysis, higher HCV RNA titer at 2 weeks after LDLT ($P = 0.026$) was the only significant factor associated with having cholestatic hepatitis C. The other factors identified in univariate analyses, including earlier peak of HCV RNA titer ($P = 0.317$), larger GV/SLV ($P = 0.382$) and cytomegalovirus infection ($P = 0.936$) were not significantly associated with cholestatic hepatitis C after LDLT. Receiver–operator curve (ROC) analysis showed that HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after LDLT was the optimal cut-off for discriminating cholestatic hepatitis C after LDLT. The area under the ROC for this value was 0.989 (Fig. 1).

Histological characteristics of cholestatic hepatitis C after LDLT

The histological characteristics of the five cases of cholestatic hepatitis C are summarized in Table 1. Although hepatocyte ballooning was prominent in all of the five patients (Fig. 2), portal infiltration and cholestasis were relatively minor or absent, despite the high serum bilirubin level. Perivenulitis was observed in four cases and was significantly more common in patients with recurrent cholestatic hepatitis C than in patients with recurrent non-cholestatic hepatitis C (80.0% vs 20.5%, $P = 0.004$, Table 2). Ductular reaction was observed in four cases.

Table 2 Factors associated with cholestatic hepatitis C

Factors	Cholestatic hepatitis		*P*-value
	No ($n = 44$)	Yes ($n = 5$)	
Recipient age (years)	57.4 ± 8.0	58.2 ± 7.7	0.839
Recipient sex, male	22 (50.0)	1 (20.0)	0.203
Hepatocellular carcinoma, yes	31 (70.5)	3 (60.0)	0.631
MELD score	14.8 ± 7.0	14.4 ± 4.3	0.908
History of IFN treatment, yes	34 (80.9)	3 (60.0)	0.602
Donor age (years)	34.5 ± 10.9	29.2 ± 10.0	0.302
Donor sex, male	31 (70.5)	3 (60.0)	0.631
ABO incompatible, yes	5 (11.4)	2 (40.0)	0.083
Graft type, left lobe	17 (38.6)	2 (40.0)	0.952
GV (g)	461 ± 91	502 ± 61	0.341
GV/SLV (%)	39.2 ± 5.9	45.0 ± 7.3	0.049
Splenectomy, yes	42 (95.5)	5 (100.0)	0.626
Cold ischemic time (min)	100 ± 62	83 ± 43	0.551
Warm ischemic time (min)	39 ± 10	37 ± 9	0.631
Operative time (min)	793 ± 136	740 ± 107	0.404
Blood loss (L)	4.5 ± 6.5	4.9 ± 3.2	0.894
Recipient IL-28B genotype, T/T	23 (60.5)	4 (80.0)	0.393
Donor IL-28B genotype, T/T	27 (64.3)	4 (80.0)	0.483
HCV genotype 1, yes	34 (80.9)	3 (60.0)	0.279
HCV RNA titer (log_{10}IU/mL)			
Before LDLT	5.4 ± 1.2	5.2 ± 0.7	0.813
At 2 weeks after LDLT	5.8 ± 1.3	7.7 ± 0.4	0.002
Peak titer	6.8 ± 1.3	7.9 ± 0.1	0.089
Time to peak HCV RNA titer (weeks)	9.4 ± 5.6	3.7 ± 2.3	0.031
Viral response (%)	22 (64.7)	5 (100.0)	0.110
Tacrolimus use, yes	22 (50.0)	1 (20.0)	0.202
Acute rejection, yes	1 (2.3)	0 (0.0)	0.733
Bile duct stenosis, yes	8 (18.2)	2 (40.0)	0.251
Cytomegalovirus infection, yes	12 (27.2)	4 (80.0)	0.017
Central perivenulitis on biopsy, yes	9 (20.5)	4 (80.0)	0.004

GV, graft volume; HCV, hepatitis C virus; IL, interleukin; LDLT, living-donor liver transplantations; MELD, Model for End-Stage Liver Disease; SLV, standard liver volume; SNP, single nuclear polymorphism; VR viral response.

DISCUSSION

IN THE CURRENT study, HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after transplantation was the only predictive factor for recurrent cholestatic hepatitis C after LDLT. None of the other donor or recipient factors, including IL-28B (rs8099917) genotypes were associated with this severe disease in multiple regression analysis. Cholestatic hepatitis C was diagnosed in all five patients based on early extensive viremia and histological findings (e.g. pan-lobular hepatocyte ballooning). VR was achieved in all of the cases following immediate treatment with PEG IFN with ribavirin.

Although cholestatic hepatitis C is an uncommon (2–5%) form of HCV recurrence, it is usually associated with rapid progression of cholestasis with fibrosis, and often results in graft failure within 1 year after transplantation.[3–6] Early and accurate diagnosis of cholestatic hepatitis C and immediate treatment is essential to save the transplanted grafts, although diagnosis is often difficult.[14–16] The difficulties in diagnosis are mainly due to the differential diagnoses, including acute rejection, biliary stenosis or primary graft dysfunction, for which the treatments are opposite or are very different from those used for cholestatic hepatitis C.[3] We think that the combination of HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after LDLT and pan-lobular ballooning of the hepatocytes are key factors for identifying cholestatic hepatitis C.

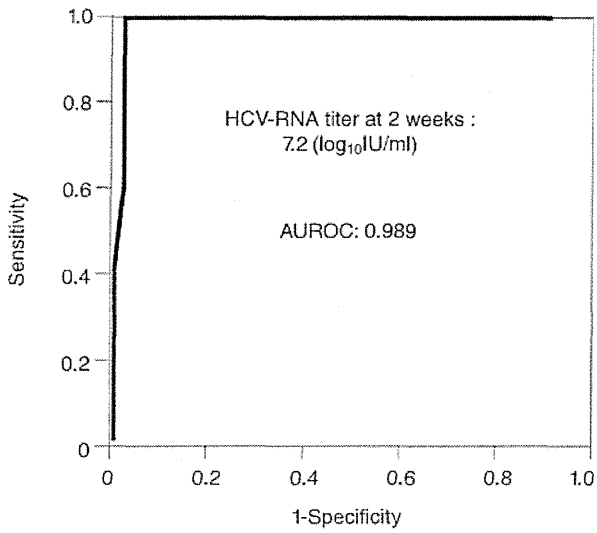

Figure 1 Receiver–operator curve analysis showed that HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after LDLT was the optimal cut-off for discriminating cholestatic hepatitis C. AUROC, area under the receiver–operator curve; HCV, hepatitis C virus; LDLT, living-donor liver transplantations.

Extensive HCV infection in hepatocytes and the direct cytopathological effects of HCV, together with a relative absence of inflammation, are thought to be the major mechanisms involved in the development of cholestatic hepatitis C.[17] Therefore, a very high HCV RNA titer was proposed as one of the diagnostic criteria for cholestatic hepatitis after LT in a consensus statement published in 2003.[13] However, the cut-off level for a very high HCV RNA titer was not reported in that consensus statement. More recently, Shackel *et al.*[18] reported that a peak HCV RNA titer of more than 7.0 log_{10}IU/mL within 1 year of LT was a predictor of HCV-associated graft failure. Moreover, Granziadei *et al.*[5] showed that HCV RNA titer of more than 6.0 log_{10}IU/mL 2 weeks after transplantation is the most significant risk factor for the development of cholestatic hepatitis. However, they did not report how they selected this value. We used ROC analysis and found that a HCV RNA titer of more than 7.2 log_{10}IU/mL at 2 weeks after LDLT was the optimal cut-off for predicting cholestatic hepatitis C after transplantation.

Histological features are also important for the diagnosis of cholestatic hepatitis C.[3,14] Hepatocyte ballooning with limited inflammation is considered to be a typical finding, and it was observed in all of our cases with pan-lobular distribution. However, the international consensus criteria stated that ballooning predominantly occurred in the perivenular zone.[14] In LDLT, perivenular hepatocyte ballooning with cholestasis is often observed in dysfunctional grafts associated with small graft size, older donor or systemic inflammation.[19] Hepatocyte cholestasis was apparent in just one case (20%) in our series, and it might be attributed to the early biopsy before becoming fully established and irreversible.

Perivenulitis with centrilobular hepatocyte dropouts is a distinct histopathological process that could occur after LT, and is associated with post-transplant processes, including cytotoxic drugs, acute or chronic rejection, recurrent or de novo autoimmune hepatitis, and viral hepatitis.[20] Recent research focused on its immunological significance with significant graft injuries.[21] In hepatitis C after LT, Khettry *et al.*[22] reported that perivenulitis was significantly recognized in cases with severe recurrent hepatitis C associated with other pathological features with autoimmune hepatitis. Antonini *et al.*[23] reported that this phenomenon was more common in cholestatic patients than in non-cholestatic patients (36% vs 4%). Taking into account that cholestatic type recurrent hepatitis C causes significant hepatocyte injuries with vigorous cytokine production with unspecified immune reactions,[20–23] perivenulitis could be a significant pathological marker in cholestatic hepatitis C.

Interleukin-28B genotyping is an important predictor for the viral response to IFN. We previously reported that the T/T genotype of rs8099917 in donors and recipients is a positive predictor of the response to IFN after LDLT for hepatitis C.[12] In the current series, however, the T/T genotype was not associated with the recurrence of cholestatic hepatitis C. By contrast, Graziadei *et al.*[5] reported that rs12979860 genotypes, other than the favorable C/C genotype, in the recipients were significantly associated with cholestatic hepatitis C after LT, although the relevance of rs12979860 in donors has not been exclusively investigated. Hanouneh *et al.*[6] reported that the favorable T/T genotype of rs8099917 in the donor was associated with cholestatic recurrence. Based on these results, no consensus can be reached regarding the impact of IL-28B genotype on recurrence of cholestatic recurrent hepatitis C. Additionally, because there is a discrepancy between the IL-28B genotype, IL-28B transcription and the expression of IFN-stimulated genes,[24] further studies are needed to clarify the role of IL-28B in anti-HCV therapy.

It is still unclear why HCV can infect and replicate so vigorously, and cause cholestatic recurrence in a small number of patients after LT. We consider that

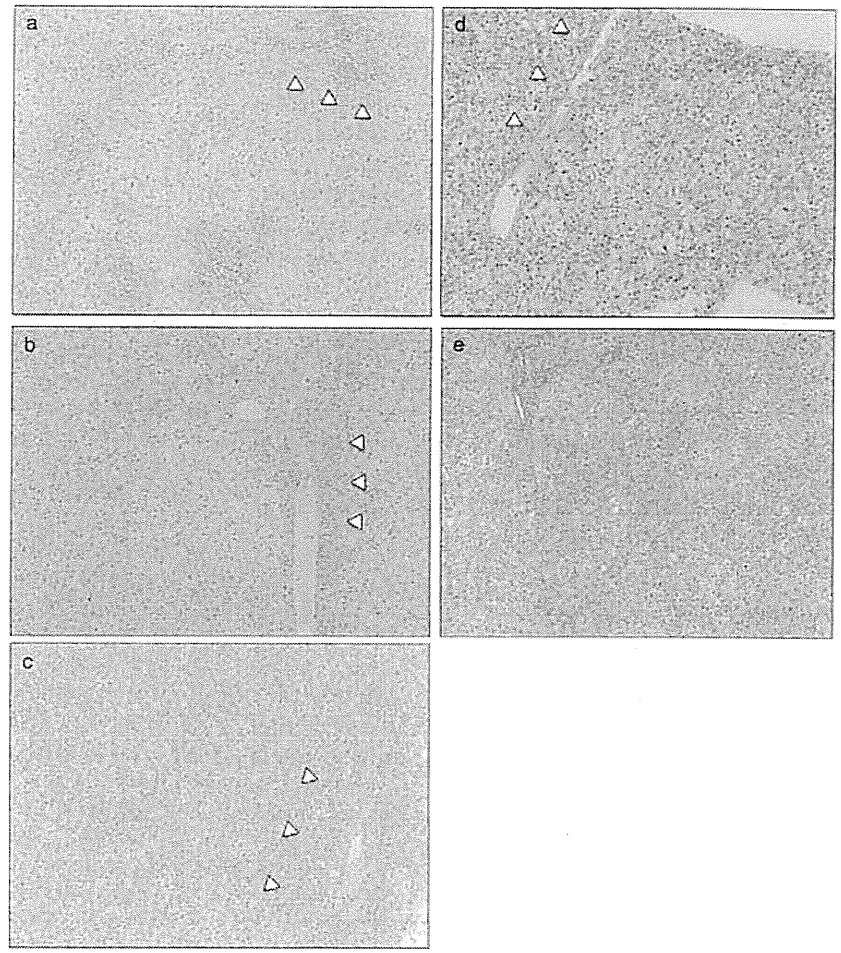

Figure 2 Histological findings of cases 1–5 (a–e, respectively) with recurrent cholestatic hepatitis C. Pan-lobular hepatocyte ballooning was prominent in all of the five patients. Perivenulitis was observed in cases 1–4 (a–d, white arrowheads) (hematoxylin–eosin, original magnification ×100).

quasispecies of HCV may play some role in this process. Previous studies showed that the number of quasispecies increased following transplantation and onset of mild recurrence, but the species distribution was more homogenous in patients with severe recurrence.[25,26] It was also reported that HCV infection becomes more severe in patients infected with HIV type 1 with decreased or homogenous quasispecies.[25,27] Because an increased number of quasispecies is thought to represent the response of HCV to a strong immune pressure, induction of the local non-specific histocompatibility independent immune system may also mediate the disease process. Although viral mutations with increased capability of antiviral drug resistance as observed in cholestatic hepatitis B may have roles,[28] we regard it as doing little in cholestatic recurrent hepatitis C after LT because it becomes evident very early after transplantation before antiviral treatment is initiated. Therefore, we regard mechanisms in higher replication property against natural immune pressure including quasispecies as playing an important role.[23–27]

In terms of treatment, we think that PEG IFN with ribavirin should be the first choice of regimen for cholestatic hepatitis C, considering its clinically relevant outcomes. Nevertheless, the important point is that antiviral treatment should only be initiated once clinical cholestasis is evident, and histological cholestasis and fibrosis are established.[4–6,14] If started too late, the tolerability of IFN may become a major problem for decompensated liver grafts. Satapathy *et al.*[4] reported that seven out of eight patients (88%) with cholestatic hepatitis discontinued IFN because of decompensation or complications. The important key step to initiate early antiviral treatment for cholestatic hepatitis C is the accurate

pathological diagnosis differentiating acute rejection, although it is not an easy task. Bolus steroids for severe hepatitis C could terminate a transplanted graft.[29] Therefore, we maintain an appropriate immunosuppression level for the first 3 months after LT for HCV-associated liver diseases and never perform rapid tapering, making pathological interpretation easier. If treatment is started early, routine splenectomy of HCV patients during LDLT is reported to increase their tolerability of intense antiviral therapies.[9]

In conclusion, HCV viremia of more than 7.2 $\log_{10}$IU/mL at 2 weeks after transplantation was the predictor of recurrent cholestatic hepatitis C after LDLT in this study. IL-28B (rs8099917) genotype and other donor and recipient factors were not associated with its recurrence. Early diagnosis followed by antiviral treatment using PEG IFN with ribavirin is important to achieve VR and graft survival.

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RESEARCH ARTICLE Open Access

Effect of laparoscopic splenectomy in patients with Hepatitis C and cirrhosis carrying IL28B minor genotype

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Abstract

Background: IL28B and ITPA genetic variants are associated with the outcome of pegylated-interferon and ribavirin (PEG-IFN/RBV) therapy. However, the significance of these genetic variants in cirrhotic patients following splenectomy has not been determined.

Methods: Thirty-seven patients with HCV-induced cirrhosis who underwent laparoscopic splenectomy (Spx group) and 90 who did not (non-Spx group) were genotyped for IL28B and ITPA. The outcome or adverse effects were compared in each group. Interferon-stimulated gene 15 (ISG15) and protein kinase R expression in the spleen was measured using total RNA extracted from exenterate spleen.

Results: Sustained virological response (SVR) rate was higher in patients carrying IL28B major genotype following splenectomy (50% vs 27.3%) and in patients carrying minor genotype in the Spx group compared to non-Spx group (27.3% vs 3.6%, $P < 0.05$). Pretreatment splenic ISG expression was higher in patients carrying IL28B major. There was no difference in progression of anemia or thrombocytopenia between patients carrying each ITPA genotype in the Spx group. Although splenectomy did not increase hemoglobin (Hb) level, Hb decline tended to be greater in the non-Spx group. In contrast, splenectomy significantly increased platelet count ($61.1 \times 10^3/\mu l$ vs $168.7 \times 10^3/\mu l$, $P < 0.01$), which was maintained during the course of PEG-IFN/RBV therapy.

Conclusions: IL28B genetic variants correlated with response to PEG-IFN/RBV following splenectomy. Splenectomy improved SVR rate among patients carrying IL28B minor genotype and protected against anemia and thrombocytopenia during the course of PEG-IFN/RBV therapy regardless of ITPA genotype.

Keywords: IL28B, ITPA, Splenectomy, Liver cirrhosis

Background

Hepatitis C virus (HCV) chronically infects over 170 million people worldwide, with 3–4 million individuals newly infected each year. Liver cirrhosis may progress in 30% of chronic hepatitis C patients, and even hepatocellular carcinomas develop in 7–8% of cirrhotic patients annually [1,2]. Although the current standard therapy for HCV is combination of pegylated-interferon plus ribavirin (PEG-IFN/RBV), sustained virological response (SVR), defined as negativity for HCV RNA for 24 weeks after cessation of therapy, can be achieved for only 50% of chronic hepatitis patients, and for <30% of cirrhotic patients [3,4]. In addition to low efficacy, many adverse effects, especially cytopenia, are extremely detrimental to cirrhotic patients.

Splenectomy is recommended for cirrhotic patients with thrombocytopenia to enable them to receive PEG-IFN/RBV therapy safely [5]. Although patients with liver cirrhosis have a high risk of hemorrhage due to dilated collateral vessels, splenomegaly, and poor liver function, we have demonstrated the safety and feasibility of laparoscopic splenectomy (LS) since its first report in 1992 [6,7]. In particular, it could be very effective for cirrhotic patients who have failed induction of PEG-IFN/RBV therapy due to low platelet counts [8].

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A genome-wide association study (GWAS) has revealed two striking single nucleotide polymorphisms (SNPs), IL28B and ITPA, which are correlated with the outcome or adverse effects of PEG-IFN/RBV therapy [9-11]. A lower VR rate has been shown in patients carrying the minor genotype (TG or GG) at rs8099917 near the IL28B locus, and treatment-induced hemolytic anemia has been reported more often in patients carrying the major genotype (CC) at rs1127354 in the ITPA gene. In addition, ITPA genetic variants have also been reported recently to correlate with platelet reduction during PEG-IFN/RBV therapy [12].

However, the significance of these two SNPs among patients who have undergone LS has yet to be determined. For more than a little patients could not achieved virological response or would failed the incessancy of PEG-IFN/RBV even after splenectomy, it would be very beneficial to predict the outcomes of anti-HCV therapy following splenectomy. In the current study, we demonstrated the association of IL28B and ITPA SNPs with the outcome of PEG-IFN/RBV therapy following LS.

Methods

Patients

From August 2004 to March 2009, 117 consecutive type C cirrhotic patients underwent LS at our institute for the induction of PEG-IFN/RBV therapy, and DNA was available for genotyping from 37 of these. They were compared to 90 cirrhotic patients who did not undergo splenectomy before induction of PEG-IFN/RBV therapy in the same period at the Department of General Internal Medicine, Kyushu University. Patients in the control group were selected on account of their platelet count $<10^5$/ml or by METAVIR fibrosis stage F4 [13]. Therefore, 127 patients were enrolled in the current study, which was approved by the Ethics Committee of Kyushu University.

DNA extraction and genotyping

Genomic DNA was extracted from the patients' spleen tissues obtained at operation, or from peripheral blood mononuclear cells in the control group. IL28B genetic polymorphism (rs8099917) and ITPA genetic polymorphism (rs1127354) were genotyped using StepOnePlus™ real-time PCR system (Applied Biosystems, Carlsbad, CA, USA).

Definition of outcomes of PEG-IFN/RBV therapy

VR was defined as a lack of HCV RNA in response to the treatment regimen, regardless of whether a relapse occurred when treatment was terminated. SVR was defined as undetectable HCV-RNA 24 weeks after the end of the therapy. Patients who had achieved VR but had showed relapse were defined as end of treatment response (ETR)-relapse. Hemoglobin (Hb) level or platelet count was assessed before splenectomy, at induction of therapy, and every 4 weeks during the course of PEG-IFN/RBV therapy.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from exenterate spleen specimens using ISOGEN (Nippon Gene, Tokyo, Japan) and reverse transcription was performed using SuperScriptIII (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed using SYBR® Green assay (Applied Biosystems) on the LightCycler®480 Real-Time PCR system (Roche Applied Sciences, Indianapolis, IN, USA). Specific primers for ISG15 were as follows: 5′-agcgaa ctcatctttg-3′ for sense primer 5′-cagctctgacaccgacatgga-3′ for antisense primer. Specific primers for protein kinase R (PKR) were 5′-acgtgtgagtcccaaagcaac-3′ for sense and 5′-ctgagaccattcataagcaacg-3′ for antisense. β-Actin expression was used for endogenous control with 5′-ct ggcaccacac cttctacaatg-3′ for sense primer and 5′-ggcgtacagggatagcacagc-3′ for antisense primer.

Statistical analysis

All data were analyzed using JMP® statistical software (SAS Institute, Cary, NC, USA). A χ^2 test was performed for qualitative variables and a Wilcoxon test was performed for quantitative variables.

Results

Patients' characteristics

Characteristics of patients who underwent splenectomy (Spx) and those who did not (non-Spx) are shown in Table 1. Although patients in the non-Spx group were selected on account of their platelet count $<10^5$/μl, count before surgery in the Spx group was lower than that in the non-Spx group (6.1×10^4/μl vs 8.7×10^4/μl, respectively, $P < 0.0001$), as well as their Hb level (12.5 g/dl vs 13.2 g/dl, respectively, $P = 0.03$). Both alanine aminotransferase and γ-glutamyl transpeptidase levels were significantly higher in the non-Spx group (55 IU/l vs 91 IU/l, $P = .001$, 45 IU/l vs 60 IU/l, $P = 0.02$, respectively), but albumin level was higher in the non-Spx group (3.3 mg/dl vs 3.9 mg/dl, $P < 0.0001$). The progression of cirrhosis was considered to be more severe in the Spx group. Neither pretreatment viral load nor the frequency of HCV genotype differed among these groups. The allele frequency of both IL28B (rs8099917) and ITPA (rs1127354) genetic polymorphisms was similar in these groups.